Plant Tissue Cult. & Biotech. **34**(2): 115-121, 2024 (December) **ISSN 1817-3721, E-ISSN 1818-8745**
DOI: https://doi.org/10.3329/ptcb.v34i2.78906

PTC&B

Direct *In vitro* Regeneration of *S*ugarcane var. China (*Saccharum officinarum* L.) Using Leaf-Sheath Explants

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Key words: Direct plant regeneration, *In vitro*, Leaf sheath explants, Sugarcane, MS

Abstract

An efficient and reproducible *in vitro* regeneration protocol was developed for sugarcane var. China using leaf-sheath explants. Direct multiple shoot formation without the intervention of callus from leaf-sheath was achieved on MS medium supplemented with 10.0 mg/l NAA. Ninety percent of the explants were found to induce shoots following a 90-day culture period. After 90 days of cultivation, each explant produced an average of 30.0 ± 2.50 shoots having a mean shoot length of 7.2 ± 0.50 cm on this medium. Within 30 days of being exposed to 1/2 strength of MS containing 2.50 mg/l NAA, 90% of the regenerated shoots produced roots. The average root length in this medium was 7.50 ± 2.30 cm, and there were 15.0 ± 1.25 roots produced from each shoot. Ninety percent of the *in vitro* raised plantlets were found to survive in the soil under a natural environment.

Introduction

Sugarcane is a large, sugar-rich grass that belongs to the Graminaceae family. Its stalk has the unique ability to retain sucrose, a crystalliable sugar. It is one of the most significant cash crops of several countries including Bangladesh and is used to make sugar, gur, and juice, among other everyday necessities. About 70% of the sugar produced worldwide comes from sugarcane (Arjumand et al. 2009). It is generally considered as a commercially significant crop in Bangladesh and many other tropical and subtropical regions of the world (Chatenet et al. 2001). It is also a well-liked export oriented product in many developing countries (Kambaska and Santilata 2009). Sugarcane also produced several effective byproducts are molasses, stock feed, alcoholic beverages, cane wax, and more. It is an important crop for the generation of ethanol fuel from biomass (Gallo et al. 2000). Three-budded sets are the vegetative propagation method for this perennial herb. However, the lack of a mechanism for quick seed

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multiplication, the lengthy breeding cycle for selection. Moreover, its poor yield is associated with infestation by fungi, viruses, bacteria and mycoplasma which reduces its yield by up to 70% and these are considered as the limitations of traditional methods of propagation of propagation (Imtiaz et al. 2006, Siddiqui et al. 1994, Xue and Chen 1994, Oropez et al. 1995, Bhavan and Gautam 2002).

It is reported that sugarcane acreage and production in Bangladesh have been reduced drastically over the past 20 years as the farmers are struggling to make a consistent profit from this crop. It is also understood that sugarcane sugarcane cultivation is very much associated with a superior quality of seed sugarcane production. Therefore, when compared to traditional methods, *in vitro* seed cane production is a suitable and effective system for rapid propagation. It is also an effective way to create genetic variants, performing *in vitro* mutagenesis, transformation of genetic material, conducting conservation, and exchange of germplasm internationally. Thus there is a continuous demand for the production of superior quality of plantlet production through *in vitro* techniques.

The techniques of plant tissue culture has been considered as a powerful tool for the improvement of crop (Carison 1975). Many researchers have developed protocols for sugarcane plant regeneration through *in vitro* technique using callus culture, axillary bud, shoot tip, and leaf sheath culture, addressing various cultivars (Lee 1986, Lee 1987, Hu and Wang 1983, Hendre et al. 1983, Nagai 1988, Milton and Alien 1995, Baksha et al. 2002, Roy and Kabir 2007, Gopitha et al. 2010). The aim of the current study is to create a reproducible procedure for *in vitro* mass propagation for sugarcane varieties and to create a possible approach for further research on *in vitro* mutagenesis and genetic transformation of sugarcane in order to address the issues of sugarcane cultivation with a desire towards the enhancement of sugar production.

Materials and Methods

The plant materials of sugarcane var. China used in this study were collected from the Sugar Crop Research Institute in Ishurdi, Bangladesh, and were allowed to grow in the research field adjacent to the Institute of Food and Radiation Biology, Atomic Energy Research Establishment, Bangladesh Atomic Energy Commission, Savar, Dhaka. The three-month-old leaf sheaths of the plant under study were taken as explants and sanitized in a laminar air flow cabinet under aseptic circumstances by rubbing them with 70% alcohol (v/v). The most common plant growth medium i.e. MS was utilized in this experiment.

The explants about 1.5 × 0.5 cm were then excised and inoculated on MS media supplemented with different concentrations of NAA alone and in combinations with BA + NAA for multiple shoot induction as well as maximum shoot proliferation. For root initiation, healthy shoots were removed one at a time and moved to half-strength MS-supplemented media containing varying amounts of IBA, IAA and NAA. Before

autoclaving, the pH was brought down to 5.8 and the medium were prepared using 3% sucrose (table sugar). White fluorescent tubes provided illumination for 16 hours at a rate of 21.8 mol/cm^2/s while the cultures were incubated at 25 ± 2°C. Following their removal from culture vessels, *in vitro*-grown plantlets were carefully cleaned to get rid of any remaining nutrient media, put to poly bags, and allowed to acclimate outdoors. Data were recorded on different parameters at 90 days for shoot induction and multiple shooting, while root induction at 30 days of the inoculation period was considered. Every other day, cultural observations were conducted. A completely randomized design (CRD) was used for the trials, with five explants per replication and three replications for each treatment. Every experiment was conducted twice. The recorded data was used for a descriptive analysis. The mean ± standard errors are represented by each value.

Results and Discussion

Browning of the cultured explants was witnessed within the inoculation period of 7 days. This might be brought on by the explants' phenol exudation and the circumstances of the medium type (Fig. 1b). Maximum multiple shooting was observed on MS + 10.0 mg/l NAA from the leaf sheath tissue within 90 days of inoculation period (Fig. 1c). Among the media used, at a concentration level of 10.0 mg/l NAA was found to be the most responsive and optimum, in which 90% of explants showed multiple shooting (Table 1).

Table 1. Effects of different concentrations of BA, NAA and BA + NAA with MS supplemented media on shoot formation from leaf sheath explants of sugarcane var. China at 90 days.

Plant Growth Regulators	Concentrations (mg/l)	% of explants forming shoots	Average number of shoot/explants Mean ± SE	Average shoot length (cm.) Mean ± SE
BA	0.5	-	-	-
	1.0	-	-	-
	1.5	-	-	-
	2.0	-	-	-
	2.5	20	2.0 ± 0.10	2.7 ± 0.30
NAA	2.5	-	-	-
	5.0	-	-	-
	7.5	50	5.0 ± 0.30	3.2 ± 0.40
	10.0	90	30.0 ± 2.50	7.2 ± 0.50
	12.5	40	4.0 ± 0.60	3.7 ± 0.70
BA + NAA	0.5 + 0.5	-	-	-
	1.0 + 0.5	-	-	-
	1.5 + 0.5	-	-	-
	2.0 + 0.5	-	-	-
	2.5 + 0.5	30	2.0 ± 0.10	2.0 ± 0.30

On MS media with 10.0 mg/l NAA, the average length of the shoots was 7.2 ± 0.50 cm and the average number of shoots per explant was 30.0 ± 2.50 (Table 1 and Fig. 1d). Shoot formation from various sugarcane explant tissues has been previously documented by numerous authors (Aftab et al. 1996, Gandonou et al. 2005, Heinz and Mee 1969, Tripathi et al. 2000). They demonstrated that different cultivars of sugarcane responses with different concentrations in single and in combinations of cytokinin and auxin for morphogenic responses. These results also consistent with the study reported by the authors (Imtiaz 2006, Sabaz et al. 2009, Roy and Kabir 2007).

Auxin concentrations had varying effects on the rooting responses of shoots grown *in vitro*. Ninety percent of the shoots rooted within 30 days of the inoculation period, indicating that NAA was the most responsive auxin and that 2.5 mg/l NAA demonstrated optimum response (Table 2). In the medium of 1/2 strength of MS + 2.5 mg/l NAA, an average of 15.0 ± 1.25 roots were formed per shoot, and the average length of the roots was 7.50 ± 2.30 cm (Fig. 1e). Aftab et al. (1996), Chen et al. (1998), Roy et al. (2011) and Kambaska and Santilata (2009) observed rooting from shoots using various auxin combinations and concentrations.

Table 2. Effects of IBA, IAA and NAA on 1/2 strength of MS media in root induction from *in vitro* raised shoots of sugarcane var. China at 30 days.

Plant Growth Regulators	Concentrations (mg/l)	% of shoots inducing roots	Average number of root induced / shoot (Mean ± SE)	Average root length (cm) Mean ± SE
IBA	0.5	-	-	-
	1.0	60	10.0 ± 0.70	4.5 ± 0.50
	1.5	40	6.0 ± 0.40	4.0 ± 0.30
	2.0	20	4.0 ± 0.20	4.0 ± 0.60
	2.5	20	3.0 ± 0.60	4.0 ± 0.70
IAA	0.5	-	-	-
	1.0	-	-	-
	1.5	20	2.0 ± 0.20	2.0 ± 0.20
	2.0	-	-	-
	2.5	-	-	-
NAA	0.5	20	4.0 ± 0.60	4.5 ± 0.20
	1.0	20	4.0 ± 0.20	4.5 ± 0.30
	1.5	70	7.0 ± 0.50	6.5 ± 0.60
	2.0	80	10.0 ± 0.90	7.5 ± 0.90
	2.5	90	15.0 ± 1.25	7.5 ± 2.30

By carefully washing the healthy *in vitro* raised rooted shoots under running water, the agar was removed from the culture vessels. To maintain moisture, the *in vitro* rooted plantlets were then placed in poly bags filled with a 2 : 1 soil and compost combination and covered with plastic films with a clear polyethylene top. After seven days, the plastic

films were removed. These results also show that rooting response also depends on competent media composition with the various genotypes of sugarcane and the type of explants used. The plantlets were misted twice daily and maintained in a shaded environment (Fig. 1f). After 30 days of adaptation, almost 90% of the plantlets started growing again in the field (Fig. 1g and 1h).

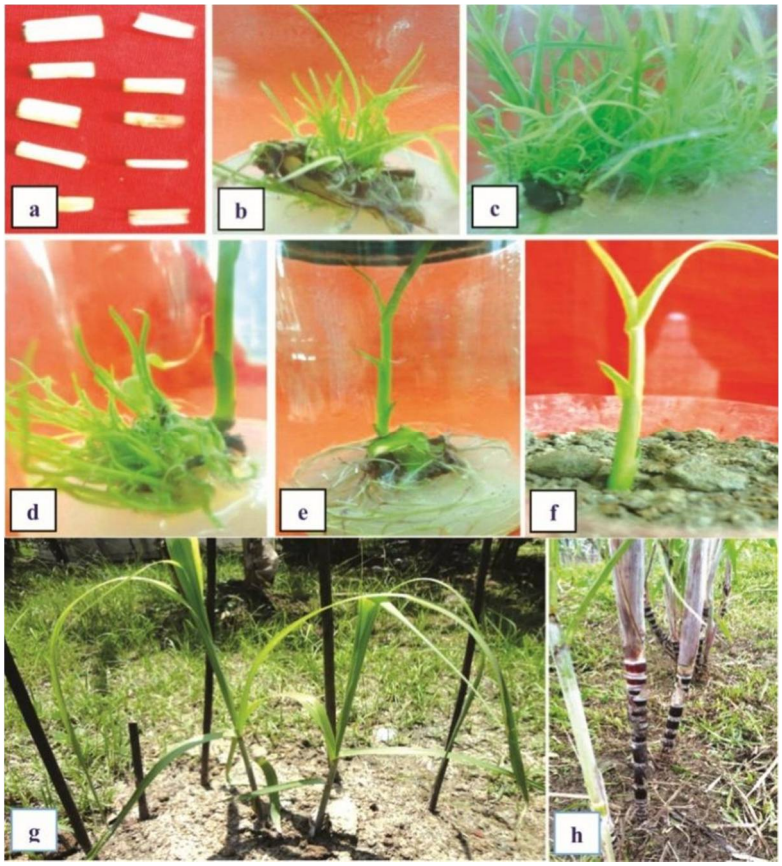

Fig. 1(a-h). Direct *in vitro* plant regeneration of sugarcane (*Saccharum officinarum* L.) var. China through leaf-sheath culture a) Leaf-sheath explants collected from the field of Plant biotechnology and genetic engineering Division, AERE, Savar, Dhaka. b) Direct shoots obtained from the explants on MS + 10.0 mg/l NAA after 30 days. c) Shoots proliferation occurred in the same medium after 90 days. d) Elongated and healthy shoot proliferation occurred after sub-culturing in the same medium. e) Root induction on 1/2 strength of MS + 2.5 mg/l NAA inoculation after 30 days. f) Fifteen days old *in vitro* raised plant resumed new growth in the polybag. g) Acclimatized sugarcane sowed in the field h) Mature *in vitro* raised sugarcane plant in PBGED, AERE experimental field.

Although the method outlined in this work is repeatable and provides disease-free propagules for conservation and international germplasm exchange, the country's requirement for enough elite clone planting material for commercial agriculture raises concerns.

According to this study, the most responsive and successful growth regulator for direct shoot induction of sugarcane var. china was NAA at a dosage of 10 mg/l, whereas the greatest results for root induction from shoots were observed at 2.5 mg/l. *In vitro* mutagenesis, genetic transformation programs, and the selection of beneficial soma-clonal variations of a variety of sugarcane cultivars could thus be facilitated by this procedure.

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(Manuscript received on 28 October, 2024; revised on 19 December, 2024)