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IN VITRO DIRECT PLANT REGENERATION FROM CULTURED YOUNG LEAF SEGMENTS OF SUGARCANE (*SACCHARUM OFFICINARUM* L.)

S. Ali, M. S. Khan* and J. Iqbal**

Department of Botany, GC University, Lahore. *CABB, University of Agriculture, Faisalabad. **School of Biological Sciences, University of the Punjab, Lahore.
Corresponding author:safdaralimirza@gmail.com

ABSTRACT

Rapid and efficient *in vitro* regeneration system that minimizes somaclonal variation is prerequisite for the genetic transformation and clonal mass propagation of commercial sugarcane varieties. Single step *in vitro* direct regeneration of sugarcane plantlets from immature leaf explants of sugarcane is reported through this research work. Transverse sections (1–2 mm thick discs) obtained from young leaf rolls, orienting with distal end facing the medium (directly in contact with medium) were critical for maximum plantlet regeneration. Adventitious shoot production occurred directly on the proximal cut surface of the explant. Shoot regeneration was observed as early as 3 weeks on MS medium supplemented with casein hydrolysate (500 mg/L) and different concentrations of BAP (0.5-2.5 mg/L) and NAA (0.1-1 mg/L). Fifty shoots or more could be generated from a single leaf disc segment in sugarcane variety SPF-213. These shoots regenerated roots on the same medium and successfully established after transplanted to pots. Large number of plantlets can be regenerated from single leaf roll rapidly, using this single step direct plant regeneration system. Greencane Seedlings, thus produced, have significant practical applications for clonal mass propagation of new cultivars in shorter period of time and with maximum genetic fidelity.

Key words: sugarcane, direct regeneration, greencane.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) as an important industrial crop of tropical and subtropical regions is cultivated on 20 million hectares in more than 90 countries because of its high trade value (Naz, 2003). It accounts for around 70% of the world's sugar (Khan *et al*., 2004).The importance of sugarcane has increased in recent years because it comes to be a good candidate as industrial raw material for sugar allied industries such as acetic acid, paper, plywood, industrial enzymes and animal feed and as source of renewable energy (De Oliveira *et al.,* 2005). Sugarcane is among the largest cash crops of Pakistan with the country's sugar industry solely dependent on the fate of this crop (Khan *et al*., 2005), all the efforts concerning the improvement in economic traits/increase in sugarcane crop production are of major importance.

The unavailability of very specific climate for flowering of sugarcane in Pakistan hinders the conventional breeding for incremental improvement of traits. Moreover the absence of certain economic traits from the genetic pool of sugarcane like insect and herbicide resistance requires adoption of genetic transformation as means of improvement. Usually due to lack of multiplication procedures, it requires 10 to 15 years to get an improved variety for commercial cultivation. The time spent for this multiplication is considered a serious economic problem, mainly in view of the higher yields that would be obtained by planting the new variety earlier on a large commercial scale (Biradar *et al.,* 2009), because the propagation of improved or modified clones of sugarcane through sets is very slow, usually 1–10 in a year, this is a major hurdle in the rapid spread of improved varieties. During vegetative propagation, the biotic and abiotic stresses generation after generation results in the decline of the varietal vigor (Sani and Mustapha, 2010). In this regard, the innovative approaches of cell and tissue culture possess significant promise for successful sugarcane clonal propagation by controlling a lot of problems which are faced during conventional breeding practices and multiplication procedure. In general, the use of callus based systems often cause genetic instability and lead to somaclonal variation also called tissue culture-induced variations to more specifically define the inducing environment (Kaeppler *et al.,* 2000). Genetic and phenotypic variation among clonally propagated plants of a single donor clone is not required when purpose is only clonal propagation in plants like sugarcane. Thus intervening callus phase is not desirable during micro-propagation of improved clones. Therefore in the present work we report the development of an efficient and rapid single step *in vitro* method for clonal propagation through induction of direct plant regeneration without intervening callus phase, from cultured young leaf segments of sugarcane.

MATERIALS AND METHODS

Procurement of germplasm: Two elite varieties SPF-213 and HSF-240 were used. Germplasm was procured in form of sugarcane tops with immature leaf rolls excised from nine months old field grown sugarcane plants, provided by Sugarcane Research Institute, Faisalabad.

Culture Medium: MS (Murashige and Skoog, 1962) medium was used as basal culture medium with supplementations such as CH (500 mg/L) and different concentrations of 2,4-D, BAP (0.5-2.5 mgl/L) and NAA (0.1-1 mgl/L) for each genotype.

Explant preparation and Culture: The outer whorls of mature leaves from cane tops were removed. Explants were washed thoroughly under running tap water for 10 min. followed by treatment with a few drops of tween-20 (Polyoxyethylene sorbitan monolaurate) for one min. with constant shaking by hand, followed by three successive washings again with distilled water. The surface sterilization was carried out with 0.1% $HgCl_2$ for 2 minutes with gentle shaking. After thorough washing with sterile distilled water, these surface sterilized immature leaf rolls 12 to 15cm long and more or less 1cm in diameter were used in *in vitro* culture experiments. The outer two to three whorls of leaves were removed and the innermost whorls, the immature leaf rolls were cut into transverse sections/segments (Ali & Iqbal 2012). The discs obtained from immature leaf rolls were aseptically transferred/cultured on Murashige and Skoog (1962) medium solidified with agar (40 ml in 150 ml jars).

Culture conditions: The pH of the medium was adjusted to 5.8 prior to all autoclaving. The cultures were incubated under white florescent light with intensity varying from 2000-3000 lux/m^2/s and 16/8 h light/dark regimes at 27°C ±1 (Ali *et al*,. 2010)

***In vitro* plantlet regeneration:** The basic MS medium as culture medium was supplemented with casein hydrolysate (CH) (500 mg/L) and growth regulators such as 2,4-D, BAP and NAA in different concentrations and combinations for induction of direct and indirect regeneration and multiplication of shoots and root induction in *in vitro* regenerates of sugarcane varieties under focus.

Hardening and acclimatization: Plantlets with well developed root system were separated, washed properly under running tap water to remove any adherent gel and transferred to Enamel trays containing peat moss covered with transparent polythene sheet to ensure high humidity and irrigated with tap water daily. Hardening and acclimatization was done under diffuse light conditions (16:8 h photoperiod). Polythene sheet was removed gradually in order to acclimatize plantlets, after 4 weeks they were successfully transferred to earthen pots containing garden soil and maintained in green house under normal day length conditions.

Data collection and statistical analysis: All the experiments were conducted in triplicate with 100 explant segments per treatment. Data for plantlet regeneration was recorded after 8 weeks of culture. The data were expressed as a mean ± SE of three repeated experiments.

RESULTS

Response of tissue type to growth regulators for regeneration:

Direct and Indirect Regeneration of Shoots: Different media tried in this research on DR-1,2 and 3 medium, solidified with agar, the immature leaf discs of 1-2 mm size showed swelling of the explant in the first few days of culture. The immature leaf segments on DR-2 medium oriented with distal end facing the medium, initiated development of primordia from their cut ends in sugarcane variety SPF-213 after 2 weeks of culture without intervening callus phase. During the fourth week of incubation, cultured leaf segments exhibited direct shoot regeneration which at the age of five weeks developed into prominent shoots (Fig. 1B) and the shoot development was maximum i.e 80 percent (Table 1). These directly regenerated shoots start developing roots on the same medium after sixth week. The same shoot development was observed on DR-1 medium and DR-3 medium in SPF-213 with the difference that the shoot development was delayed by more or less one and a half week and the percentage of shoot development was much less as compared to DR-2 medium (Table 1). The only medium found effective for direct regeneration of shoots in HSF-240 was DR-2 medium and the maximum frequency of shoot regeneration was 10 percent. In addition, several other media were used to compare regeneration efficiencies, containing MS salts supplemented with different concentrations and combinations of plant growth regulators. In cultures where negative factors were eliminated, shoot regeneration reached up to 70 shoots on the DR-2 medium from each leaf disc section of sugarcane variety SPF 213. Depending on the varieties used, direct regeneration varied (SPF 213 ≥ HSF 240) on culture medium DR-2 (MS salts supplemented with 1.5mg/L BAP and 0.5 mg/L NAA). The leaf discs orienting with proximal end facing the medium were unable to respond for direct regeneration. It was interesting to note that maximum number of shoots that regenerated from one leaf segment were 50–70 in number.

Among different media tried for regeneration in these varieties IDR-1 and IDR-2 (Table-1) were found effective for indirect shoot regeneration via callus intervening phase. The time span to develop shoots was

longer by five weeks. The effect of IDR-1 and 2 was found vice versa in SPF-213 and HSF-240 for shoot induction percentage (Table 1).

Root Induction: During the sixth week of incubation, profuse rooting occurred from the base of shoots in DR-2 medium, complete separable plantlets were recovered during the 8th week of incubation, the recovery of complete plantlets was late in DR-1 and DR-3 medium by two weeks.

The shoots on IDR medium developed roots when transferred to half strength simple MS medium solidified with agar. Roots developed more conveniently from shoots on MS medium supplemented with NAA at levels of 4-5 mg/L along with 500 mg/L CH.

Hardening and Acclimatization of Regenerated Plantlets: Rooted shoots when removed from the culture jars and transferred to Enamel trays continued to survive and did not wilt under polythene cover. During hardening of plantlets secondary and tertiary roots were seen to develop. Hardened plantlets transferred to polythene bags in the greenhouse were healthy and green and 80-90% survival was recorded.

Table 1. Response of sugarcane varieties to Regeneration.

Sugar-cane Variety	Number of Leaf (discs) Employed	Medium Code with Growth Regulators/ Concentration	Direct Regeneration Frequency	Indirect Regeneration Frequency
SPF-213	100	DR-1: MS salts, MS vit., CH (500 mg/L), BAP + NAA (1+ 0.5 mg/L)	10 ± 0.0	---
		DR-2: MS salts, MS vit., CH (500 mg/L), BAP+NAA (1.5+0.5 mg/ L)	80 ± 0.8	---
		DR-3: MS salts, MS vit., CH (500 mg/L), BAP + NAA (2+ 0.5 mg/L)	10 ± 0.0	---
		IDR-1: MS salts, MS vit., CH (500 mg/L) 2,4-D (0.5 mg/L)	---	80 ± 0.8
		IDR-2: MS salts, MS vit., CH (500 mg/L) 2,4-D (1 mg/L)	---	60 ± 0.8
HSF-240	100	DR-2: MS salts, MS vit., CH (500 mg/L), BAP+NAA (1.5+ 0.5 mg/L)	10 ± 0.0	---
		IDR-1: MS salts, MS vit., CH (500 mg/L), 2,4-D (0.5 mg/L)	---	60 ± 0.8
		IDR-2: MS salts, MS vit., CH (500 mg/L), 2,4-D (1 mg/L)	---	85 ± 0.8

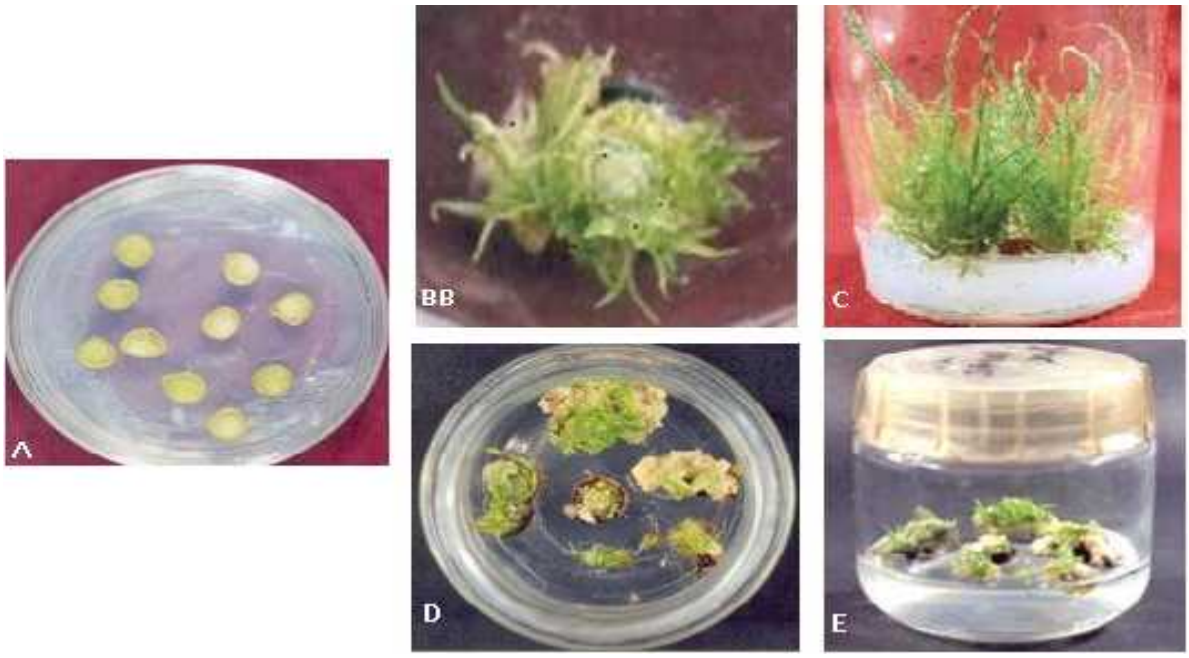

Fig. 1. One step direct regeneration and indirect regeneration in sugarcane. A) Transverse sections (1–2 mm thick discs) of SPF-213 obtained from young leaf spindle rolls, orienting with distal end facing the medium, one day old culture (1X). B) Direct shoot regeneration from single leaf disc of SPF-213 on DR-2 medium, culture of five weeks age (3X). C) Shoot proliferation and root induction on the same DR-2 medium at eight weeks age (1X). D) Leaf discs of SPF-213 in Petri plate showing indirect shoot regeneration on IDR-1 medium, cultures at the age of (1X) E) Leaf discs of HSF-240 in jar showing indirect shoot regeneration on IDR-2 medium (1X).

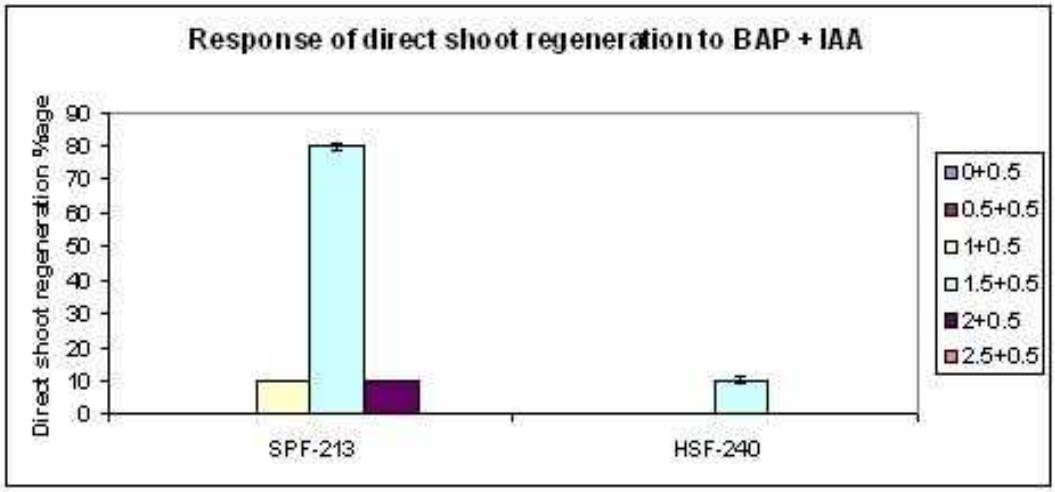

Fig. 2: Bar graph showing response of direct shoot regeneration to different concentrations of BAP with 0.5 NAA.

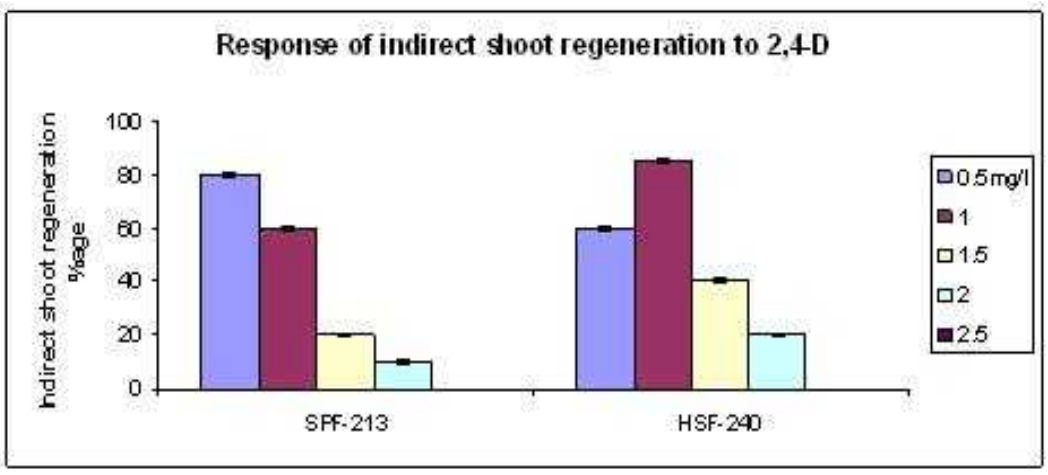

Fig. 3. Bar graph showing response of indirect shoot regeneration to different concentrations of 2,4-D.

DISCUSSION

In vitro plant regeneration is often the most important step for successful implementation of various biotechnological strategies for crop improvement. In sugarcane, there are many reports available on regeneration and multiplication via callus formation. In these studies, embryogenic calluses were induced in the presence of 2,4-D or picloram and regeneration was obtained either by reducing the concentration of the auxin or deleting it from the medium (Mozdhorst *et al*., 1997; Snyman, 2000; Sahasrabudhe *et al*., 2000; Eudes *et al*., 2003) or by media supplementation with thidiazuron (Gallo-Meagher *et al*., 2000), while there are only few reports dealing with direct regeneration from different explants (Gill *et al*., 2006), A simple, fast and efficient protocol for i*n vitro* SSDR of plantlets from immature leaf explants of sugarcane is reported without the intervening callus phase, thus exclusive of the occurrence of somaclonal variations.

In this study, using a transverse thin cell layer culture system, there have been identified some of the developmental constraints that limit high-frequency of regeneration in sugarcane leaf tissue. Tissue polarity and consequently the orientation of the explant in culture, size of explant, and concentration and growth regulators play a significant role in determining the direct regeneration potential of leaf tissue eliminating callus phase in *in vitro* culture. The leaf discs orienting with proximal end facing the medium were unable to respond for direct adventitious shoot production/regeneration and only the leaf discs facing the distal end to the medium were able to respond to direct regeneration. Direct adventitious shoot production occurred on the proximal cut surface of the explant. Generally decrease in regeneration gradient was observed from the basal to the distal end in the leaf roll, the addition of NAA to the culture medium, reduced this spatial developmental constraint as described by Lakshmanan *et al.* (2006) that transverse sections /leaf roll discs orienting with distal end facing the medium

(directly in contact with medium) were found critical for maximum regeneration.

We identified proper combinations of growth regulators for the induction of direct regeneration of plantlets in sugarcane varieties concerned. Many different media were used for SSDR studies (Table 1). The developmental pattern that the regenerating tissue of an explant will follow is determined by exogenously applied growth regulators. Application of BAP in induction of multiple shoots on shoot tip explant of sugarcane has established its role (Birader *et al.,* 2009). Combination of auxin and cytokinin with particular importance of the auxin and cytokinin types and their balance (Table 1) played a critical role to permit direct shoot induction, the addition of BAP + NAA to the MS medium was necessary for direct regeneration (Behera and Sahoo 2009). This response was greatly affected by the concentration of growth regulators in the combination in the nutrient medium employed. In this study, the best results were obtained in the presence of 1.5 mg/L BAP + 0.5mg/L NAA. The positive effect of plant growth regulators of BAP and NAA, especially in combination may be due to the fact that this combination may have changed the balance of endogenous growth regulators and thus enhanced direct shoot regeneration. The ease and simplicity of the SSDR protocol lies in the fact that shoot elongation and root induction did not require separate media and induction of direct regeneration from immature leaf segment yielded a large number of plants in a short time (Gill *et al*., 2006). Healthy and well-grown rooted plants were obtained within eight weeks. The type of combination (BAP + NAA) of growth regulators used in the medium had a marked effect on shoot proliferation also. The regenerated plants were transferred to plastic pots for acclimatization and into the field for evaluation. The regeneration of sugarcane plants via direct organogenesis from transverse thin layer sections of leaves is currently the most efficient method of achieving fast and large scale production of disease free verities (Lakshmanan *et al.,* 2006) Micropropagated sugarcane plants are disease free, vigorously growing, and superior to seed cane's yield and sugar recovery under field agronomic practices. (Sood *et al*., 2006).

Plants obtained through *in vitro* cultures on IDR-1and 2 medium (Table 1) were with intervening callus phase showed phenotypic variability which may be due to true genetic changes (Orton, 1980) as was obvious through limited RAPD studies (data not shown). Aneuploids may have lower or higher number of chromosome and plantlets regenerated from these cells could express different genetic behaviour. Direct regeneration method enables the exploitation of existing genetic heterogeneity present within cells in the form of different cytotypes. For an efficient application of the direct regeneration system, according to Lakshmanan *et al.,* 2006, regeneration of plants directly from explants presents an effective strategy to avoid or substantially reduce somaclonal variation as was clearly shown by the clones regenerated through SSDR. Our primary objective was to develop a simple and reproducible protocol for shoot regeneration directly from the explant tissue excluding the callus phase. RAPD studies (data not shown) carried out on limited scale to investigate the genetic variation in sugarcane plantlets derived from SSDR method supported the hypothesis of genetic stability found in directly regenerated clones (Pandey *et al.,* 2012). However the SSDR protocol is genotype specific due to the variable genotype of the different varieties.

In conclusion, in the present investigation, culturing of leaf discs from young leaf roll was found to be a quick, effective and reproducible procedure for establishing *in vitro* culture in order to use either for micropropagation/rapid clonal propagation of desired sugarcane genotypes.

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