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Disease-free Production of Sugarcane Varieties (*Saccharum officinarum* L.) Using *in vitro* Meristem Culture

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Abstract: The aim of present study is to find-out the meristem size of sugarcane varieties related to disease-free with a high recovery percentage. *In vitro* plantlets of four sugarcane varieties, Khonkan1, K97-29, KPS94-13 and K84-200, were cultured on modified-MS media. Meristem-tips were excised under stereomicroscope in different sizes, 0.5, 1.0 and 1.5 mm and cultured on modified-MS media under darkness. The meristem tips (0.5-1.5 mm) in all varieties were eliminated from bacteria and fungi, except those tips of K97-29 in size 1.0 and 1.5 mm showed 1.65 and 2.5% bacteria contamination. Recovery percentages were positively related to meristem size of K97-29 (R^2 = 0.60), KPS94-13 (R^2 = 0.77) and K84-200 (R^2 = 74) varieties, but Khonkan1 variety dose not correlated (R^2 = 0.23). In the largest size, the recovery percentages of KPS94-13 and K84-200 varieties were higher than those of Khonkan1 and K97-29 varieties for 2 folds. It should be concluded that the sizes of sugarcane meristem tips and varieties would play a key role on the disease-free production.

Key words: Contamination, *in vitro*, meristem size, meristem tip, recovery percentage

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is a tall growing monocotyledonous crop plant, belonging to Poaceae family. It has been cultivated as a cash crop in the tropical and subtropical regions of the world for its ability to store high concentrations of sucrose, or sugar, in the internodes of the stem (Cox *et al*., 2000). Normally, sugarcane crop is primarily grown as a source of sugar that directly extracted to syrup from sugarcane mills and evaporated to clarify crystal (Mackintosh, 2000). In addition, there are several by-products from crushing sugarcane i.e., bagasse (fibre) to produce fuel ethanol (Alien *et al*., 1997) and molasses to use for alcohol fermentation, a stock feed supplement and a bio-fertilizer (Mackintosh, 2000). However, there are many obstacles to limited on sugarcane productivity, especially biotic stresses including pests and diseases (McLeod *et al*., 1999). Sugarcane diseases caused by bacteria i.e., ratoon stunting (*Clavibacter xyli* subsp. *xyli*) and leaf scald (*Xanthomonas albilineans*) and fungi i.e., orange rust (*Puccinia kuehnii*), sugarcane rust (*Puccinia melanocephala*), root rot (*Pythium arrhenomanes*), smut (*Ustilago scitaminea*) play as a major biotic problem to loss of productivity (Magarey and Bull, 2003; Croft *et al*., 2000; Frison and Putter, 1993).

Disease-free production using *in vitro* meristem culture is an alternative channel to eliminate the bacteria and fungi from the host plant. There are many plants species to escape from diseases by meristem culture such as garlic, shallot (Walkey *et al*., 1987), lily turf (Strandberg, 1993), ginger (Sharma and Singh, 1997), sweet potato (Kuo *et al*., 1985), sugarcane (Homhual *et al*., 1999) and banana (Ko *et al*., 1991). Meristematic tissues of shoot tip are well known as disease-free zone. Consequently, there are many obstacles on disease-free production through meristem technology such as the size of meristem, disease-free assay, endogenous infection and long-term recovering growth (Hamill *et al*., 1993; Yin *et al*., 1993). The objective of this study is to investigate the meristem size cutting related to sugarcane varieties for disease-free production through *in vitro* meristem culture.

MATERIALS AND METHODS

Sterilization and shoot proliferation: Four varieties of sugarcane, Khonkan1, K97-29, KPS94-13 and K84-200, were used as initial material. Nodes of sugarcane were cut and then put into plastic bag containing soil for new shoot initiation. The new shoots 10±2 cm in length without leaf blade were rinsed by tap water in several

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times and then pre-sterilized twice by incubation in hot water (50°C) for 20 min. Plant materials were cut in size 5±0.5 cm and the 2-3 leaf sheaths were removed. Shoots were surface-sterilized by alcohol 70% for 10 sec, shook by 20% Clorox solution (The Clorox Company, USA) for 15 min and then washed by sterilized-distill water for 10 min. The shoots were secondly sterilized by 10% Clorox for 10 min and then washed by sterilized-distill water for 10 min. The leaf sheaths in outside were removed, thirdly sterilized by 5% Clorox for 10 min and then washed thrice by sterilized-distill water for 30 min. The shoot apex in size 1.5±0.2 cm was excised and cultured on MS (Murashige and Skoog, 1962) media containing with 8.88 μM benzyl amino purine (BA), 15% Coconut Water (CW) and 88 mM sucrose for shoot proliferation. All shoot apexes were incubated under 25±2°C air temperature, 60±5% Relative Humidity (RH), 60±10 μmol m^{-2} sec^{-1} Photosynthetic Proton Flux (PPF) with 16 h/day photoperiod provided by fluorescence lamps.

***In vitro* pretreatment and meristem cutting:** *In vitro* sugarcane plantlets 5±0.5 cm in length were pre-treated by cultured on MS media containing with 0.288 μM Gibberellic Acid (GA) and then incubated under the dark condition with 40°C air temperature in Plant Growth Chamber (EYELA, Japan) for 10 days. Meristem-tips in various sizes, 0.5, 1.0 and 1.5 mm were cut under stereomicroscope (40x) and cultured on modified MS media containing with 15% coconut water (CW) and 88 mM sucrose for 14 days. All cultures were incubated under the conditions as a shoot proliferation.

Data collection and experimental design: The contamination and recovering growth percentage were recorded after cultured on fresh media for 14 days. The experiment was designed as a factorial in Completely Randomized Design (CRD) with 10 replications and 10 plantlets per replication. The means of parameters in all treatments were compared by Duncan's New Multiple Ranged Test (DMRT) and analyzed using SPSS software.

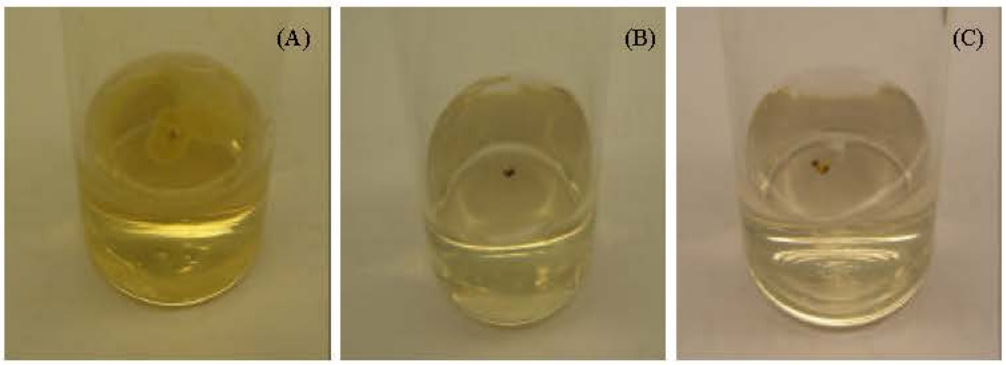
RESULTS

Multiple shoots of four sugarcane varieties were elongated on media containing 0.288 μM GA and then incubated under darkness and heat treatment with 40°C air temperature for 10 days. Leaf sheaths of pretreated-sugarcane were removed and then meristems in sizes 0.5, 1.0 and 1.5 mm were cut under stereomicroscope. Meristem tips in all sizes of Khonkan1, KPS94-13 and K84-200 varieties showed a 100% aseptic explants on culture media for 14 days, except line K97-29, in sizes 1.0 and 1.5 mm were contaminated with bacteria for 1.65% and 2.5%, respectively (Table 1). The bacteria contamination represented in Fig. 1A was evaluated using *in vitro* culture assay by modified MS media supplemented with 88 mM sucrose and 15% coconut water (w/w). Greenish color of meristem tips cultured on the media in one-week incubation was identified as plantlet development

Table 1: Contamination percentage of sugarcane varieties and various meristem sizes cultured on modified MS media containing 15% coconut water and 88 mM sucrose for 14 days

	Contamination (%)		
Lines	Size 0.5 (mm)	Size 1.0 (mm)	Size 1.5 (mm)
Khonkan1	0	0	0
K97-29	0	1.65	2.50
KPS94-13	0	0	0
K84-200	0	0	0
ANOVA	NS	NS	NS

NS: Represents on not-significance in statistic analysis



Bacteria contamination Brownish color meristem Whitish color meristem

Fig. 1: Bacteria contamination (A), brownish (B) and whitish colors (C) of meristem tip cultured on modified MS media containing 15% coconut water and 88 mM sucrose for 10 days

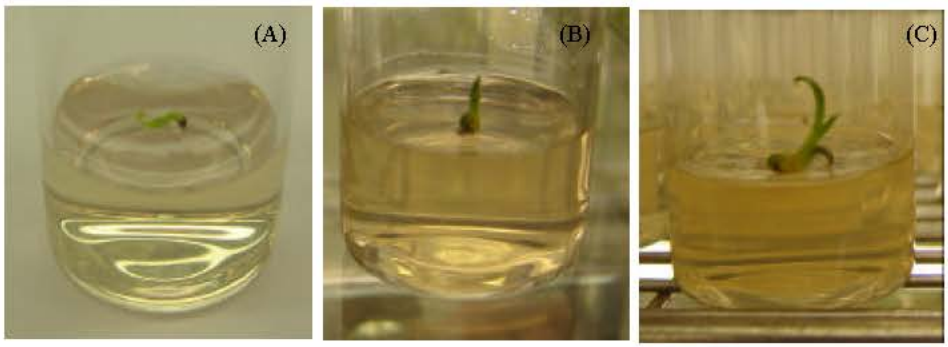


Fig. 2: Growth and development of KPS94-13 sugarcane variety cultured on modified MS media containing 15% coconut water and 88 mM sucrose for 1 (A), 2 (B) and 3 weeks (C)

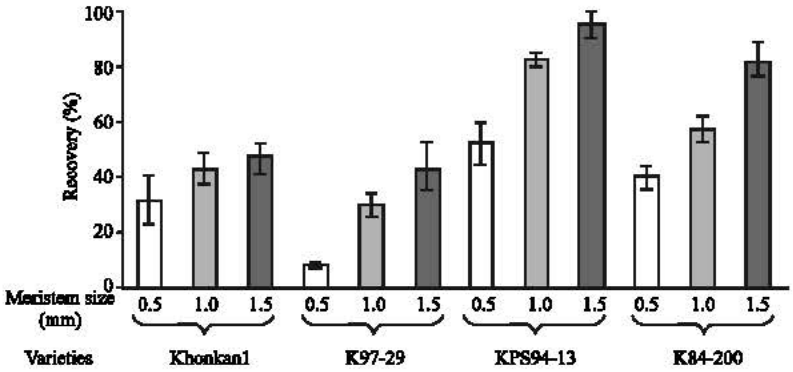


Fig. 3: Recovery percentage of Khonkan1, K97-29, KPS94-13 and K84-200 sugarcane varieties and various meristem sizes, 0.5, 1.0 and 1.5 mm, cultured on modified MS media containing 15% coconut water and 88 mM sucrose for 14 days

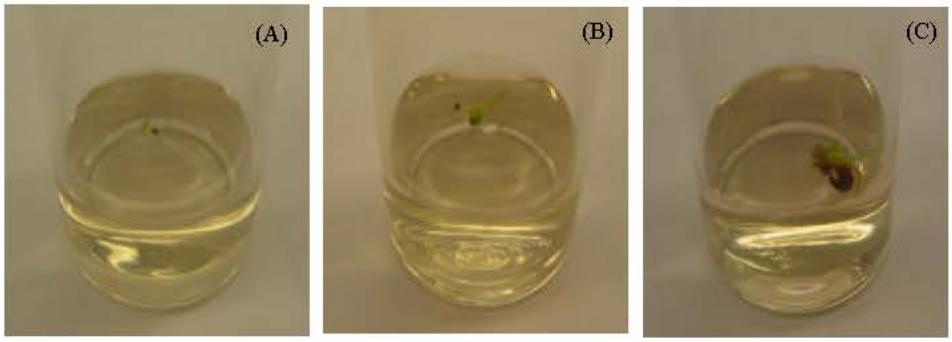


Fig. 4: The morphological characteristics of sugarcane plantlet development in various meristem sizes; 0.5 (A), 1.0 (B) and 1.5 mm (C) inoculation of Khonkan1 variety cultured on modified MS media containing 15% coconut water and 88 mM sucrose for 14 days

(Fig. 2A), while the brownish (Fig. 1B), whitish and translucent colors (Fig. 1C) were classified as a death meristem. The greenish meristems were continuously developed to new plantlets in two and three weeks on the culture media, presenting on Fig. 2B and C, respectively.

In addition, meristem tips of all varieties in size 1.5 mm were expressed the highest recovery percentage after culture on the media for 14 days. It should be noted that the recovery percentage of meristem culture was depended on sugarcane varieties and meristem sizes.

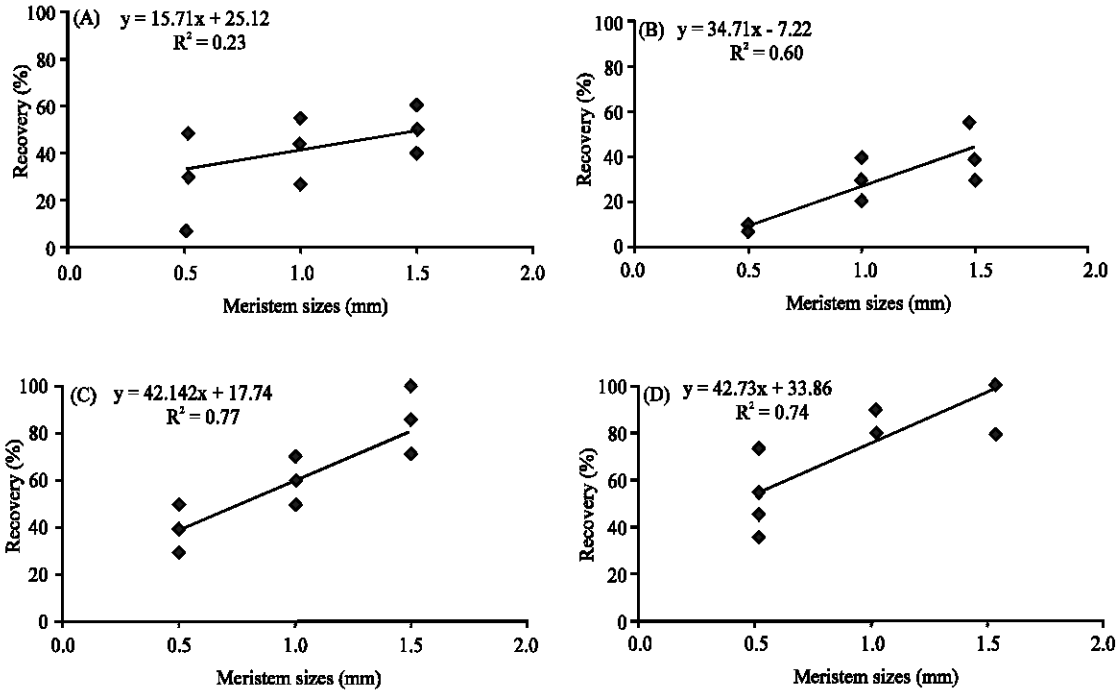


Fig. 5: Relationship between meristem sizes and recovery percentage of Khonkan1 (A), K97-29 (B), KPS94-13 (C) and K84-200 (D) sugarcane varieties cultured on modified MS media containing 15% coconut water and 88 mM sucrose for 14 days

The KPS94-13 and K84-200 varieties in a largest size illustrated a more efficient recovery percentage than those Khonkan1 and K97-29 varieties (Fig. 3). The recovering percentage was positively related to meristem size cutting of K97-29 (R^2 = 0.60), KPS94-13 (R^2 = 0.77) and K84-200 (R^2 = 0.74) varieties (Fig. 5). A large size of meristem was effective way to produce a green plantlet in rapid procedure (Fig. 4), while the virus diseases would be escaped and contaminated on plantlets derived from this technique. On the other hand, the small meristem size, especially 0.5 mm, was an effective size for disease-free production, whereas it sensitively damaged with a low recovering percentage.

DISCUSSION

In vitro pretreatment by heat, GA and darkness conditions have been applied for shoot elongation in an initial step of meristem culture in many plant species *i.e.* sugarcane (Flynn *et al*., 2005), bean (Grum *et al*., 1998) and black wattle (Beck *et al*., 2000). Meristem tips are simply excised from elongated shoot and eliminated from pathogenic diseases, including bacteria and fungi (Grum *et al*., 1998; Beck *et al*., 2000). There are many publications to recommend on 0.1-0.5 mm in length of meristem sizes for pathogenic virus disease-free (Nagib *et al*., 2003; Alam *et al*., 2004; Hosokawa *et al*., 2006) as well as 0.5-2.0 mm for bacteria and fungi disease-free production (Grum *et al*., 1998; Beck *et al*., 2000; Prehn *et al*., 2003; Ray *et al*., 2006), depending on plant species (Zapata *et al*., 1999; Chiari and bridgen, 2002; Verma *et al*., 2004). In our experiment, the small meristem sizes, 0.5-1.0 mm, are effectively excised for sugarcane disease-free production in all varieties. In *Alstroemeria* plant, the 0.7 mm meristem size from shoot tips is eliminated from *Alstroemeria mosaic potyvirus* (AlMV) for 90-95% in laboratory and 74% in the field trial (Chiari and Bridgen, 2002). As well as, the 0.3-0.4 mm meristem size of chrysanthemums is eradicated from *Cucumber mosaic virus* (CMV) for 76-84% using double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) and 60-72% using reverse transcriptase polymerase chain reaction (RT-PCR) assays (Verma *et al*., 2004). It would be confirmed that a small meristem cutting is efficacy process for disease-free plant production through meristem culture technique. In addition, the different varieties are directly related to recovery percentage as reported on cotton (Zapata *et al*., 1999), potato (Nagib *et al*., 2003) and tomato (Alam *et al*., 2004).

CONCLUSIONS

Meristem cutting in sugarcane would be effectively applied to produce disease-free plant, depending on

varieties and sizes. The recovery percentages of KPS94-13 and K84-200 varieties were higher than those Khonkan1 and K97-29 varieties. A large meristem size of sugarcane was successfully recovered after cultured for 14 days, whereas it should be possibly contaminated with bacteria. On the other hand, a small meristem size was exactly eliminated from bacteria with a low recovery percentage. This study would be intensively applied for disease-free production and large-scale micropropagation as well as further detected on virus-free.

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