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RESEARCH ARTICLE

PROTOCOL OPTIMIZATION OF SUGARCANE (*SACCHARUM OFFICINARUM* L.) CV. SP-70-1284 BY IN-VITRO MERISTEM CULTURE

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ABSTRACT

Traditionally Sugarcane accounts approximately 75% of the world's sugar and it is economically important cash crop in tropical and sub-tropical regions of many countries. Tissue culture of sugarcane has got a considerable research attention because of its economic importance as a cash crop. In Ethiopia, sugarcane is grown as an important cash and industrial crop. It is not an ideal crop for conventional breeding and it lacks rapid multiplication procedures to commercialize newly released varieties within a short period of time. The research was carried out at Tigray Biotechnology Center Plc, Mekelle, Ethiopia in 2017. The objective of this study, therefore, was to optimizea reproducible micro propagation protocol for SP-70-1284in completely randomized design with factorial treatment combinations arrangements. Using meristem explants and assessing the in vitro multiplication potential of this plant up to the fifth sub-culturing stage on MS medium. BAP with full MS medium was used for culture initiation, the survival percentages of the cultured shoots with healthy morphology was 75.8% in0.5 mg/l BAP +30 g sucrose/l and 5 g agar/l. SP-70-1284 showed the highest number of shoots per explants (34.8 ± 0.837) with 14.04 ± 0.089 average numbers of leaves per shoot and 9.4 ± 0.008 cm shoot length,was obtained after 30 days of sub culturing, in 1 mg/l BAP combined with 0.5 mg/l IBA. The maximum number of roots and the longest root length were 13 ± 0.69 and 4.5 cm in media supplemented with half MS and3.0 mg/l IBA alone. Finally, the rooted shoots were planted on substrate called coco peat; the survival rate of the acclimatized plantlets in the greenhouse was 98.4%. On the other hand the survival rate of field planted well hardened SP-70-1284 plantlets was nearly 96.5%. Therefore, the optimized protocol can be used for rapid micro propagation of SP-70-1284 planting materials for commercializing and large scale production.

Key words: In-vitro, meristem, micro propagation, Sugarcane.

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INTRODUCTION

Sugarcane (*Saccharumofficinarum*L.) is a monocotyledonous crop plant that belongs to the family *Poaceae* (Magness, 2014).Is tall perennial grass species of the genus *Saccharum*L native to the warm temperate to tropical regions of South Asia and Indonesia, and used for sugar production. Sugarcane is also known as noble cane, due to its high sucrose content and low fiber content (Kadam *et al*., 2008). Sugarcane stores its sugar (sucrose) in its stalk instead of storing in seed heads like the grain crops (Bull, 2000). Sugarcane is considered the world's most valuable crop estimated to be worth US $ 143 billion (Mendoza, 2000). Sugarcane accounts for approximately 75% of the world's sugar and is an economically important cash crop in the tropical and sub-tropical regions of many countries (Chengalrayan and Gallomeagher, 2001).

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Sugarcane is cultivated in over 110 countries and 50% of the production occurs in Brazil and India (Mokennen *et al*., 2014). Brazil is the largest producer of sugarcane in the world followed by India, China, Thailand, Pakistan, and Mexico (Godheja*et al.,* 2014). In Africa, South Africa is the leading producer followed by Sudan, Kenya and Swaziland. Peru gives the highest (135 ton/ha) yield of sugarcane followed by Egypt, Senegal, Malawi, Sudan, Ethiopia, Zambia and Tanzania (103) ton/ha (FAO, 2010). Sugarcane is one of the most widely grown crops in Ethiopia and its production was started in 1954/55(ANRS, 2008). Most sugarcane production is intended for sugar. It is also a source of other products like animal feed, antibiotics, particle board, bio-fertilizer and raw material for generating electricity, and it has lately emerged as an important base material for bioethanol production. There are also other by-products which are produced from crushing sugarcane, such as molasses and bagasse. Molasses is used widely as the main raw material for alcohol and alcohol-based industries, as additive in livestock feed; bagasse is also used as a fuel, as raw material in the paper industry and as animal feed.

The sugarcanes are used to establish most sugar industries around the world (Bull, 2000) and furthermore it provides job opportunity. Sugarcane hybrid cultivars have been arisen through intensive selective breading of species within the *Saccharum genus* (Sundara, 2000). The basic breeding concept involves combination and improvement of vigorous, disease resistant and high sucrose containing varieties. However, sugarcane is not an ideal candidate for crossing in conventional plant breeding due to its characteristic non-synchronous flowering and low sexual seed viability (Selman-Housein *et al*., 2000). Moreover, sugarcane breeding for improved varieties is a time consuming process taking upwards of ten years from initial crosses to final agronomic assessment of elite varieties (Cox *et al*., 2000). As a result, various techniques have been developed in different countries including alternation of photo period which can induce flowering. Sugarcane is propagated vegetatively by stem cutting with each cutting have two or three buds. It is a clonally propagated crop from which multiple annual cuttings of stalks are typically obtained from each planting .However, this conventional propagation method has been a serious problem in sugarcane that is lack of rapid multiplication. Conventional means of propagation takesa long time to multiply the sugarcane. During this long time, deterioration of various yield and quality characteristics is inevitable prior to commercial use on account of systemic infections during vegetative multiplication. This crop is especially vulnerable to diseases and propagation from cuttings facilitates the spread of pathogens and may results in epidemics (Schenck and Lehrer, 2000). Important reason for low yield in sugarcane is also its susceptibility to attacks by pathogens such as fungi, virus, bacteria and mycoplasma which can cause up to 70% in yields reduction (Xue and Chen., 1994; Oropez *et al*., 1995; Bhavan & Gautam, 2002 as cited in Yadav *et al*., 2012).

As sugarcane is mostly propagated by vegetative means containing two or three buds, once a plant becomes infected by a pathogen it can easily transfer the pathogen from one generation to another (Yadav *et al*., 2012). Virus infection is also the major problem in commercial sugarcane crop. Viruses cause heavy loss of sugarcane plants by reducing quality and yield. 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. Its growth is closely related to temperature [1]. Sugarcane, being a vegetatively propagated crop, has a low seed multiplication rate, which means one hectare of seed cane suffices six to eight hectare of commercial plantation. Lack of flowering potential and multiplication procedures has long been a serious problem in sugarcane breeding programs [2]. Therefore, tissue culture technology is considered as an appropriate and efficient option to overcome this problem, since which also has the advantages of speed and optimized use of plant materials. It also results in production of a large number of disease free and true to type sugarcane platelets within a shorter period of time (Usman, 2015). The meristem-tip technique can be linked with heat therapy for improving of the efficacy of disease elimination, or antiviral, chemotherapeutic agents (Grout, 1999). Tissue culture generally refers to the growth of cells from a tissue of a multicellular organism *in vitro* via use of a liquid, semi-solid, or solid growth medium, such as broth or agar. Plant tissue culture in particular is concerned with the growing of entire plants from small pieces of plant tissue (explants), cultured in medium under sterile condition (Jane *et al*., 2011). It has been exploited to create genetic variability from which crop plants can be improved, to produce healthy planting material and to increase the number of desirable germplasms available to the plant breeders (Tripathi *et al*., 2007). In tissue culture, a single explant can be multiplied into several thousand plants in less than a year. So, this allows fast commercial propagation of new cultivars. The most popular plant tissue culture application, that is an alternative to vegetative propagation of plants, is micro propagation. It is the best method for propagation as it produces plants phenotypically similar to the mother plant and gives much more rapid multiplication rate. It is being used in some sugarcane industries for the development of disease free clones, mostly to facilitate their safe and speedy movement through quarantine (Ali *et al*., 2008). Various protocols have been developed for rapid multiplication of sugarcane through callus culture (Behera and Sahoo, 2009), shoot-tip culture (Godheja *et al*., 2014; Mokennon *et al*., 2014), and auxillary bud culture and api cal meristem (Eun *et al*., 2012; Udhuth *et al*., 2016). However, in Ethiopia, the in vitro propagation of sugarcane through meristem culture protocol was not done so far. To overcome problems associated with conventional means propagation, tissue culture is the only realistic method as the meristematic tissue remains free from virus disease (Usman, 2015). Therefore, the objective of this study was to optimize effective and reproducible in-vitro protocol for sugarcane micro propagation (*saccharumofficinarum* l.) cv. SP-70-1284 via meristem culture.

MATERIALS AND METHODS

Description of the study area: The study was conducted at Tigray Biotechnology center; plant tissue culture laboratory which is located at Mekelle town, near to Tigray Agricultural Research Center that is located at latitude of 13°29'N, longitude of 39°28'E and altitude of 2076 meters above sea level.

Plant material and explant preparation: Mother plants of variety SP-70-1284 that were used as a source of explants were raised from stem cuttings (setts) obtained from meteharasugar factory. Before planting, the explants were treated with hot water at 50°C for 2 hours. The actively growing shoot tips with apical meristem were collected to use as explants source. Then the explants were planted on the substrate so called coco peat to give more active micro shoots for one months. The explants were excised properly and washed thoroughly under running tap water to remove the debris. Thereafter, 0.25 g of kocide, Bayleton and Redimol and three drops of tween 20 were collectively mixed to 100 ml of distilled water and continuously shacked to form homogenous solution. Then the explants were placed to the solution and continuously shacked for 15 minutes. Then the explants were washed 2 times with distilled water to remove the chemical residues over the surface of the explants. Then 5 % of NaOCl (Barakina) solution was prepared and the explants were placed to the solution and shacked thoroughly for 15 minutes again. Finally 0.25g per 100 ml of DH_2O $HgCl_2$ (Mercury chloride) were prepared and the explants were placed to the solution for 10 minutes to remove the systemic contaminants. Then after, the explants were washed through DH2O for 5, 3 and 1 minutes respectively and excised to 2 -3 cm properly and inoculated to their respective media formulation accordingly.

Table 1. Effect of BAP on shoot proliferation

Treatment Code	Hormone(BAP)	% response of explants(mean)	Date of response(mean)
T1	0	67	15
T2	0.25	52	15
T3	0.5	75.8	11
T4	0.75	57	13
T5	1	71	14

Table 2. Effect of BAP and IBA on shoot multiplication stage after five consecutive sub culturing

Treatment code	Hormone		Number of shoot/explants (Mean)	Average length of shoots/explants (cm) mean	Leaf Number per explants (mean)	AMF (Mean)
T1	0 BAP		21.33	6.67	8.00	1.33
T2	1 mg/l BAP+0.5 mg/l IBA		34.8	9.4	14.06	6.63
T3	1.5 mg/l BAP+0.5 mg/l IBA		22.67	6.67	10.00	3.33
T4	2 mg/ L BAP+0.75 mg/ l IBA		26.33	6.33	11.33	4.00
T5	2.5mg/l BAP		29.50	8.33	10.00	3.67
	Total		26.93	7.52	10.66	3.79
		N	15	15	15	15
		Std. Deviation	5.166	1.364	2.277	1.91

Culture media preparation: Full strength Murashige and Skoog (MS) basal medium Murashige and Skoog [Murashige *et al*, 1962) were used as a culture medium. MS basal medium consisted of 30 g/l sucrose and 5.0 g/l agar were used for all in vitro stages. The pH of the medium was adjusted to 5.8 using 1 N NaOH and HCl before autoclaved at 121°C, 15 psi for 20 minutes. The agar were mixed with MS salt solutions at about 90-95 °C to melt effectively. Then the solution was divided in to glass jars in to 40 ml of solution. Then the glass jars with 40 ml of media were sterilized at 121°C, 15 psi for 20 minutes. Finally the media were incubated for three days at 4 °C for further inspection of microorganisms. Treatments were arranged in a factorial CRD with three replications.

Media formulation of Initiation

T1=MS+30 g/l sucrose+5.0 g/l agar+0.0 mg/l BAP(Control)
T2=MS+30 g/l sucrose+5.0 g/l agar+0.25mg/l BAP
T3=MS+30 g/l sucrose+5.0 g/l agar+0.5 mg/l BAP
T4=MS+30 g/l sucrose+5.0 g/l agar+0.75 mg/l BAP
T5=MS+30 g/l sucrose+5.0 g/l agar+1 mg/l BAP

After the plants were inoculated to their respective media formulations the platelets were incubated at 25±2 °C and 8 and 16 dark/light photoperiod and 2500 Klux light intensity. Those media combination were evaluated for the next five sub culturing.

Treatment combination for multiplication stage

T1 =MS +30g/l Sucrose+5g/l agar Control
T2 =Ms+1 mg/l BAP+0.5 mg/l IBA+30g/l Sucrose+5g/l agar
T =MS+1.5 mg/l BAP+0.5 mg/l IBA+30g/l Sucrose+5g/l agar
T4= MS+2 mg/ l BAP+0.75 mg/ l IBA+30g/l Sucrose+5g/l agar
T5= MS+2.5mg/l BAP+30g/l Sucrose+5g/l agar

Rooting media formulation

T1= ½ MS+ +30g/l Sucrose+5g/l agar Control
T2 =½ Ms+30g/l Sucrose+5g/l agar +1.5 mg/l IBA
T3= ½ MS+30g/l Sucrose+5g/l agar + 1.5 mg/l IBA+0.5 mg/l NAA
T4= ½ MS+30g/l Sucrose+5g/l agar +2 mg/ L IBA+0.75 mg/ l NAA
T5 =½ MS+30g/l Sucrose+5g/l agar+3mg/l IBA

Data were collected based on The performances of the plantlets (Good, medium and poor) were evaluated after 4 weeks of sub culturing as the quality and parameters of the plant performance, height of plants (cm), number of shoots per explant, leaf number per explant, root length per explant, root number per explants, dry and fresh weight of the roots based on their stages. Data were analyzed using of SPSS version20 soft were. Treatments Means were separated by using of LSD at 0.05 level of confidence.

RESULTS AND DISCUSSION

Percent of initiated culture- Initiation culture from the apical meristem explants was observed within two weeks after inoculation of the explants on MS medium containing five different concentrations of BAP (0.0, 0.25 and 0.5, 0.75 and 1mg/l). The results showed that shoot apical meristem culture initiation or establishment responses on the used variety was variable across the different BAP concentrations. Among the different concentrations of BAP tested, 0.5 mg/l BAP gave the highest initiation culture responses (75.8%). As overall interaction effect on shoot number per plant height (Cm) and leaf number per explant and on its average multiplication factor full MS medium supplemented with 1 mg/l BAP and 0.5 mg/l IBA were produced good result on average shoot number per explants were (34.8 ± 0.837) with 14.04 ± 0.089 average numbers of leaves per shoot and 9.4 ± 0.008 cm shoot length, was obtained after 30 days of sub culturing.

Survival rate in green house for acclimatization: When all the important data in rooting stage were collected and evaluated the cultures were deflasked to greenhouse. When the plants were planted in greenhouse the relative humidity and the environment of the greenhouse were maintained properly. Then the first one week the relative humidity of the greenhouse were maintained 80 -90 % relative and 25±2 degree Celsius temperature and 12 000 lux light intensity were maintained. The leaves of the plantlets were dark green, had no sign of hyper-hydration (verification), and they could attain small leave size, besides plantlets had short and hairy roots, strong and lignified stem, with no microbial contamination.

Table 3. Effect of IBA and NAA for root development

Treatment code		Hormone(mg/l)	Root length(Cm) mean	Number of roots per explant(mean)	Plant Height (cm) (mean)	Leaf Number per explant(mean)	Root fresh weight per explant(g)(mean)	Root dry weight per explant(g)(mean)
T1		0	.00	.00	3.00	7.00	0	0.0
T2		1.5 mg/l IBA	3.00	9.00	4.00	6.00	1.00	1
T3		1.5 mg/l IBA+0.5 mg/l NAA	3.00	8.00	3.00	7.00	1.00	.50
T4		2 mg/ L IBA+0.75 mg/ l NAA	3.00	8.00	4.00	11.00	1.00	.45
T5		+3mg/l IBA	4.5	13.69	5.00	13.00	1.00	.65
Total	N	5	5	5	5	5	5	5
	Mean		2.60	7.80	3.80	8.80	.80	.32
	Std. Deviation		1.517	5.020	.837	3.033	.447	.301

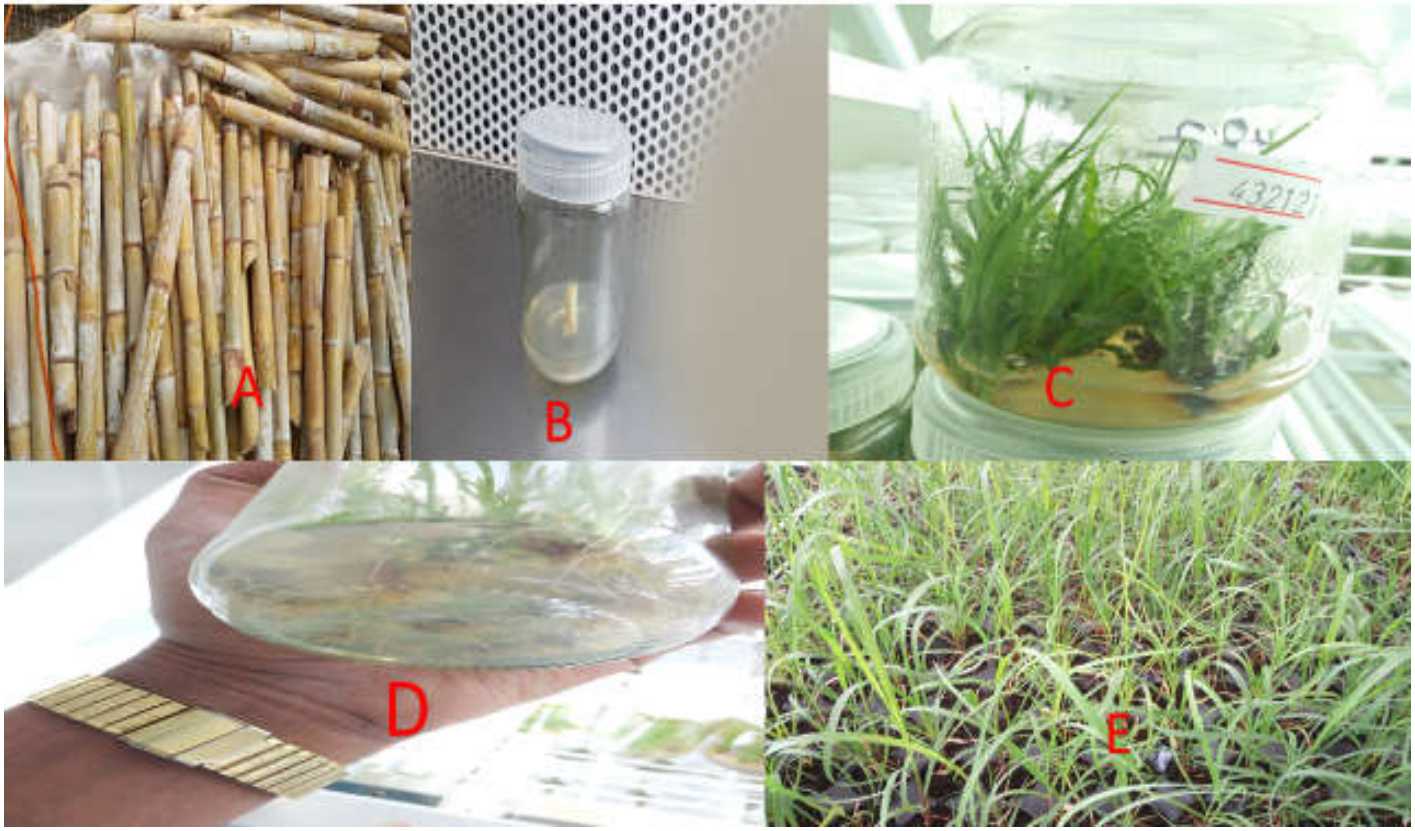

Figure 1. A.Mother plants to be plantedin the green house for explant establishment. B. Initiation of cultures on full MS+0.5 mg/l BAP C. Best performed cultures of variety SP-70-1284 on shoot multiplication stage (MS+1.5 mg/l BAP+1.0 IBA media).D. best rooted cultures of variety SP-70-1284 on rooting stage (1/2 MS+3 mg/l IBA media).E. sugarcane variety of SP-70-1284 on coco peat at primary acclimatization

The survival percentage of the specified variety in greenhouse was 98.4% on the substrate so called coco peat alone. On the other hand the survival rate of field planted well hardened SP-70-1284 plantlets was nearly 96.5%. Mother plants to be planted in the green house for explant establishment. B. Initiation of cultures on full MS+0.5 mg/l BAP C. Best performed cultures of variety SP-70-1284 on shoot multiplication stage (MS+1.5 mg/l BAP+1.0 IBA media).D. best rooted cultures of variety SP-70-1284 on rooting stage (1/2 MS+3 mg/l IBA media).E. sugarcane variety of SP-70-1284 on coco peat at primary acclimatization.

Conclusion and Recommendation

Conventional means of propagation faced problems, which leads to low production and productivity of the target crop even up to crop failure. Hence, developing an efficient protocol is a pre-requisite for successful use of this in vitro propagation to support the natural regeneration. The overall objective of this research was to optimize an in vitro propagation protocol and to secure the production of (S*accharumofficinarum* L.) cv. sp-70-1284. Four experimental stages were conducted in this study (initiation, shoot induction, root induction and acclimatization). The experiments were laid out in CRD in three replications. Meristem were used as source of explants. Disinfectants like kocide, Bayleton, Redimol mercuric chloride Tween-20, commercial detergent (soap solution), and sterile distilled water at different concentration levels with different duration of sterilization period were used for explants sterilization. Full MS media supplemented with BAP at (0, 0.25, 0.5, 0.75 and 1mg/l) was used for shoot proliferation and (0, 0.5, 0.75) IBA and (0, 1.00, 1.5, 2.00 and 2.5) BAP for shoot proliferation, half strength of MS and supplemented with NAA (0, 0.5, 0.75) and IBA at (0, 1.5, 2.00, and 3.00 mg/l) were used for root induction. Hardening off in-vitro derived plantlets of the tested variety was performed on coco peat and resulted in 98.4% of survived plants. The optimized an in vitro protocol of sugarcane variety of Sp-70-1284 has been commercialized and used for mass propagation at Tigray Biotechnology Center, Mekelle, Tigray, Ethiopia and found effective. As a result of this: Full MS medium supplemented with 0.5 mg/l BAP for shoot proliferation, 1mg/l BAP and 0.5mg/l IBAfor shoot multiplication, half MS medium supplemented with 3 mg /l IBA for root induction and coco peat for primary hardening (Acclimatization) were the best protocol for in vitro propagation of (S*accharumofficinarum* L.) cv. SP-70-1284. Further study is needed, special genotype based protocol optimization and refinement should be the next work of researchers on of sugarcane variety.

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