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OPTIMIZATION OF THE PROTOCOLS FOR CALLUS INDUCTION, REGENERATION AND ACCLIMATIZATION OF SUGARCANE CV. THATTA-10

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Abstract

A very efficient protocol for the micropropagation of indigenous sugarcane cultivar Thatta-10 is presented here. The axillary buds and shoot apices taken as an explant were surface sterilized with 20% Sodium hypochlorite solution for 20 minutes followed by initiation on simple MS medium. The initiated plantlets were then used for the callus induction experiment in which high amount of calli (100%) were produced using 2,4-D in the concentration of 3.0 mg/L with MS medium. The induced calli were further evaluated for the regeneration. Medium comprised of MS basal salts and vitamins with BAP (6-Benzyl Amino Purine) in the concentration of 1.0 mg/L, was found best in terms of highest number of shoot regeneration (31.50±6.23). Maximum shoot elongation (10.52±1.88) alongwith the highest number of root emergence (6.51±2.41) was observed on the medium composed of MS salts and vitamins with GA (Gibberellic Acid) in the concentration of 3.0 mg/L. The *In vitro* grown plantlets were then transferred to green house conditions where they survived successfully with the rate of 96% (calculated after four weeks of transfer) when farm yard manure was used with garden soil in the ratio of 2:8 as a potting mixture.

Introduction

Sugarcane (*Saccharum officinarum*.L), belongs to the family Poaceae, is a tropical grass of high polyploidy (2n=36-170). Sugarcane accounts for approximately 70% of the world's sugar and is an economically important cash crop in tropical and sub-tropical region of many countries (Chatenet *et al*., 2001). Properties such as an efficient photosynthesis and efficient biomass production make this an excellent target for industrial processing and a valuable alternative for animal feeding and the production of by-products. It is also a prime candidate as a fuel crop (production of ethanol) because of its efficient biomass production. Some other byproducts from sugarcane include Molasses, Stock feed, Alcoholic drinks, Bagasses and cane wax etc (Gallo-Meagher *et al*., 2000).

Sugarcane is a clonally propagated crop from which multiple annual cuttings of stalks are typically obtained from each planting. This crop is especially vulnerable to diseases and propagation from cuttings facilitates the spread of pathogens and may results in epidemics (Schenck & Lehrer, 2000). Sugarcane stalks can be infected by various pathogens without exhibiting any symptoms, and therefore there is a high risk of disease transfer during exchange and transport of sugarcane cuttings (Parmessur *et al*., 2002).

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In Pakistan, sugarcane is grown in the three zones which include the tropical Sind, subtropical Punjab and the temperate Peshawar valley. It is an important and the second largest cash crop of Pakistan grown over an area of 963,000 hectares with an average yield of 47 tons/hectare, which is the lowest among all the sugarcane producing countries (Khan *et al*., 2006). Every year a large amount of our sugarcane crop is lost due to several factors like less availability of advanced techniques and damage to the crops by several bacterial, fungal and viral diseases which cause up to 70% of yield reduction (Parmessur *et al*., 2002).

Due to its global importance as an agricultural commodity, much research has been focused on sugar cane crop improvement through breeding and recently through biotechnological approaches like micropropagation (Hoy *et al*., 2003). The use of plant tissue culture techniques for the propagation of sugarcane has overcome many problems associated with the traditional breeding methods by ensuring the disease free multiplication of the plants and reducing the time period required for the multiplication (Khan *et al*., 2006).

The presented study was targeted to optimize a complete tissue culture protocol (callus induction, regeneration, multiplication and acclimatization) suitable for the micropropagation of disease and virus free plants of a sugarcane variety Thatta-10.

Materials and Methods

All the plant material was provided by the Pakistan Agricultural Research Council, University of Karachi, Pakistan. Axillary buds and shoot apices were taken as an explant. Sterilization was performed according to Khan *et al*., 2006, under Laminar Flow Cabinet.

The *In vitro* cultures of sugarcane cv. Thatta-10 were initiated on the simple MS medium, supplemented with 25gm/l sugar and 8% agar for solidification with pH value in between 5.75 to 5.80 before medium sterilization by autoclaving at 121^{o}C at 15 psi for 15 minutes. The sterilized explants were transferred to the medium in an upward orientation and the cultures were kept under 16 hours of photoperiod (1000 Lux) at 25±1 °C. The cultures were kept under the same conditions for at least two weeks.

For callus induction, 8 different media formulations were used. All the formulations comprised of MS (Murashige & Skoog, 1962) medium supplemented with different concentrations of IBA and 2,4-D (Table 1) with 25gm/l of sugar. Agar (8%) was used as a solidifying agent in all formulations. Media were autoclaved at standard temperature and pressure. The previously established *In vitro* grown cultures were used as a source for explants. *In vitro* grown shoots were transferred aseptically to all the media formulations (Table 1). The cultures were kept under complete darkness for four weeks and data were recorded on weekly basis. All the media trials were performed in 5 replicates with 3 explants per replication.

For the regeneration of plant from callus, 16 different media were analyzed (Table 1) which composed of MS (Murashige & Skoog, 1962) as a basal medium with different concentrations of NAA and BAP. Sugar was added in the concentration of 25 gm/l and 8% agar was used as a solidifying agent in all the formulations. Nearly equal amount of callus was transferred to all the media (in 5 replicates) and the cultures were kept under 16 hrs photoperiod provided by white fluorescent tube lights (1000 Lux) at 25±1°C for four weeks. Data on number of shoots were recorded after every week.

Table 1. Media formulations used in different experiments used in callus induction, shoot multiplication and shoot elongation experiment.

Shoot multiplication experiment			Callus induction experiment			Shoot elongation experiment		
Media codes	PGR (Conc. in mg/L)		Media codes	PGR	Conc. (mg/L)	Media codes	PGR	Conc. (mg/L)
	BAP	NAA						
S1	0.00	0.00	C1	IBA	1.00	E1		
S2		0.50	C2		2.00	E2	GA	0.00
S3		1.00	C3		3.00	E3		1.00
S4		2.00	C4		4.00	E4		2.00
S5	0.50	0.00	C5	2.4-D	1.00	E5		3.00
S6		0.50	C6		2.00			4.00
S7		1.00	C7		3.00			
S8		2.00	C8		4.00			
S9	1.00	0.00	C9	No PGR	.000			
S10		0.50						
S11		1.00						
S12		2.00						
S13	2.00	0.00						
S14		0.50						
S15		1.00						
S16		2.00						

The regenerated shoots were then transferred to shoot elongation medium. Different concentrations of Gibberellic acid (GA) were used (Table 1) to study their effect on shoot elongation. The multiplied shoots were than transferred to the shoot elongation medium and the cultures were kept under 16 hrs photoperiod of 1000 Lux for two weeks. The length of shoots were measured and recorded after every week. Same medium was used to induce roots and data against number of roots were also recorded after four weeks.

The sufficiently rooted, *In vitro* grown plantlets were then transferred to the green house conditions for acclimatization. For hardening purpose, most common and easily available potting mix was used which comprised of farm yard manure and garden soil in the ratio of 2:8 (v/v).

Results and Discussion

A very efficient and reliable protocol for the *In vitro* propagation of Sugarcane cv. Thatta-10 has been optimized. The results produced by the above experiments are discussed in the following sections.

For callus induction, 8 different media formulations were used (Table 1) and on the basis of our observations, media contained 3.0 mg/L of 2,4-D (C7) was found best for the induction of callus with 100% callus induction rate (Fig. 1). This response is same as many other plants because 2,4-D is the primary auxin which is used for the callus induction. The medium contained 2,4-D in the concentration of 4.0 mg/L (C8) also produced the same result but with much less regeneration potency as compared to C7 medium. Higher amount of this auxin may result in the loss the regeneration potential of the callus as observed in this experiment. The selected medium was found very much efficient and after three to four weeks, all the explants were fully covered (100%) with the light yellow mass of undifferentiated cells.

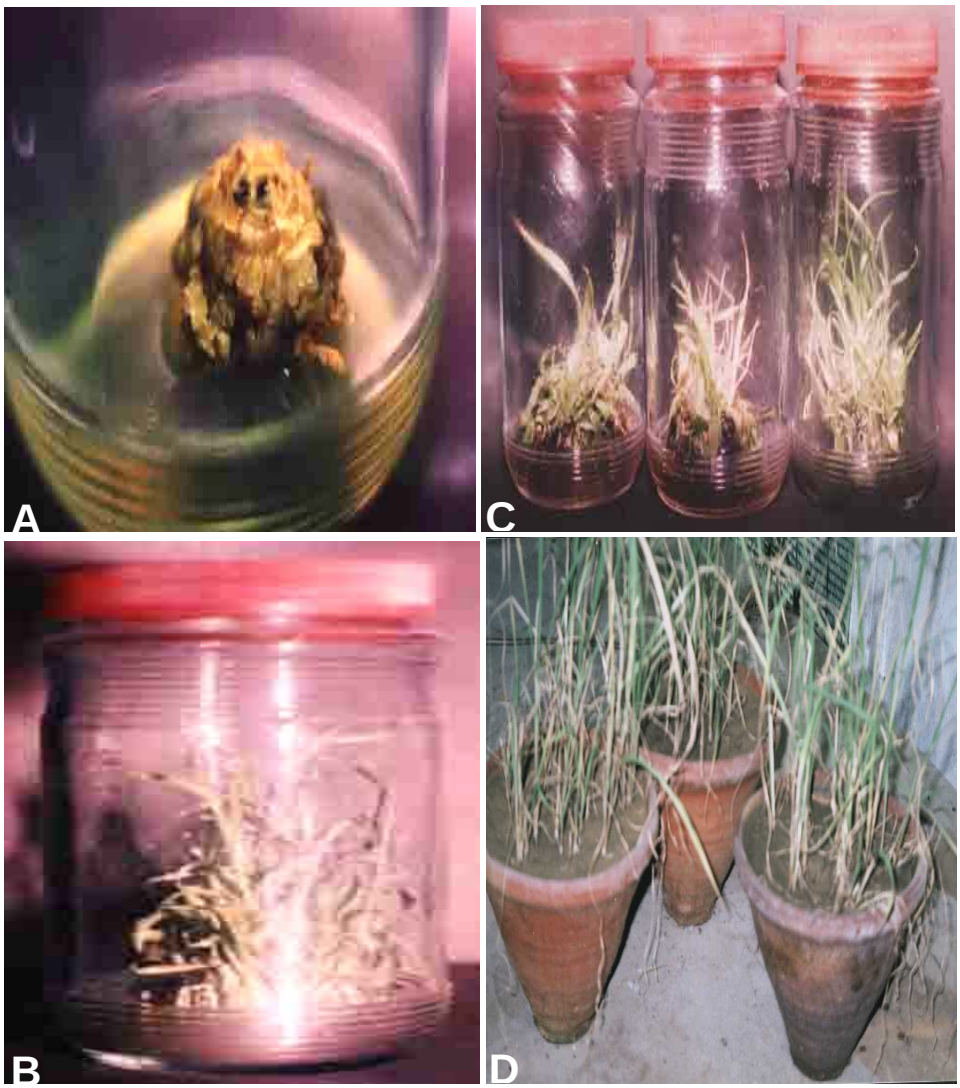

Fig. 1. Different stages of Micropropagation of sugarcane. (**A**) Initiation and callus induction. (**B**) Shoot Multiplication. (**C**) Root induction. (**D**) Acclimatization.

The calli produced were then evaluated for regeneration. In this regard 16 different media formulations were used (Table 1). A very high number of shoots emergence (31.50±6.23) was observed with 1 mg/L of BAP (S9) (Fig. 3). Our observations suggest that the use of both cytokinin and auxin, BAP and NAA respectively, have no synergistic effect on shoot regeneration. This high number of shoot formation also indicates that the induced callus had a very high regeneration potential. Fig. 2 also indicate that the higher concentration of BAP also produced nearly the same results but in order to reduce the overall cost of the protocol and to minimize the risk of variation caused by high concentration of PGR, medium with lower BAP concentration (S9) was selected.

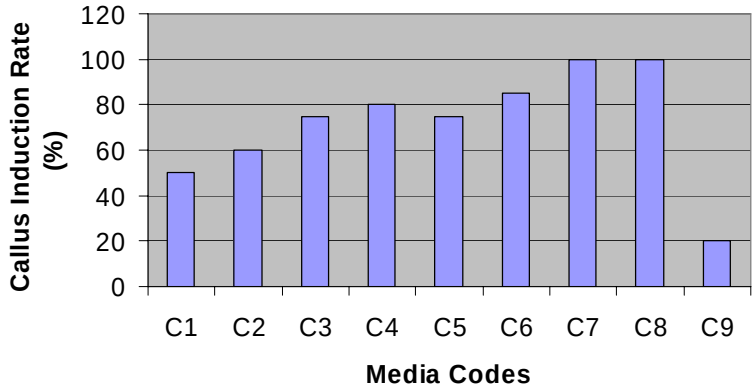

Fig. 2. Callus induction in sugarcane using different media formulations.

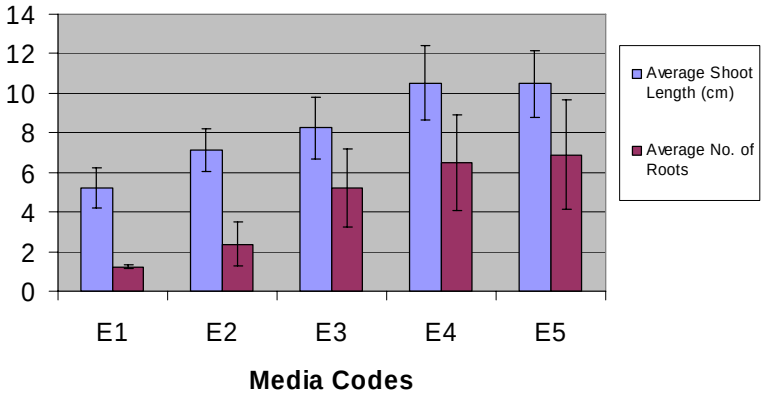

Fig. 3. Shoot elongation and root induction using different concentrations of GA.

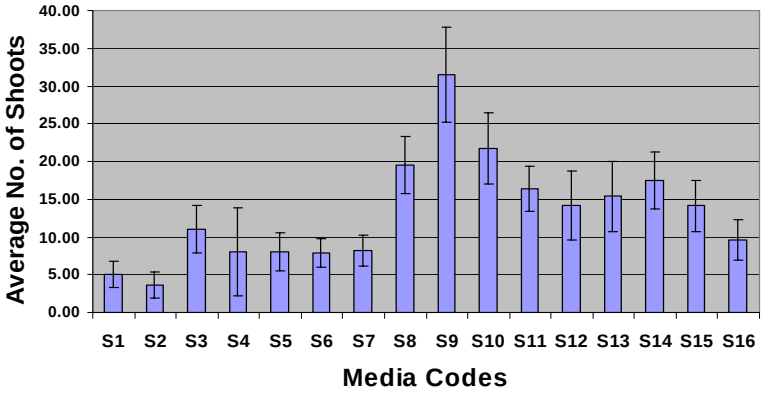

Fig. 4. Shoot regeneration of sugarcane using different media formulations.

Note: For media formulations see Table 1.

Gibberellic acid (GA) is involved in several important biochemical and morphogenetic responses which include the promotion of elongation in axial organs, such as stems and flower pedicels, along with the stimulation of root growth (Srivastava, 2005). The effect of GA on shoot elongation of *In vitro* grown sugarcane was evaluated in this study. Medium contained GA in the concentration of 3.0 mg/L showed maximum elongation (10.52±1.88) within two weeks. Sufficient numbers of roots were also developed (6.51±2.41) on the same medium (Fig. 2). Very limited data is available regarding the use of GA for the shoot elongation and root induction of *In vitro* grown sugarcane and hence the results obtained through this study are of high importance. The *In vitro* grown plantlets were then transferred to the green house conditions and a very low cost and easily available potting mix was used for the hardening purpose. The potting mix used for the acclimatization contained farm yard manure and garden soil in the ratio of 2:8 (v/v). The transferred plantlets perform well under these conditions with the survival rate of 96% (calculated after four weeks of transfer).

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