Standardizing Sterilization Protocol and Establishment of Callus Culture of Sugarcane for Enhanced Plant Regeneration in vitro

Satish Tiwari, Arvind Arya and Sandeep Kumar

1National Institute of Engineering and Technology, NIMS University, Jaipur
2Department of Biotechnology, Meerut Institute of Engineering and Technology, Meerut, India

Corresponding Author: Arvind Arya, Department of Biotechnology, Meerut Institute of Engineering and Technology, Meerut, India

ABSTRACT

The present investigation is an attempt towards finding an effective sterilization protocol for two, field grown sugarcane varieties, CoS 88230 and CoS 767 from Meerut region and to establish the callus culture protocol. Four sterilizing agents EtOH, NaOCl, HgCl₂ and H₂O₂ were used alone and in combination. Our finding suggests that the use of only one sterilizing agent is not successful for the sterilization of leaf sheath explant of sugarcane. HgCl₂ (0.1%) for 5 min and EtOH (90%) for 10 min was found best when used along with prior washing and surface sterilization with 20 and 4 per cent. An increased response of callus initiation was achieved on MS medium supplemented with 2,4-D (1 mg L⁻¹) and antibiotic amoxicillin (2 mL L⁻¹). Present research attempt will allow mass multiplication of economically important sugarcane varieties through tissue culture technology can be used further to complete the demand of farmer for the planting material of the genetically superior sugarcane varieties.

Key words: Sterilization, contamination, callus, regeneration, sugarcane

INTRODUCTION

Clonal propagation through tissue culture is one of the most widely accepted tools in the field of large scale production of genetically improved plants (George and Sherrington, 1984; George, 1993). In tropical and sub-tropical part of the country, Sugarcane (Saccharum officinarum L.) is an important cash crop in agriculture and used for the production of majority of sugar in the country and in the world (Guimaraes and Sobral, 1998). Common multiplication of sugarcane involves rooting of stem cutting. Such methods are always prone to systemic disease and often result in loss of production (Nand and Singh, 1994). For the large scale production of disease free superior planting material tissue culture is the only alternative left (Lal and Krishna, 1994; Barba et al., 1977). However, in vitro culture establishment from field grown plant is also prone to contamination and also the most important reason for losses during in vitro culture of plant. Microbial contaminants viz., viruses, bacteria, yeast and fungi are found on surface as well as inside the plant body (Omanor et al., 2007). However, surface sterilization is not sufficient some time as the internal contamination is usually not removed by this approach (Hennerty et al., 1988), especially when explants are taken from field grown plants (Savela and Uosukainen, 1994) and transferred to in vitro culture.
There is a competition between microbes and the in vitro plants for nutrients. Mortality of the in vitro cultures increases as a result of microbial contamination and also shows the reduced growth and necrosis of cultures (Kane, 2003). In result of microbial contamination a great loss between 3-15% is facing by most of the commercial and scientific plant tissue culture laboratories (Leifert et al., 1989). The time and efforts required for decontaminating the explants are also some time not economical.

The success of plant tissue culture protocol depends on explant sterilization (Dodds and Roberts, 1985). Selection of sterilizing agent and time period of exposure is also critical because the living material should not lose their biological activity and only contaminants should be eliminated during sterilization. Past investigations suggest that sodium hypochlorite (NaOCl) is the best choice for surface sterilization as it is readily available and can be diluted to proper concentrations. However, a balance between concentration and time must be determined empirically for each type of explant because of phytotoxicity. Ethanol (EtOH) is also used as powerful sterilizing agent but also extremely phytotoxic. Therefore, very short exposure of few minutes or seconds is generally given to explants. Surface sterilization with heavy metal salts is another way of sterilization in which Mercuric chloride (HgCl₂) is the famous one and always been a major choice but because of is toxicity and unsafe nature for both researcher and environment it is usually replace by other disinfectants.

Optimization of sterilization protocol for two sugarcane varieties viz., CoS 88230 and CoS 767 from Meerut region is an attempt towards enhanced regeneration of sugarcane through tissue culture. Therefore, the present investigation was undertaken to ensure that large numbers of clean explants should survive sterilization. In the present study different sterilization agent viz., Commercial bleach NaOCl, HgCl₂, H₂O₂ and EtOH were used. Attempts were also made to establish a callus induction protocol for further fast multiplication of sugarcane varieties.

MATERIALS AND METHODS
The primary establishment of the aseptic in vitro culture needs surface sterilization of the explants form ex vitro or filed grown plants. The leaf sheath explant was obtained from field grown sugarcane varieties CoS 88230 and CoS 767. The outer mature leaves were removed till a spindle of about 1 cm in diameter was obtained. The spindle (3.0-4.0 cm) was then excised and thoroughly washed under running tap water for 30 min The explants were treated with aqueous solution of bavistin (A fungicide; BASF India Limited) (1% w/v) and few drops of tween-20 for 20 min outside the laminar hood. Explants were again washed with sterile double distilled water for 10 min and taken inside the laminar hood for further sterilization. For initiation of callus cultures, MS (Murashige and Skoog, 1962) medium was used supplemented with 2,4-D and antibiotic amoxicilin (2 mL L⁻¹).

Treatment of sterilizing agent: Four sterilizing agents were used for surface sterilization of leaf sheath explant of both the sugarcane varieties. Different concentrations of NaOCl (0.5, 1.0, 1.5 and 2.0%) were applied for 5, 10 and 15 min prior to inoculation the explant was rinsed 7-8 times with autoclaved distilled water to remove any traces of hypochlorite solution.

EtOH is a powerful sterilizing agent but also phytotoxic in nature. Different concentrations of ethanol (70, 85, 90 and 95%) were used for 5, 10 and 15 min and a final wash with autoclaved distilled water 5-8 times was added to remove the alcohol.
HgCl₂ is extremely toxic to both plants and humans and hence, a proper concentrations and time of exposure is important when used as a surface disinfectant. HgCl₂ (0.1-0.4%) for 5, 10 and 20 min was applied and after the treatment explants were rinsed 8-10 times with distilled water to remove the traces of HgCl₂.

H₂O₂ is easily available and one of the useful sterilizing agent in lab as well as in field. Different concentrations (10-40%) were used to disinfect the explant material.

All the above mentioned sterilizing agents were used alone as well as in combinations to get the optimum results of surface sterilization of explant material.

Establishment of callus culture: For the establishment of callus culture the sterilized leaf sheath explants were inoculated on agar solidified basal MS medium supplemented with 2,4-D (1-5 mg L⁻¹) and amoxicillin (2 mL L⁻¹). Cultures were incubated in the controlled conditions of temperature (25±2°C) and light intensity (2000-2500 lux for 16 h); the experiment was performed 4 times with 5 replicates per treatment.

RESULTS AND DISCUSSION

It is always a big challenge to avoid contamination and establishment of aseptic cultures from the field grown plants which are always at high risk of internal and external contamination (Hennerty et al., 1988; Misaghi and Donndelinger, 1990). The present investigation was carried out to optimize sterilization protocol for fast multiplication of sugarcane varieties (CoS 88230 and CoS 767). The explants were procured from the field grown plants of sugarcane and the prior washing with bavistin and mild 20 was performed. To ensure the complete sterilization, explants were again treated with different sterilizing agents in laminar air hood. The two-step procedure by the two reagents for sterilization of explant (Mathews and Duncan, 1999) is always a cumbersome process. However the use of one sterilizing agent is not always effective. From the economical prospective of the tissue culture a simple, cheap and effective sterilization protocol is needed. Present investigation highlighted the importance of different sterilizing agents for the surface sterilization of leaf explants of Sugarcane.

At lower concentrations EtOH (70-85%) when used alone showed failure in sterilizing the explants. However at high concentrations (90-95%), EtOH treatment resulted toxic for the explant when treated for 10-15 min but at 90% concentration for 5 min it was found satisfactory and also in line with the findings of Chen et al. (1988) (Fig. 1a). HgCl₂ at different concentrations was far much better than EtOH. But because of the toxic effect of this heavy metal it is usually avoided. Explant was found not responsive when treated with higher concentrations of HgCl₂ (0.2, 0.3 and 0.4%) (Fig. 1c). However, lower concentration of HgCl₂ (0.1%) gives satisfactory results (Mamun et al., 2004). We found that spindles turned black when leaf explants were sterilized with 0.1% HgCl₂ for 8-10 min In present investigation NaOCl (1%) for 10 min was found more effective for sterilization and further in vitro response of explant (Fig. 1b). The past findings also suggests that NaOCl is an effective sterilizing agent at a concentration of 0.5% for 20 min and a good sterilizing agent for leaf explant (Chengalrayan et al., 2005). H₂O₂ at all concentrations was found toxic and resulted in the blackening of leaf sheath (Fig. 1d). Our finding suggests the use of EtOH (90%) for extended time at least for 10 min either or HgCl₂ (0.1%) for very short time period to obtain aseptic cultures of sugarcane.

Fig. 1(a-d): Effect of various concentrations of sterilizing agent (a) EtOH, (b) NaOCl, (c) HgCl₂ and (d) H₂O₂ on contamination percentage

Fig. 2: Effect of 2,4-D supplementations in MS basal medium on percentage of explants response towards callus formation

Basal MS medium supplemented with 2,4-D (1-5 mg L⁻¹) was tested for induction of callus in two varieties of sugarcane (CoS 88230 and CoS 767). Maximum callus induction response (75 and
79%) by leaf explants was found on 2,4-D (1 mg L⁻¹) in CoS 88230 and CoS 767, respectively (Fig. 2). Tarique et al. (2010) also reported 2,4-D as best hormone for callus induction in sugarcane. It is also evident from the results that 2,4-D is more responsive towards callogenesis at lower concentrations. However, some reports found whitish callus formation at lower concentration of 2,4-D and brown callus in sugarcane at higher concentrations (Barba et al., 1977; Mannan and Amin, 1999). To avoid the late contamination in cultures, amoxicillin (2 mL L⁻¹) was added in MS medium along with different concentrations of 2,4-D (1-5 mg L⁻¹). The antibiotic addition showed positive response and the percentage response of explants was found increased from 75% (without antibiotic; Fig. 2) to 85% (with antibiotic; Fig. 3) for CoS 88230 and from 79% (without antibiotic; Fig. 2) to 90% (with antibiotic; Fig. 3) for CoS 767. Higher concentration of 2,4-D (5 mg L⁻¹) also showed significant increase in the number of explants response. At higher concentration of 2,4-D (5 mg L⁻¹), amoxicillin addition raised the percentage response of explants from 20-40% for CoS 88230 and from 27-48% for CoS 767 (Fig. 2 and 3). 2,4-D (1 mg L⁻¹)+ amoxicillin (2 mL L⁻¹) showed 94% callusing in CoS 88230 (Fig. 4) and resulted in the formation of brownish white callus (Fig. 5). However, 92% callusing from leaf sheath explants was obtained in CoS 767 (Fig. 4) with the formation of brown callus (Fig. 6). Calli thus produced were successfully proliferated and further differentiated into shoots.

![Graph 1](image1.png)

**Fig. 3**: Effect different concentrations of 2,4-D supplemented with 2 mL L⁻¹ amoxicillin in MS basal medium on explant response

![Graph 2](image2.png)

**Fig. 4**: Effect of different concentrations of 2,4-D supplemented with 2 mL L⁻¹ amoxicillin on callus percentage
Fig. 5: Induction of callus from leaf sheath on MS+2,4-D (1 mg L\(^{-1}\))+amoxicillin (2 mL L\(^{-1}\)) of sugarcane (CoS 88230)

Fig. 6: Multiplication of callus on MS+2,4-D (2 mg L\(^{-1}\))+amoxicillin (2 mL L\(^{-1}\)) of sugarcane (CoS 767)

These findings will provide a good base for effective and quick sterilization of sugarcane explants especially when they are procured from field grown plants. Callus induction form the spindle of sugarcane is a faster way of plant multiplication and is vital for researches related to genetic improvement of sugarcane.

REFERENCES


