

Full Length Research Paper

An alternative safer and cost effective surface sterilization method for sugarcane (*Saccharum officinarum* L.) explants

Tilahun Mekonnen^{1*}, Mulugeta Diro² and Manju Sharma³

¹Addis Ababa University, Institute of Biotechnology, Addis Ababa Ethiopia

²Capacity Building for Scaling up of Evidence-based Best Practices in Agricultural Production in Ethiopia (CASCAPE) Project, Addis Ababa, Ethiopia

³Jimma University College of Agriculture and Veterinary Medicine, Jimma, Ethiopia. *Corresponding author. E-mail: mekonnetilahun27@yahoo.com.

Accepted 27 September, 2013

Regardless of its serious health effect, mercury chloride is frequently utilized for surface sterilization to mitigate microbial contamination in sugarcane tissue culture. The current study aimed at finding an alternative safer and cost effective sterilization method to substitute mercury chloride. In the study, sugarcane shoot tip blocks were treated with three concentrations (1, 3, and 5% active ingredient of chlorine) of local bleach (Berekina) for varying exposure time (10, 15, 20, 25 and 30 min). Surface sterilization with 0.1% mercury chloride for 10 min was used as standard check. Combinations of the surface sterilization treatments were applied to explants of two sugarcane genotypes in completely randomized design. Data were collected on contamination and survival percentage of explants after 15 days of *in vitro* culturing on Murashige and Skoog (MS) medium supplemented with 2 mg l⁻¹ 6-benzylamino purine + 0.5 mg l⁻¹ indole-3- butyric acid. Data were subjected to three way analysis of variance. The study verified that surface sterilization with Berekina 5% ingredients of chlorine for 25 min exposure time is optimal for sugarcane shoot tip decontamination and this treatment combination can replace sterilization with 0.1% mercury chloride for 10 min.

Key words: Berekina, mercury chloride, exposure time, shoot tip, *in vitro*.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is a monocotyledonous crop plant that belongs to the family Poaceae (Sharma, 2005; Cha-um et al., 2006). It has chromosome number of 2n = 80 (Daniels and Roach, 1987; Asano et al., 2004). It is a tall perennial crop that tillers at the base, grows three to four meters tall and about five cm in diameter (Singh, 2003). Today, the crop is grown in over 110 countries and 50% of the production occurs in Brazil and India (FAO, 2008). The 2010/11 global annual sugar production was estimated to be more than 174.3 million tons (Czariniow, 2010) and sugarcane accounts nearly for 70% of the production (Sengar, 2010). Sugarcane has been cultivated in the tropical and subtropical regions of

the world for its multiple uses. The sugar juice is used for making sugar (Cox et al., 2000). Molasses (thick syrupy residue) is used in the production of ethanol (blended for motor fuel) and as livestock feed. The bagasse (fibrous portion) is burned to provide heat and electricity for sugar mills and green tops can be used as livestock feed (Mackintosh, 2000).

Sugarcane is one of the commercial cash crops of Ethiopia. Teklemariam (1991) stated that sugar industry development in Ethiopia has great contribution to the development of the livelihood of the society and the national economy in many ways. These contributions are concerned with production and consumption of sugar

income generation, employment creation, revenue contribution, foreign exchange earnings and savings, electric power contribution, skill and know how development, capital formation, agriculture and other industries development, urbanization and marketing development benefits. Hence, improving sugarcane production capacity has a paramount importance in enhancing the economic prosperity of the country.

Varieties of sugarcane are highly heterogeneous and generally multiplied vegetatively by stem cutting. However, the conventional seed cane production method where stem cuttings with two or three nodes are used as planting material, has various limitations. The seed multiplication rate is too low (1:6 to 1:8) which makes the spread of newly released varieties slow, taking over 10 years to scale up a newly released variety to the commercial level (Cheema and Hussain, 2004; Sengar, 2010), and also facilitates the spread of pathogens and may result in epidemics (Schenck and Lehrer, 2000). Moreover, the method requires large nursery space: one hectare nursery for 10 to 15 hectares field planting (Sundara, 2000). Therefore, it is imperative to find out and implement a technological intervention that circumvents the problems associated with the conventional propagation methods.

Plant tissue culture (micropropagation) is a tool for obtaining rapid, mass multiplication of disease free, true to type planting material (Singh, 2003). Ali et al. (2004) stated that sugarcane micropropagation has the benefits of rapid propagation of new cane varieties, reduction in seed use, regeneration of large number of true to type plantlets from a small tissue, elimination of pathogens and storage of plant germplasm under aseptic condition. Explants surface sterilization is one of the critical steps in plant tissue culture.

During sterilization, the living materials should not lose their biological activity and only contaminants should be eliminated; explants need to be surface sterilized only by treatment with disinfectant solution at suitable concentrations for a specified period (Oyebanji et al., 2009). In line with this, mercury chloride solution is frequently utilized to mitigate microbial contamination in sugarcane tissue culture (Ali et al., 2004; Gosal et al., 2006; Behera and Sahoo, 2009; Kanwar, 2009; Lal et al., 2009).

However, mercuric chloride (HgCl_2) is reported to be a widespread environmental and industrial pollutant, which induces severe alterations in the tissues of both animals and men (Lund et al., 1993; Mahboob et al., 2001). Various reports depicted that mercury chloride results in a variety of undesirable health effects including neurological, renal, respiratory, immune, dermatological, reproductive and developmental sequela (Risher and Amler, 2005; WHO, 2005; Sharma et al., 2007; Durak et al., 2010).

Akin-Idowu et al. (2009) also stated that mercury chloride is difficult to dispose off. Hence, it is imperative to find an alternative safer surface sterilant that can replace the highly deleterious environmental pollutant

mercury chloride. In line with this, the use of locally available bleach or Berekina makes the sterilization process simple, rapid and cost effective (WHO, 2006; Oyebanji et al., 2009). Thus, targeting to standardize both the concentration and length of exposure time for the local bleach (Berekina) for surface sterilization of sugarcane explants helps to sterilize explants at reasonable cost and in safer condition. Therefore, the present study was initiated to develop an alternative safer and cost effective surface sterilization procedure for aseptic shoot tip culture of sugarcane.

MATERIALS AND METHODS

The study was conducted at plant tissue culture laboratory of Jimma University College of Agriculture and Veterinary Medicine, Ethiopia. Two sugarcane genotypes, Co449 and Co678, were used in this study. They were obtained from Matahara Sugar Estate, operating under Ethiopian Sugar Corporation. To reduce contamination from explants, the stock plants were raised by planting seed canes under greenhouse condition. For *in vitro* studies, shoot tips were excised from tops of three to four months-old actively growing sugarcane raised in the greenhouse. The leaves were removed and the shoot blocks were taken to the laboratory. In the laboratory, surrounding leaf sheaths were carefully removed one by one until the inner white sheaths were exposed. Then, 10 cm long tops were collected by cutting off at the two ends, locating the growing point somewhere in the middle of the top. The shoot tip blocks were washed under running tap water for 30 min with soap solution and treated with 0.3% kocide (fungicide solution) for one and half hour under laminar air flow cabinet. After decanting kocide solution, shoot tip blocks were washed three times with sterile distilled water and further immersed in 70% ethanol for 30 s and rinsed three times with sterile distilled water to remove ethanol.

To increase efficacy, two drops of Tween-20 solution was added into Berekina and HgCl_2 solutions. Tween-20 solution is a wetting agent added to the disinfectants to reduce surface tension and allow better surface contact. Decanting the sterilizing solutions under safe condition, the explants were washed three times each for 5 min with sterile distilled water and left for 10 min to make the surface dry. Thereafter, leaf sheaths damaged during sterilization were removed using sterilized forceps. Finally, 2 cm long shoot tips were excised with sterilized scalpels and cultured on MS basal medium supplemented with 2 mg l^{-1} benzyl amino purine (BAP) +

0.5 mg l^{-1} indole-3-butyric acid (IBA), 3% sucrose, and 8% agar

(Bakesha et al., 2002). Cultures were transferred to growth chamber with environmental conditions: Temperature of $25 \pm 2^\circ\text{C}$, 16 h light photoperiod, relative humidity of 70-80%, and fluorescent light intensity of 2500 lux. For the two sugarcane genotypes (Co449 and Co678), three concentrations levels of Berekina (1, 3 and 5% active ingredient of chlorine) and five levels of exposure time (10, 15, 20, 25 and 30 min) with a treatment combination of $2 \times 3 \times 5 = 30$ plus one standard check (0.1% (w/v) mercury chloride (HgCl_2) for 10 min). Experiments were set up in a completely randomized design (CRD). Fifteen explants were randomly assigned to each treatment combination. One explant was used per culture jar. Data were recorded on the number of contaminated and survived (clean) cultures per treatment combinations after 15 days of inoculation or culturing. The data were converted into percentages and subjected to three way analysis of variance using Statistical Analysis System (SAS) software version 9.2 (SAS Institute Inc., 2008). Statistical significance was computed at a 5% probability level and treatment means were separated using procedure of REGWQ (Ryan, Elinot, Gabriel, and Welsh)- multiple range test.

Table 1. ANOVA summary for the effect of Berekina and explant exposure time on contamination and survival level of cultured explants.

Source of variation	DF	Mean square	
		Contamination percentage	Survival Percentage
Gen	1	23.85 ^{ns}	24.20 ^{ns}
Ber	2	19621.47 ^{***}	14994.57 ^{***}
Time	4	5224.96 ^{***}	4074.57 ^{***}
Gen * Ber	2	14.07 ^{ns}	13.83 ^{ns}
Gen * Time	4	19.41 ^{ns}	16.79 ^{ns}
Ber * Time	8	446.20 ^{***}	346.42 ^{***}
Gen * Ber * Time	8	5.19 ^{ns}	6.42 ^{ns}
CV (%)		4.06	7.91

***, Very highly significant ($P \leq 0.0001$) at $\alpha=0.05$ significance level; ns, non significant ($p > 0.05$) at $\alpha=0.05$ significance level; DF, degree of freedom; Gen, sugarcane genotypes; Ber, Berekina (local bleach).

Table 2. Effect of different concentrations of Berekina and length of exposure time on *in vitro* sugarcane explants contamination and survival percentage.

(v/v) % of Chlorine in Berekina	Time of Exposure		Explant percent	
	(minutes)		Contaminated	Clean and survived
1	10	100.00 ^a	0.00 ^j	
1	15	93.33 ^b	6.67 ^l	
1	20	86.67 ^c	13.33 ^h	
1	25	80.00 ^d	20.00 ^g	
1	30	73.33 ^e	26.67 ^f	
3	10	93.33 ^b	6.67 ^l	
3	15	80.00 ^d	20.00 ^g	
3	20	73.33 ^e	26.67 ^f	
3	25	60.00 ^f	40.00 ^e	
3	30	53.33 ^g	46.67 ^d	
5	10	73.33 ^e	26.67 ^f	
5	15	53.33 ^g	46.67 ^d	
5	20	40.00 ^h	60.00 ^c	
5	25	13.33 ⁱ	86.67 ^a	
5	30	6.67 ^l	66.67 ^{bc}	
Control (HgCl ₂)	10	13.33 ⁱ	73.33 ^b	
CV (%)		4.06	7.91	

Means in column with the same letter are not significantly different by Ryan-Einot-Gabriel-Welsch Multiple Range Test at $\alpha = 5\%$ significant level.

RESULTS AND DISCUSSION

Analysis of variance (ANOVA) revealed that the main effect, concentration of Berekina (Ber) and time of explant exposure (Time), and the interaction effect of concentration of Berekina by length of time of exposure (Ber*Time) had very highly significant ($p < 0.0001$) effect on both the contamination and survival level of sugarcane shoot tip *in vitro* culture (Table 1). The ANOVA also showed that genotype had non-significant ($p > 0.05$) effect

on establishment of aseptic culture indicating that shoot tip surface sterilization using Berekina does not depend on sugarcane genotypes.

The highest explant contamination (100%) and the least culture survival (0%) were recorded when explants were surface sterilized with 1% active chlorinated Berekina for 10 min (Table 2). This might be due to the insufficiency of the concentration of active chlorine in Berekina and length of exposure time to kill culture contaminants. Fungi and bacteria are the most commonly

observed culture contaminants. The least culture contamination (13.33%) and the highest culture survival (86.67%) were recorded when explants were decontaminated with 5% active chlorinated Berekina for 25 min, which is similar rate of culture contamination (13.33%) but greater rate of culture survival compared with those treated with standard check, 0.1% mercury chloride (HgCl_2) for 10 min (Table 2). Lesser survival rate (73.33%) of culture treated with the standard check might be due to the phytotoxic effect of mercury chloride.

It was also observed that an increase in the percentage of active chlorine in Berekina from 1 to 5% when explants treated for 10 min decreased the culture contamination by 26.67% and increased the culture survival by the same percentage. Whereas an increase in exposure time from 10 to 30 min for 1% concentration of chlorine in Berekina had decreased culture contamination to 73.33% and increased culture survival by 26.67%. Generally, it was observed that as the concentration of chlorine in Berekina increased from 1 to 5% and the time of exposure increased from 10 to 25 min, the percent of contamination decreased and the percent of culture survival increased. This might be because of synergetic effect of chlorine concentration and length of exposure time that had a killing effect on culture contaminants.

However, surface sterilization with 5% active chlorinated Berekina beyond 25 min resulted in less contamination but more death of explants. This could be due to the phytotoxic effect of 5% chlorinated Berekina at longer exposure time. This implies that during sterilization, the living materials(explants) should not lose their biological activity and only contaminants should be eliminated; explants are surface sterilized only by treatment with disinfectant solution at suitable concentrations for a specified period (Oyebanji et al., 2009). Therefore, in the present study local bleach or Berekina with 5% ingredients of chlorine for 25 min exposure time was found to be optimal for sugarcane shoot tip sterilization and this treatment combination can replace sterilization with 0.1% mercury chloride for 10 min.

The current result is consistent with the findings of Ali et al. (2004) who reported 60-75% contamination free cultures as best results when explants of two wild relatives of sugarcane (*Erianthus 3854* and *SES 089*) are treated with 0.1% HgCl_2 for 10 min but disagree with the results of Yilekal (2011) who reported 72.23% contamination free culture using 5% active chlorinated Berekina for 20 min. The deviation might be due to the fact that the author did not expose explants to Berekina beyond 20 min and also due to difference in explant type.

Tesfaye (2011) reported 60% culture survival rate using 5% active ingredient of Berekina for 20 min which was exactly similar to the result obtained in the present study at the same (5%) concentration of Berekina for 20 min explant exposure time. Chaudhry et al. (2007) also reported 70-90% contamination control (culture growth) using clorox (commercial bleach) in surface sterilization of apical meristem of three sugarcane cultivars: HSF-240,

CP-77-400 and CPF-230. Therefore, Berekina (with 5% active ingredient of chlorine) which is affordable, widely available in local shops and supermarkets, environmentally friendly, less toxic compared to HgCl_2 , and does not require special handling and waste disposal precautions (Emongor et al 2010) can be used to surface sterilize sugarcane explants particularly shoot tips. Surface sterilization with local bleach is a safer option for both researchers and the environment (Emongor et al 2010).

Conclusion

Based on the current result, it is possible to deduce that, we have developed a safer and cost effective alternative procedure for surface sterilization of sugarcane explants, which can use Berekina as surface sterilizing agent as a substitute for mercury chloride. Hence, Berekina with 5% active ingredient of chlorine for 25 min exposure time is the appropriate combination to use for surface sterilization of sugarcane shoot tips.

ACKNOWLEDGEMENTS

The authors would like to thank Ethiopian Sugar Corporation for financing the research and Jimma University College of Agriculture and Veterinary Medicine Department of Horticulture and Plant Science for availing tissue culture facilities.

REFERENCES

- Akin-Idowu PE, Ibitoye DO, Ademoyegun OT (2009). Tissue culture as a plant production technique for horticultural crops. *Afri. J. Biotechnol.* 8(16):3782-3788.
- Ali S, Hassan SW, Razi-ud-Din S, Shah S, Zamir R (2004). Micro-propagation of sugarcane through bud culture. *Sarhad J. Agric.* 20(1):79-82.
- Asano T, Takahashi S, Tsudzuki T, Shimada H, Kadowaki K (2004). Complete nucleotide sequence of the sugarcane (*Saccharum Officinarum*) chloroplast genome: A comparative analysis of four monocot chloroplast genomes. *DNA Research* 11:93-99.
- Bakesh R, Alam R, Karim MZ, Paul SK, Hossain MA, Miah MAS, Rahman ABMM (2002). *In vitro* shoot tip culture of sugarcane (*Saccharum officinarum*) variety lsd 28. *Biotechnol.* 1(2-4):67-72.
- Behera KK, Sahoo S (2009). Rapid *in vitro* micro propagation of sugarcane (*Saccharum L. cv-Nayana*) through callus culture. *Nat. Sci.* 7(4):1-10.
- Chaudhry Z, Khan SA, Rhashid H, Chaudhry MF (2007). Optimization of explant Sterilization condition in sugarcane cultivars. *Pakistan J. Agric. Res.* 20: 3-4.
- Cheema KL, Hussain M (2004). Micropropagation of sugarcane through apical bud and axillary bud. *International J. Agric. Biol.* 6(2):257-259.
- Cox M, Hogarth M, Smith G (2000). Cane breeding and improvement .In: Manual of cane growing, Hogarth, M. and P. Allsopp (eds). Bureau of Sugar Experimental Stations, Indooroopilly, Australia. pp. 91-108.
- Czarinkow G (2010). A First Look at the 10/11 Balance Sheet: A Return to Surplus?. Global sugar production expected to reach record. [www.czarnikow.com/resource_library/documents/en/0007_A First Look at the 10 11 Balance Sheet A Return to Surplus .asp](http://www.czarnikow.com/resource_library/documents/en/0007_A_First_Look_at_the_10_11_Balance_Sheet_A_Return_to_Surplus_.asp).

- Accessed on 24/6/2011.
- Lund BO, Miller DM, Woods JS (1993). Studies on Hg (II)-induced H₂O₂ formation and oxidative stress *in vivo* and *in vitro* in rat kidney mitochondria. *Biochem. Pharmacol.* 45:2017- 2024.
- Daniels J, Roach BT (1987). Taxonomy and Evolution. Chapter 2. In: D.J. Heinz (ed). Sugarcane improvement through breeding. Elsevier publication, Amsterdam, Netherland, 11:7-84.
- Durak D, Kalender S, Gokce F, Demir F, Kalender Y (2010). Mercury chloride-induced oxidative stress in human erythrocytes and the effect of vitamins C and E *in vitro*. *Afri. J. Biotechnol.* 9 (4):488-495.
- Emongor Q, Main SM, Sharma KK, Gichuki TS, Gathaara M, De- Villies MS (2010). Surface sterilant effect on the regeneration efficiency from cotyledon explants of groundnut (*Arachis hypogaea* L.) varieties adapted to eastern and Southern Africa. *Afri. J. Biotechnol.* 9(20):2866-2871.
- FAO (2008). Food and Agriculture Organization of the United Nations: The State of Food and Agriculture. FAO, Rome, Italy.
- Gosal SS, Sood N, Gupta PK , Srivastava RK (2006). Comparative Studies on Field Performance of micropropagated and conventionally propagated sugarcane plants. *Plant tissue cult. Biotechnol.* 16(1):25-29.
- Kanwar RS (2009). New Sugarcane Production Technology. <http://www.sugarcaneindia.com/> Accessed on 12/6/2011.
- Lal M, Pathak S, Sharma ML, Tiwari AK (2009). Effect of growth regulators on *in vitro* multiplication and rooting of shoot cultures in sugarcane. *Short Communication, Sugar Tech.* 11(1):86-88.
- Mahboob M, Shireen KF, Atkinson A, Khan AT (2001). Lipid peroxidation and oxidant enzymes activity in different organs of mice exposed to low level of mercury. *J. Environ. Sci. Health, B* 36:687-697.
- Makintosh D (2000). Sugar milling. *In: Manual of cane growing.* Hogarth M , Allsopp P (Eds.). Bureau of sugar experimental stations. Indooroopilly, Australia, pp.369-377.
- Oyebanji OB, Nweke O, Odebunmi O, Galadima NB, Idris MS, Nnodi UN, Afolabi A S, Ogbadu GH (2009). Simple, effective and economical explant surface sterilization protocol for cowpea, rice and sorghum Seeds. *Afri. J. Biotechnol.* 8 (20):5395-5399
- Risher JF, Amler SN (2005). Mercury exposure: evaluation and intervention, the inappropriate use of chelating agents in diagnosis and treatment of putative mercury poisoning. *Neurotoxicol.* 26(4): 691-699.
- SAS Institute Inc. (2008). SAS/STAT[®] 9.2 User's Guide. Cary, NC: SAS Institute Inc.
- Schenck S, Lehrer AT (2000). Factors affecting the transmission and spread of sugarcane yellow leaf virus. *Plant Dis.* 84(10):1085–1088.
- Sengar K (2010). Developing an efficient protocol through tissue culture technique for sugarcane micropropagation. *Bio InfoBank.* 18:56.
- Sharma M (2005). *In Vitro* Regeneration Studies of Sugarcane. M.Sc. Dissertation Submitted To Thapar Institute Of Engineering and Technology, Patiala, India.
- Sharma MK, Sharma A, Kumar A, Kumar M (2007). *Spirulina fusiformis* provides protection against mercuric chloride induced oxidative stress in Swiss albino mice. *Food Chem. Toxicol.* 45:2412-2419
- Singh R (2003). Tissue Culture Studies of Sugarcane. An M.Sc. Thesis submitted to Thapar Institute of Engineering and Technology, Patiala, India.
- Sundara B (2000). Sugarcane cultivation. Vikas Publications Pvt. Ltd., New Delhi, India. Pp 302.
- Tesfaye F (2011). *In Vitro* Microrrhizome Regeneration of Termeric (*Curcuma domestica* Val.). An M.Sc. Thesis submitted to School of Graduate Studies, Jimma University, Ethiopia.
- WHO (2005). Mercury in drinking water . WHO.
- WHO (2006). Collecting, preserving and shipping specimens for the diagnosis of avian influenza A(H5N1)
- Yilekal B (2011). Protocol Optimization for *in Vitro* Mass Propagation of Vanilla (*Vanilla planifolia* Andr.). virus infection Guide for field operations. Guide for field operations. www.who.int/csr/resources/publications/surveillance/CDS_EPR_ARO2006_1.pdf. Accessed on 14/5/2011. An M.Sc. Thesis, submitted to School of graduate studies, Jimma University, Ethiopia.