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GENOTYPE INDEPENDENT *IN VITRO* REGENERATION SYSTEM IN ELITE VARIETIES OF SUGARCANE

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Abstract

Nine sugarcane genotypes were tested for callus induction and subsequent regeneration on MS medium supplemented with different combinations of hormones. Genotype-independent plant regeneration was developed from calli induced from young leaf roll discs on MS medium supplemented with seven different levels (1-5mgl^{-l}) of 2, 4-D. Further, five levels (1-5mgl^{-l}) of 2, 4-D alongwith 1 mgl^{-1} of Kinetin for callus induction were used. All the genotypes responded well to 2, 4-D alone as well as in combination with Kinetin. Nevertheless, high percentage of regeneration was observed on MS medium supplemented with 500mg l^{-1} casein hydrolysate. Regenerated shoots developed profused roots on NAA (4-5mgl^{-l}) and casein hydrolysate (500mgl^{-l})-containing MS medium which were otherwise delayed on a medium containing full as well as half strength MS salts.

Introduction

Sugarcane is one of the major sources (70%) of world sugar (Lakshmanan *et al*., 2002). In addition, natural pharmaceutical compounds are also derived from sugarcane (Menéndez *et al*., 1994). Further, agricultural and industrial by-products of the sugar production process are extensively employed for animal nutrition, food processing, paper manufacturing and fuel (Patrau, 1989). Area under this crop is continuously increasing because of compelling demand of refined sugar and the use of its by-products for various purposes.

It is one of the most important cash crops of Pakistan with annual production of 63.92 million tones of cane cultivated on 1241 thousand hectares of land (Anon., 2008). Pakistan ranks second and eighth with respect of cane acreage (1241, 000 ha) and yield (~48.37 tonnes per hectare) respectively, in the world**.** In Pakistan, the crop is facing a lot of problems like insect, pests and disease attacks. Worldwide losses due to pests and diseases have been estimated at 37% of the agricultural production with 13% due to insects (Gatehouse *et al*., 1992). Moreover, due to alarming increase in the population of the world, there is a continuing need to increase food production, particularly in the developing countries of Asia, Africa and Latin America. To help these people, it is needed to increase the yields of major crops grown on existing cultivable lands. The one way of achieving greater yields is to provide disease-free cane seed to farmers. In this regard conventional biotechnology plays a major role by producing disease-free seed through *in vitro* techniques but the difficulties in (i) maintenance of totipotent callus or embryogenic callus because regeneration potential of callus in sugarcane decreases along with subcultures (Chen *et al*., 1988) (ii) prediction of suitable time for transformation of embryogenic callus or somatic embryos and (iii) unsynchronized growth and development of somatic embryos (iv) the occurrence of somaclonal variations with increasing age of callus (Heinz & Me, 1971) resulting in plants of heterogenous nature

(v) involvement of longer time scale in the recovery of plants are the issues which make the success very expensive and labour intensive. In view of these perspectives, tissue culture studies were undertaken to develop an efficient, rapid and reproducible genotype independent *In vitro* regeneration system for the nine promising sugarcane varieties.

Materials and Methods

Plant material and explant preparation: The study was initiated with nine elite sugarcane genotypes. Healthy cane tops of sugarcane varieties viz., S96SP-302, S96SP-571, S96SP-574, HSF-240, CP72-2086, CP77-400, SPF-213, S97US-183 and S97US-102 were kindly provided by Sugarcane Research Institute, Faisalabad, Pakistan. The cane tops collected from 9 month old cane plants were surface sterilized with ethanol. As apical , sub-apical region of the apical meristem and young leaves are tightly wrapped by 6-8 older leaves, so in general there was no need to surface sterilize the explant and only outer leaf surface sterilization was sufficient. Immature leaf rolls above the first node, excised in about 1-2mm thick cross-sectional slices were used as explants (Heins & Mee, 1969; Fitch & Moor, 1993).

Callus induction and culture conditions: MS (Murashige & Skoog, 1962) medium was used as basal culture medium with pH 5.8 and various modifications in growth regulator-supplementations such as auxins {2,4-D (1-5 mgl^{-1}), NAA (5 mgl^{-1})} and cytokinins {BAP (mgl^{-1}), Kinetin ($1mgl^{-1}$)} for each genotype. Cultures were maintained at temperature (27±2°C) for all treatments. For callus induction the cultures were placed in dark conditions and during regeneration studies these cultures were exposed to 16h photoperiod with light (2000-3000 lux).

Plantlet regeneration: Five weeks old calli were subjected to both regeneration and subcultured to increase callus masses. Five weeks old *In vitro* shoots were transferred to rooting medium. Rooted plantlets were shifted to pots or 2x4 inches polythene bags to acclimatize for field plantation.

Results

Ex-plant types and callogenesis: Among different explants used for callus induction, immature leaf proved better explant source for all genotypes producing maximum callus masses (Fig. 3) from young whorled leaf discs. Shoot apical meristem and nodal explants excreted more phenolic compounds in the culture medium compared to leaf explants in all genotypes except S96SP-302.

Callus induced within 8-10 days of culture from leaf explants of all varieties (Fig. 1) on MS medium supplemented with 2,4-D under dark. Further calli were light brown to brownish in colour. All varieties responded to seven levels of 2,4-D (Fig. 1), nevertheless callus initiation as well as proliferation was significantly vigorous only at levels, 2.5 and 3mg l^{-1} (Table 2). Two types of calli were observed on cultured explant tissues; one, embryogenic compact and/or friable and two, non-embryogenic sticky. Calli from all varieties were embryogenic compact or friable however, in some cases sticky non-embryogenic calli were also observed on explant tissues. The embryogenic calli compared to non-embryogenic were highly regenerative as for as callus maintenance and organogenesis is concerned. The statistical analysis showed that the results are highly significant (Table 2).

Table 1. The affect of 2,4-D and Kinitin on Callus formation in different sugarcane varieties.

Sugarcane variety and name	2, 4D (mg^{-1})	Best callus induction and proliferation	2, 4D + kinetin (mg^{-1})	Best callus induction
S97US-183	3	++++	2+1	++++
S97US-102	3	++++	3+1	++++
S96SP-574	3	++++	2+1	++++
S96 SP-571	2.5	++++	3+1	++++
S96SP-302	2.5	++++	3+1	++++
CP72-2086	3	++++	3+1	++++
CP77-400	3	++++	3+1	++++
SPF-213 3	3	++++	3+1	++++
HSF-240	2.5	++++	3+1	++++

MS medium containing MS salts, vitamins, myo-inositol (100 mgl^{-1}), and sucrose 3%, ++++ (best callus induction and proliferation against + for slow callus proliferation)

Table 2. ANOVA for 2,4-D concentrations and time (in days) for callus initiation in the nine sugarcane varieties.

K value	Source	*DF	Sum of squares	Mean square	F value	Prob.
2	**GR	6	458.571	76.429	38.3175	0.0000
4	***Var.	8	306.858	38.357	19.2304	0.0000
6	GRxVar.	48	407.143	8.482	4.2525	0.0000
-7	Error	126	251.321	1.995		

*Degree of Freedom; **Growth regulator; *** Varieties

Table 3. ANOVA for 2,4-D+Kinetin concentrations and time (in days) for callus initiation in all the nine sugarcane varieties.

K value	Source	*DF	Sum of squares	Mean square	F value	Prob.
2	**GR	4	216.267	54.067	43.3295	0.0000
4	***Var.	8	122.933	15.367	12.3150	0.0000
6	GRxVar.	32	113.733	3.554	2.8483	0.0000
-7	Error	90	112.302	1.248		

*Degree of Freedom; **Growth regulator; *** Varieties

Table 4. Shoot regeneration and varietal response to media composition.

Sugarcane variety and name	2, 4D (mg^{-1})	* % Age of shoot induction
S97US-183	1.5	87
S97US-102	1.5	70
S96SP-574	1.5	84
S96 SP-571	1.5	88
S96SP-302	1.5	86
CP72-2086	1.0	84
CP77-400	1.5	90
SPF-213 3	1.5	89
HSF-240	1.5	86

MS medium containing MS salts, vitamins, myo-inositol (100 mgl^{-1}), Casein hydrolysate (0.5g l^{-1}) and sucrose 3% *Average of three replicate flasks each containing six callus segments produced from separate explant discs

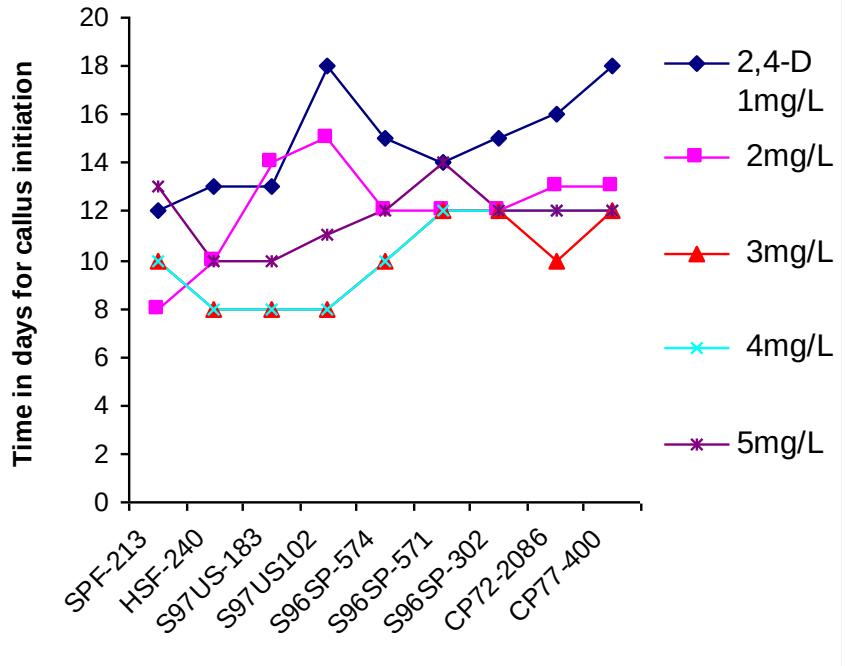

Fig. 1. Effect of 2,4-D verses time (in days) for callus initiation in the nine sugarcane varieties.

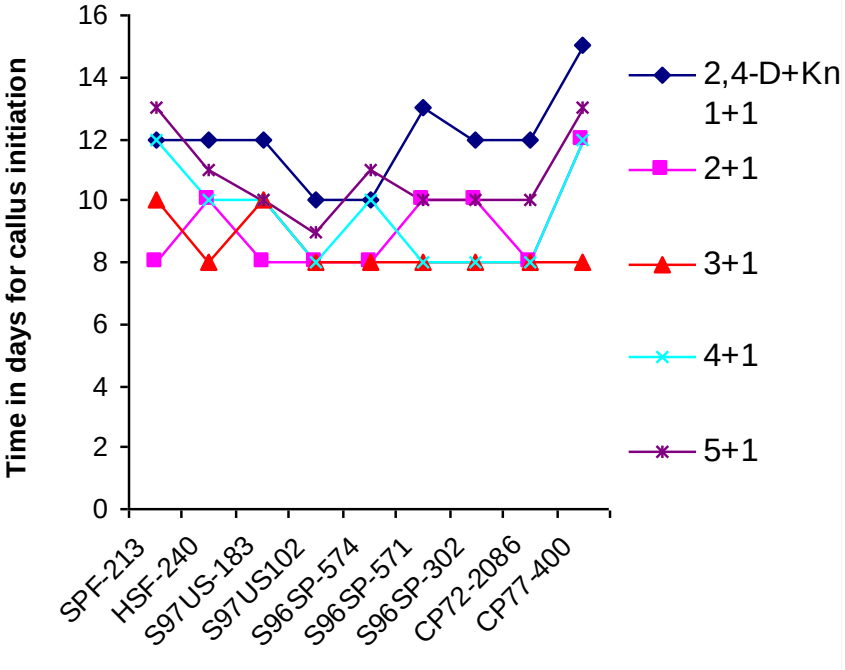

Fig.2. Kinetin with different 2,4-D concentrations verses time (in days) for callus initiation in the nine sugarcane varieties.

Table 5. Root induction in regenerated shoots of different sugarcane varieties.

Sugarcane variety and name	NAA (mg^{-1}) for best shoot induction response	* % Age of shoots in which root induction occurred
S97US-183	5	95
S97US-102	5	92
S96SP-574	5	90
S96 SP-571	5	94
S96SP-302	5	90
CP72-2086	5	95
CP77-400	5	95
SPF-213 3	5	80
HSF-240	5	90

MS medium containing MS salts, vitamins, myo-inositol (100 mgl^{-1}), Casein hydrolysate, 0.5g l^{-1} and sucrose 3% *Root formation %age was recorded five weeks after transferring regenerated shoots to root induction medium, from six individual discs the represents the average of shoots transferred.

Considerable callus masses emerged from explant tissues on almost all combinations of 2,4-D and Kinetin but maximum masses of calli were observed on medium containing combination 3 + 1 (Fig. 2). However, most of calli were friable and non-embryogenic

Response of tissue type to growth regulators for regeneration: The calli after five weeks developed shoots when transferred to regeneration medium with varying concentrations of 2,4-D. The concentration 1.5mg l^{-1} of 2,4-D appeared to be the best for shoot induction (in percentage) from all varieties except CP77-400 where 1mg l^{-1} was the effective concentration (Table 4). Roots developed more conveniently from shoots on MS medium supplemented with NAA at levels of 4-5mg l^{-1} along with 500mg l^{-1} casein hydrolysate. Nevertheless, NAA at level of 5mg l^{-1} proved best for root induction in all varieties except S96SP-302. In this variety 4mg l^{-1} NAA was observed more effective compared to 5mg l^{-1} as for as root development was concerned (Table 5).

Discussion

Callus can be induced from totipotent tissues of plants including sugarcane. In present studies, only immature leaf roll discs of almost all genotypes produced maximum callus masses on MS medium supplemented with 2,4-D at a concentration of 3mg l^{-1}. The callogenic response from dissected tissues of the leaf roll has confirmed that leaf sections beyond apical meristematic region could be used for callus induction and subsequent regeneration into plants. Guiderdoni & Demarly (1988) reported embryogenic callus production from different developmental stages of *In vitro* grown sugarcane plants. Young leaf roll here used as explant are most probably analogous to the least mature leaf sections of their *In vitro* grown plants that produced the largest amounts of calli. Calli developed in all varieties were both non-embryogenic and embryogenic in nature (Fig. 3). Among different callus types on the media, only embryogenic cells were regenerated into shoots upon transfer on regeneration medium. The addition of cytokinin alone or in combination with 2,4-D adversely affected the regenerative capability of the calli (Fitch & Moor, 1993) that was observed as inhibitory factor to subsequent embryogenic callus formation.

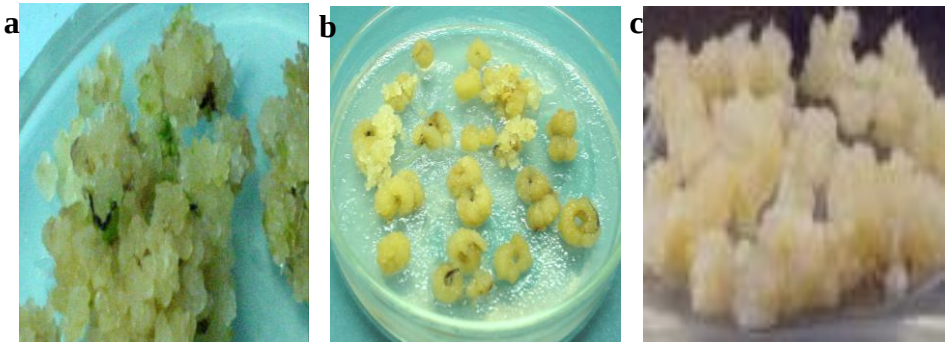

Fig.3. Types of callus developed under dark conditions in sugarcane cultivar HSF-240. **a**) Compact embryogenic callus with globular structures like somatic embryos after 8 week at MSCD medium (MS medium supplemented with casein hydrolysate and 2,4-D) **b**) Compact and friable embryogenic callus after 6 weeks at MS2 medium. **c**) Friable non-embryogenic white to yellow and sticky callus after 14 week at MS2 medium callus (magnification: **a** at 2.5 x, **b** and **c** at 1x).

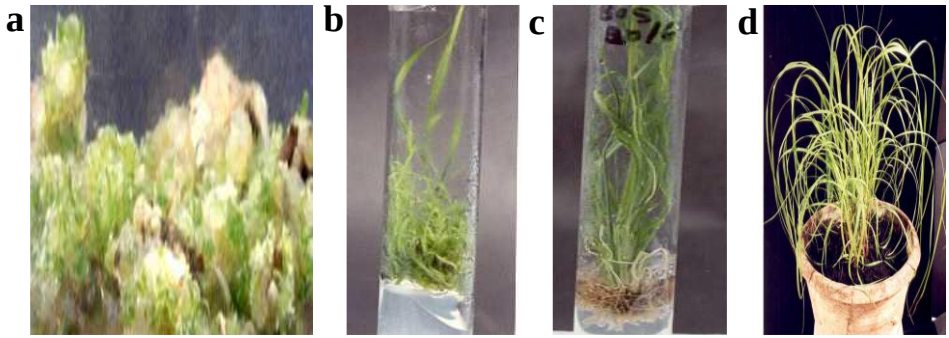

Fig. 4. Regeneration and hardening of sugarcane variety HSF-240. **a)** Regeneration initiation of calli on MSCD medium, cultures are of 8 weeks age **b)** Proliferation of regenerated shoots, photographed at the age of 12 weeks on vitamin containing MS medium at 2.5x magnification **c)** Root development of proliferated shoots on MS medium supplemented with NAA. **d)** Acclimatization of sugarcane plants with developed root system (magnification: **c** & **d** at 1x).

Plant cell's ability to regenerate into whole plant from single cell (i.e. totipotency), can be exploited by modulating culture conditions as described by Reinert (1959). So far, little is known about the mechanism (s) of totipotency, and it is mainly discussed in relation to the concentration and ratio of plant growth regulators (Toonen & De Vries, 1996) whereas the stage of *In vitro* culture has been found to influence the impact of growth regulator. The same growth regulator has different morphogenic responses at different stages of *in vitro* cultures as observed in these studies; NAA (5mg l^{-1}) at callus stage induces shoots whereas shoots developed roots when same concentration of NAA was used in the regeneration medium. Similarly 2,4-D induces callus in young leaf tissue when used in high amounts (3-4mg l^{-1}) compared to when used at lower levels (1-1.5 mg l^{-1}) and that results in organ development (Table 4). This difference of 2,4-D level is most probably involved in inducing polarization for cell elongation in tissues leading to differential growth as a result of varied response in cell membranes of growing cells. Specific auxin-binding sites have been demonstrated in the plasma membranes of maize

coleoptiles where auxin-binding protein–ABP1 was identified which functions as a putative receptor in ABP1-mediated membrane-associated ion fluxes and cell enlargement. Exogenously added purified corn ABP1 confers the competence in tobacco protoplasts to respond to auxin, while the antibodies against ABP1 block the auxin-induced hyper polarization (Barbier *et al*., 1989). The second view is that calcium ions, either themselves and or along with calcium binding proteins e.g., calmodulin, activate the protein kinase which in turn activates other proteins, including the transcription factors (Johri & Mitra, 2001). These factors presumably interact with the auxin-response elements and regulate the expression of auxin-inducible or auxin-responsive gene families such as Aux/IAA gene, SAUR gene and ACS gene families which share similar promoters consisting of two auxin-response domains (Johri & Mitra, 2001). However, the auxin-response mechanism is more complex and yet not clearly understood.

The regeneration of the plants from leaf tissues of sugarcane in *In vitro* can occur through organogenesis, (Larkin, 1982; Chen *et al*., 1988) as well as embryogenesis (Falco *et al*., 1996) or by both organogenesis and somatic embryogenesis (Taylor *et al*.,1992) depending upon the culture conditions. The fresh callus on regeneration medium will develop shoots whereas on callus proliferation medium the same cultures develop somatic embryos. Regeneration of embryos into plants require regeneration medium. The regeneration system is the key for successful stable transformation of sugarcane in our laboratory.

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