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Appraisal of nutritional status and *in vitro* mass propagation of sugarcane (*saccharum officinarum* l. Cv. Us-633) through callus culture

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Abstract: In present investigation, protocol was established for callus induction, maintenance and regeneration of sugarcane cultivar US-633. Inner leaf sheath was used as an explant for callus induction. It was observed that the best medium for callus induction and maintenance was MS medium supplemented with 2.5mg/L 2, 4-D. Callus cultures after 30days showed regeneration. The small regenerated shootlets were transferred to multiplication medium where BAP in combination with GA_3 depicted best response having average number and length of shoot was 13.00±0.49 and 20.33±0.12cm, respectively. The regenerated shoots were transferred on to the MS basal media supplemented with different concentrations of NAA alone and in combination of IBA for root induction. In case of root development 0.5mg/L NAA in combination with 1.0mg/L IBA showed good response towards root formation having maximum average number and length 19.00± 0.12 and 2.5± 0.01cm respectively. Rooted shoots were acclimatized and shifted to the field with survival rate of 80%. Nutritional status and chlorophyll contents of *in vitro* grown, *in vitro* grown acclimatized and field grown sugarcane plants of variety US-633 were also estimated during present study. The proposed technique can be used to enhance mass propagation of sugarcanes variety US-633 economically especially with the present trend of demand of sugarcanes in the region.

Keywords: Callus, BAP, Sugar cane, Indole butyric acid (IBA), Chlorophyll, Proximate, 2, 4-D.
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INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is one of the most efficient converters of solar energy into sugars and other renewable forms of energy. It is an important industrial cash crop of Pakistan and ranks fourth in acerage after wheat, cotton and rice[1]. It is an economically important, polysomatic, highly heterozygous, vegetatively propagated crop that accounts for more than 60% of the world's sugar production[2]. Sugarcane is a perennial herb and belongs to family *Graminae*. It is mainly propagated by vegetative means sets having three buds each, and a low multiplication rate (1:6 to 1:8) due to which seed material production of newly released varieties is invariably slow[3]. Further, the vegetative seed accumulates diseases and pests during several cycles of field multiplication. Hence, non-availability of disease-free, true to type planting material is a major constraint in improving sugarcane productivity.

Plant tissue culture techniques have become a powerful tool for studying and solving basic and applied problems in conventional breeding and propagation procedures[4]. Micropropagation through exploitation of tissue culture technique provides means of producing uniform high quality, disease-fee seed at a fairly faster rate. Development of tissue culture technology for rapid multiplication of disease free planting material has been an important step towards quality seed production of sugarcane. Standardization of protocols for *in vitro* multiplication of sugarcane through leaf sheath callus culture, axillary bud and shoot tip culture have been reported by many authors[5-9]. But the nutritional requirements for *in vitro* culture vary according to genotype as well as explants used[3].

The present communication demonstrates an effective high frequency regeneration method which allows for expedient multiplication of plantlets that are developed and established *in vitro* through callus culture initiated from inner soft leaf sheath used as an explant. Comparative nutritional status of *in vitro* grown, acclimatized sugarcane plantlets and field grown sugarcane plants, was also explored in current study. US- 633 was selected for this study based on its early maturity, tremendous sugar content and resistance to pests in Punjab area of Pakistan[10-11].

MATERIALS AND METHODS

Source of Explants

Sugarcane variety US633 was investigated in present study conducted from January-2010 to April-2011. Certified plant material was obtained from Ayub Agriculture Research Centre, Faisalabad and grown in the botanical garden of PCSIR Labs Complex, Lahore. Green tops were collected from 3-4 months old healthy plants of sugarcane var. US633 growing in the botanical garden of PCSIR Lahore. For explants preparation, 4-6cm long segments (above the growing point) along with young leaves were dissected removing with 5-6 outer leaves and washed under running tap water for 10 minutes.

Surface sterilization of sugarcane explants

After washing young leaves were surface sterilized by dipping in 20% Clorox (commercial bleach containing 5.25% v/v sodium hypochlorite) for 15 minutes followed by 3minutes immersion in 0.1% mercuric chloride solution containing 2-3 drops of tween-20 per 100mL. These explants were washed thrice with autoclaved distilled thereafter. The

immature folded leaves, pale yellow in color were exposed for inoculation onto semi-solid MS medium[12] supplemented with 3% sucrose along with various combinations of plant growth regulators mentioned where used) discarding the outer most whorl of leaves. Ascorbic acid (1.0mg/L) was added in the medium to control the browning of the explants due to production of phenolic compounds. The pH of the medium was adjusted to 5.8 using 0.1N HCl/NaOH prior to the addition of phytagel (2g/L bacteriological grade, Sigma) the media was homogenized by cooking it on a hotplate dissolving gelling agent. The prepared media was then dispensed in the culture tubes and autoclaved for 20 min at121ºC at 15lbs psi pressure. All the cultures were incubated in a culture room provided with a 16h photoperiod of cool white fluorescent light (3000 Lux) and the temperature was maintained at 25±1ºC with 70-75% relative humidity in the culture room. Data was recorded weekly. Each experiment consisted of 5 replicates.

Callogenesis medium

For callus induction MS[12] basal medium supplemented with different concentrations of 2, 4-dichloropenoxy acetic acid (1.0-3.5mg/L) were tried. Calli were regenerated after 30 days of incubation period under controlled conditions.

Proximate analysis

Nutritional analysis of *in vitro* grown plantlets, plants after acclimatization and field grown plants of sugarcane cultivar US-633 were carried out according to standard methods of A.O.A.C (1990)[13]. The parameters analyzed during present study were moisture, ash, crude fibre, and nitrogen. All the tests were carried out in triplicate

Chlorophyll determination

Chlorophyll content present the in leaves from all three categories of sugarcane cultivar US-633 plants (*in vitro* grown, acclimatized and field grown plants) were determined spectrophotometrically using standard procedure described earlier[14]. 100mg of crushed sugarcane leaves were dipped in to 10mL DMSO and incubated at 4ºC for 72 hours. After incubation period filter and 3mL of the extract will be used to read the absorbance at 642 and 660nm on a spectrophotometer (UV/vis.spectrophotometer, Hitachi 220S). Chlorophyll levels (expressed in mg/L) were calculated using the equations reported by Arnon (1949)[15] and is given thus:

$Chl.a = 12.7A_{660} - 2.69A_{642}$

$Chl.b = 22.90A_{642} - 4.68\ A_{660}$

$Total\ Chl. = 20.2\ A_{642} + 8.02\ A_{660}$

Statistical analysis

Results are presented as means±SD.

Shoot multiplication medium

The regenerated shoots were cultured on MS medium supplemented with different concentrations of BAP (0.5-3.0mg/L), Kinetin (0.5- 3.0mg/L) alone and in combination with GA_3 (1.0mg/L) for multiple shoot regeneration.

Rooting medium

Elongated shootlets measuring about 5-6 cm in length were excised aseptically from bunch of shoots growing in the culture tubes and transferred to MS medium supplemented with different concentrations of IBA (0.5-3.0mg/L) and NAA (0.5-3.0mg/L) individually or in combinations.

Acclimatization

Plantlets with well-developed root system were removed from the culture tubes to transfer them to plastic bags for hardening containing autoclaved defined rooting mix under a high humidity environment. The plastic bags containing plantlets to be hardened were maintained undisturbed under controlled light, temperature and high humidity for one week in the growth chamber. After first week plants were irrigated with 1/4 MS basal salt solution devoid of sucrose and every 5 days thereafter for two weeks and with water for one week. After 30 days the plantlets were transferred in to the soil in field condition.

RESULTS AND DISCUSSION

Callogenesis and caullogenesis

Effect of growth regulators on callogenesis as well as caullogenesis in sugarcane cv. US-633 was investigated in this study. Results pertaining to callogenesis are shown in table 1.

Table 1: Effect of 2, 4-D on callogenesis from folded leaf sheath explants of sugarcane cultivar US-633.

Growth regulators (mg/L)	No of explants showed callusing (%)	% of explants with callus induction	Callus characteristics		
			Color	Texture	Growth
1.0	2.00	40.0	Brownish black	Compact granular	++
2.0	3.00	60.0	Greenish brown	Compact granular	++
2.5	5.00	100	Greenish brown	Compact granular	++++
3.0	4.00	80.0	Greenish brown	Compact granular	+++
3.5	1.00	20.0	Brownish black	Compact granular	+

+ = Poor, ++ = Good, +++ = Very Good, ++++ = Excellent

Callus induction was observed within two weeks in explants grown on all investigated combinations of 2, 4-D (1.0-3.5mg/L). Cut ends of the inner leaf sheath became swollen and started to produce tiny buds after 15 days of culture. During present study the maximum callus growth was observed on medium containing 2.5mg/L of 2, 4-D with full

potential of shoot regeneration having greenish white color and compact granular texture (Table 1). Current results are in accordance with the earlier findings[16] who has reported callus induction in all explants at 2.5 mg/L of 2, 4-D supplied media for the sugarcane cultivar Nayana. Whereas present findings contradict to the Begum *et al.* (1995)[17] who found that higher quantity of 2, 4-D i.e 3.5mg/L favored highest percentage of callus induction from leaf base explant in Nagabari variety of sugarcane in Bangladesh on the contrary US-633 showed moderate callus growth on medium supplied with the same quantity of 2, 4-D, in addition to the fact that lesser callus was produced it was observed to be having less regeneration potential as compared to 2.0, 2.5 and 3.0 mg/L of 2, 4-D. This study showed that a moderate amount of 2, 4-D is more suitable for callus induction, its further growth and regeneration in US-633.

As depicted in table 2, caullogenesis was observed after 30 days growth period in greenish brown calli (shown in Figure 1A) originated from explants grown on medium containing 2.0-3.0mg/L 2, 4-D. Maximum number of shoots that is 12±0.3 was observed in cultures supplied with 2.5mg/L of 2, 4-D in the medium. Lower as well as higher levels of 2, 4-D were found to be less or not supportive for shoot regeneration as shown in table-2. It is observed during this study that explants growing on MS medium containing lowest (1.0mg/L) as well as highest (3.5mg/L) level of 2, 4-D present in the medium showed poor growth in terms of callus formation as shown in table-1 and no regeneration which is reported in table 2.

Table 2: Effect of 2, 4-D on morphogenetic potential of calli originated from sugarcane leaf sheath.

S. #	Growth regulators (mg/L)	% of calli with shoot regeneration	Callus color	Morpho-genetic potential	Shoots regenerated
1	1.0	0.00	Dark brown	Nil	_
2	2.0	40.0	Greenish brown	Shoots regeneration	6.0±0.51
3	2.5	100	Greenish brown	Shoots regeneration	12.0±0.3
4	3.0	60.0	Greenish brown	Shoots regeneration	8.0±0.24
5	3.5	0.00	Dark brown	Nil	_

In vitro shoots multiplication

Different hormonal combinations attributed significant impact on multiplication of micro shoots regenerated from callus. Among different concentrations and combinations of BA/Kin alone or combined with GA_3 for shoot multiplication of regenerated buds were tried results are shown in table 3. These experiments revealed that BA or Kin alone are less effective for shoot proliferation as compared to their cumulative effect when supplied in various ratios as presented in table 3. Combination of BA (2.0 mg/L) and GA3 (1.0mg/L) supplemented in MS medium was observed to be the most favorable among all concentrations/combinations tried with respect to the number of tillers produced as shown in table 3. In this case the average number of tillers was found to be 13.00±0.49 with mean length of the shoots 20.33±0.12cm of variety US-633 (Figure 1B, table-3). These observations indicated that the ratio of the cytokinns and GA_3 played an important role in sugarcane shoot proliferation. Sabaz *et al.* (2008)[4] reported that BA in combination with GA_3 is good for shoot multiplication of sugarcane as compared to Kin and GA_3. Present study also revealed that BA alone or in combination with GA_3 is more effective as compared to Kinetin alone or combined with GA3 (table 3). Present results are supported by the findings reported by Karim *et al.,* (2002)[18] who reported that BA played efficient role in shoot multiplication of sugarcane as compared to Kinetin. Islam *et al.,* (1982)[19] also reported the positive effects of BA combined with an auxin on shoot formation in sugarcane. Previous studies on different cultivars of sugarcane concluded that regeneration potential of callus was specific and genotype dependent phenomenon, furthermore hormonal concentration and combinations are equivalently influential for regeneration and multiplication[20]. Callus induction, proliferation and regeneration potential in sugarcane exhibited synchrony to each other[21].

Table 3: Effect of cytokinin (BAP, Kin) and GA_3 interaction on shoot multiplication of sugarcane variety US-633.

Hormonal supplements (mg/L)			Average shoot length (cm)	Average No. of tillers
BAP	Kin	GA_3		
0.5	_	_	4.5±0.05	5.00±0.37
1.0	_	_	6.0±0.03	7.00±0.11
2.0	_	_	9.53±0.21	9.00±0.04
3.0	_	_	3.50±0.13	4.00±0.06
_	0.5	_	3.11±0.62	2.00±0.43
_	1.0	_	4.90±0.39	5.00±0.21
_	2.0	_	7.21±0.22	7.00±0.11
_	3.0	_	3.50±0.94	3.00±0.04
0.5	_	1.00	12.00±0.07	9.00±0.31
1.0	_	1.00	16.10±0.04	10.00±0.43
2.0	_	1.00	**20.33**±0.12	**13.00**±0.49
3.0	_	1.00	9.50±0.15	7.00±0.07
_	0.5	1.00	10.33±0.23	6.00±0.23
_	1.0	1.00	11.03±0.25	8.00±0.21
_	2.0	1.00	12.55 ±0.16	9.00±0.04
_	3.0	1.00	8.90±0.12	5.00±0.31

In vitro rooting and acclimatization

Different types of auxins were used at different concentrations and combinations to induce adventitious roots in micropropagated shoots.

Optimal rooting was observed in MS medium supplemented with 0.5 mg/L NAA and 1.0 mg/L IBA (Table 4) and the highest number of roots observed per shoots was recorded as 19.00± 0.12, which took only 6-15 days for initiation of root primordial with average root length 2.5± 0.01cm for the variety US-633 (shown in Figure 1C and Table 3). In present study low concentrations of NAA alone or in combination with IBA was found to give better response for root formation in the shoots of sugarcane variety US-633. These results do not conform to the findings of Mamun *et al.,* (2004)[22] who obtained best response for rooting on MS medium supplemented with auxins NAA in combination with IBA in a balanced ratio i.e. 0.5 mg/L each. Similarly Sabaz *et al.,* (2008)[4] used 1.0mg/L IBA alone as the best root initiating growth hormone with highest number of roots per plant. According to Lal and Singh (1994)[23] root can be easily induced on culture shoots by their transfer to another medium with or without NAA. Baksha *et al.,* (2002)[24] also got rooting response at 0.1 - 0.5mg/l IBA along with 0.5 - 2.0mg/L BAP but they were found to be of poor quality. Present results contradict to the findings of Behera *et al.,* (2009)[16] who reported 2.5mg/L NAA to be more effective supplement for higher number of adventious root formation in sugarcane cultivar Nayana. Similarly Baksha *et al.,* (2002) [24] used 5.0mg/L NAA for best rooting response which contradict to present findings where 5.0mg/L NAA showed poor response regarding the root formation in sugarcane shoots as shown in table 4.

Table 4: Effect of different concentrations of auxins on root formation of sugarcane variety US-633.

Auxins supplements (mg/L)		No. of roots per micro shoot	Mean length of roots (cm)	Days to emergence of roots
NAA	IBA			
0.5	_	16.00±0.12	2.5±0.02	9-15
1.0	_	13.00±0.03	1.7±0.06	10-15
2.0	_	10.00±0.07	1. 2±0.11	12-15
3.0	_	11.00±0.24	1.0±0.03	12-15
5.0	_	7.00±00.44	0.75±0.01	12-15
_	0.5	7.00±0.03	0.8±0.05	12-15
_	1.0	9.00±0.12	1.0±0.31	12-15
_	2.0	13.00±0.02	1.7±0.25	9-15
_	3.0	11.00±0.16	1.5±0.07	9-15
_	5.0	8.00±0.09	0.25±0.05	11-15
0.5	1.0	19.00±0.12	2.5±0.01	6-15
1.0	1.0	14.00±0.03	1.5±0.04	10-15
2.0	1.0	11.00±0.18	1.5±0.12	10-15
3.0	1.0	9.00±0.10	1.2 ±0.09	12-15
5.0	1.0	8.00±0.05	1.0±0.03	12-15

Acclimatization

The plantlets with healthy shoots and well developed roots were successfully transplanted in soil after acclimatization with 80% survival rate in field conditions (shown in Figures 1D and E).

This protocol described in present study was repeated for several times to establish its reproducibility and robustness for long term application. Plants regenerated from callus are sometimes not true-to-types due to their chromosomal aberrations. Many new characters might be identified in the regenerated plants. If any character is found to be superior to mother plant then somaclone with this phenotype can be released as new variety. The protocol used in the present study also has great utility for rapid multiplication of sugarcane.

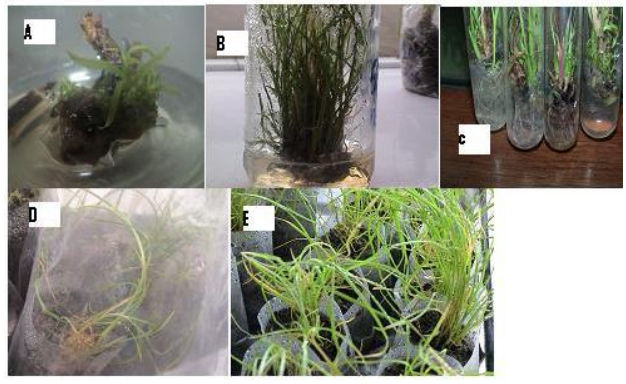

Figure 1: Developmental stages of sugarcane variety US-633 through callus under controlled conditions A. Regeneration of microshoots from callus. B. Multiplication of microshoots C. Shootlets with different auxins concentration showing the root formation (rightside 1st tube showing poor root development due to high conc. Of IBA alone) D. Plantlets covered with polythene bags to provide humid environment. E. Acclimatized plantlets ready to transfer in soil under field conditions.

Proximate analysis

The results of nutritional analysis of selected sugarcane variety US-633 from various sources e.g. *in vitro* regenerated acclimatized, and field grown plants are shown in table-5. These results show that *in vitro* grown plantlets of sugarcane had maximum level of moisture (20.33±0.05%) and total nitrogen (21.31±0.23%) whereas acclimatized and field grown plants had comparable levels of moisture among themselves. Total nitrogen was found to be lesser in acclimatized as well as field grown plants as compared to the *in vitro* plants (Table 5).

Table 5: Proximate analysis of sugarcane cultivars grown under different conditions.

Origin of samples	Moisture (%)	Total Ash (%)	Crude fiber (%)	Nitrogen (%)
In vitro grown plantlets	20.33±0.15	0.98±0.07	0.59±0.05	21.31±0.23
In vitro grown acclimatized plants	17.20±0.53	1.26±0.29	0.61±0.07	5.61±0.55
Field grown plants	17.80±0.42	5.77±0.21	0.52±0.01	4.24±0.02

Table 6: Chlorophyll contents of sugarcane leaves determined spectrophotometrically.

Origin of samples	DMSO as extraction solvent		
	Chlorophyll a	**Chlorophyll b**	**Total Chlorophyll**
In vitro grown plantlets	7.263±0.05	2.099±0.03	9.362±0.05
In vitro grown acclimatized plants	5.159±0.25	1.203±0.07	6.362±0.04
Field grown plants	6.894±0.17	1.909±0.12	8.803±0.23

Chlorophyll estimation

The chlorophyll profile of three types of plant materials investigated is presented in table 6. It was observed that chlorophyll content is highest in the *in vitro* plants (9.362±0.05) as they were growing under optimal nutritional as well as temperature and illumination conditions provided in the growth chamber. The experimental plants experience a climate change during acclimatization which affects the overall growth of the plant but it regains its normal growth after acclimatization when transferred to the field conditions.

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