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Evaluation of Sterilant Effect on *In-vitro* Culture Establishment in Sugarcane Variety Co 0118

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A B S T R A C T

In-vitro propagation is widely used as reliable alternative for true-to-type and quality seed production in sugarcane. The main obstacle under *in-vitro* propagation is growth of contaminants, resulting in failure of culture establishment that can be minimized by effective surface sterilization method. The sterilization process varied species to species and explants type thus, present study was designed to evaluate the effects of various sterilants on *in-vitro* culture establishment of sugarcane variety Co 0118 through meristem shoot tip. The explants were collected and treated with different concentrations of sterilants *viz.*, Bavistin^{TM} (0.01-0.4%), $HgCl_2$ (0.01-0.4%), NaOCl (2-10%) and EtOH (60-100%). The treated explants were inoculated on semi solid MS media supplemented with 0.5 mg/L each of BAP and Kn. The results obtained under study showed significant differences in their activity. The optimum culture survival percent and lower contamination with reduced necrosis was to be found when explants were treated with the combination of 0.1% Bavistin for 10 min followed by 0.1% $HgCl_2$ for 5 min, 6% NaOCl for 10 min and 70% EtOH for 1 min. This sterilization protocol will be contributive in efficient surface sterilization for in-vitro propagation of sugarcane though meristem shoot tip.

Introduction

Sugarcane is an economically important cash crop and used as one of the principal sources of sugar, ethanol, and jaggery in Indian subcontinent (Gallo-Meagher *et al.,* 2000). It is widely grown crop in tropical and subtropical regions of the world contributing about 70% of the world sugar production (Chatenet *et al.,* 2001). Worldwide, sugarcane is cultivated over 110 countries with an area of 25.98 million ha, and production of 1841.52 million metric tonnes. India is the second largest producer of sugarcane occupies 4.39

million ha area with the production of 306.07 million tonnes (FAOSTAT, 2017). The usefulness of this crop makes it unique for intensive and extensive field plantation.

Generally, sugarcane is propagated through multiple cuttings of stalks. The vegetative cultivation practices are results in low multiplication rate and facilitate the source of survival for many pathogens in sugarcane setts (Schenck and Lehrer, 2000; Sengar *et al.,* 2011). Sugarcane production is negatively affected by diseases mainly due to its vegetative propagation. Now days, in-vitro propagation through tissue culture has been established as an efficient and reliable strategy. Tissue culture not only reduces the time of multiplication but also produce true-to-type plants and can be used an alternative for rapid propagation of sugarcane (Jalaja, 2008; Sengar *et al.,* 2012). Thus, propagation procedure of sugarcane through tissue culture should be developed in more advanced and efficient to get higher establishment.

The plants brought from the field in laboratory have high number of contaminations on their surface. These contaminants have a negative effect on explants survival and compete with in-vitro plantlets for nutrition if the proper treatment not given for their removal. Microbial contaminations reduce the mortality of in-vitro cultures and cause necrosis. The explants propagation without using any sterilant results in very less culture survival sometimes no culture survival. The prevalence of contamination makes culture establishment vulnerable in achieving goal. In successfulness of in-vitro culture establishment primary consideration given to explants selection and secondary to the suitable sterilizing agents. The chemical agent selection should of be the type that could remove contamination properly (Panhwar, 2005), and ensure higher survival rate after treatment. Development of aseptic culture protocol is a critical step to avoid the invasion and growth of contaminants. However, many protocols had been established for in-vitro propagation of sugarcane varieties (George and Tripepi, 2001; Sawant and Tawar, 2011; Ghatge *et al.,* 2014; Belete and Kalu, 2015: Thorat *et al.,* 2016) but looking for better optimization of sterilant combination is mandatory to achieve sustainable micropropagation. Thus present study was conducted to optimize surface sterilization for in-vitro establishment of sugarcane through meristem tip culture.

Materials and Methods

Experimental plants and protocol

The present investigation was conducted at Tissue culture laboratory, Department of Agricultural Biotechnology, College of Agriculture, SVPUA&T, Meerut. The explants were collected from nine month old sugarcane plants Co 0118 in the form of upper tops from ShriRam Sugarcane Research Farm, Modipuram, Meerut, UP, India. MS (Murashige and Skoog) basal medium was prepared by supplementing 3% sucrose, 300mg/L PVP, 100 mg/L myo-inositol, agar (8g/L) as solidifying agent along with 0.5 mg/L each of BAP and Kn and pH adjusted to 5.8, autoclaved at 121°C and 15 lbs (101kpa) for 20 minutes before 3 days of inoculation. The autoclaved media was poured into pre-sterilized culture tubes (150×25 mm). After 3 days media was screened to check any contamination.

Explant sterilization and sterilants

The collected sugarcane tops were excised into 6-8 cm long spindles containing apical meristem shoot tip and thoroughly washed under running tap water for 30 min. Initially these segments were treated with Lavolene (5% Calcium Hypochlorite) for 10 min and rinsed with water to remove any traces of

detergent. Then these segments were dipped into 0.1% Polyvinyl pyrilidone (PVP) solution and further surface sterilization treatments were given under laminar air flow hood. The explants were treated with various concentrations and combination of sterilants *viz.* Bavistin^{TM} (0.01, 0.05, 0.1, 0.2 and 04%), Mercuric chloride (0.01, 0.05, 0.1, 0.2 and 0.4%), Sodium hypochlorite (2, 4, 6, 8 and 10%) and Ethanol (60, 70, 80, 90 and 100%) to optimize minimum contamination, explant survival and culture establishment (Table 1). The explants were washed three times with autoclaved de-ionized water after each treatment. The explants were prepared by carefully removing upper extended outer leaf sheaths and excised without disturbing upper meristematic shoot bud using scalpel blade in 1.5-2.0 cm. The prepared explants were immediately inoculated on semisolid MS medium containing 0.5 mg/L of each BAP and Kn and incubated in culture room under white fluorescent light intensity of 4000 lux, 16 h of light and 8 h of dark photoperiod, 50-60% RH and 25±2ºC temperature. The cultures were observed on regular interval of 3 days for contamination for four weeks and data was collected for contaminated, survived cultures.

Culture survival percent was calculated as per the formula:

$$\text{Culture survival } \% = \frac{\text{number of explant survived}}{\text{number of explant inoculated}} \times 100$$

Statistical analysis

The data was analyzed using SPSS (computer software package version 20.0) and one way ANOVA was applied to test mean differences among all treatments followed by Tukey's-b multiple range test and represented as mean±SE. The value $p \leq 0.05$ was considered statistically significant in each case.

Results and Discussion

In-vitro culture establishment is primarily dependant of sterilization procedures. The explants collected from field contain many microbes on their surface because plant faces every type of situation in field condition. The culture medium used in the tissue culture is most suitable for microbial growth thus, surface sterilization of collected explants is necessary. Under present study, various concentrations of sterilants and their combination had been optimized for higher culture survival and establishment (Table 1).

Bavistin, a fungicide widely used as sterilant in plant tissue culture studies (Altan *et al.,* 2010; Khatun *et al.,* 2016) has been used under study in different concentrations for 10 min for explants surface sterilization. Analysis of variance indicates culture survival percent range from 26.8 to 55.4% (Table 1 and Fig. 1).

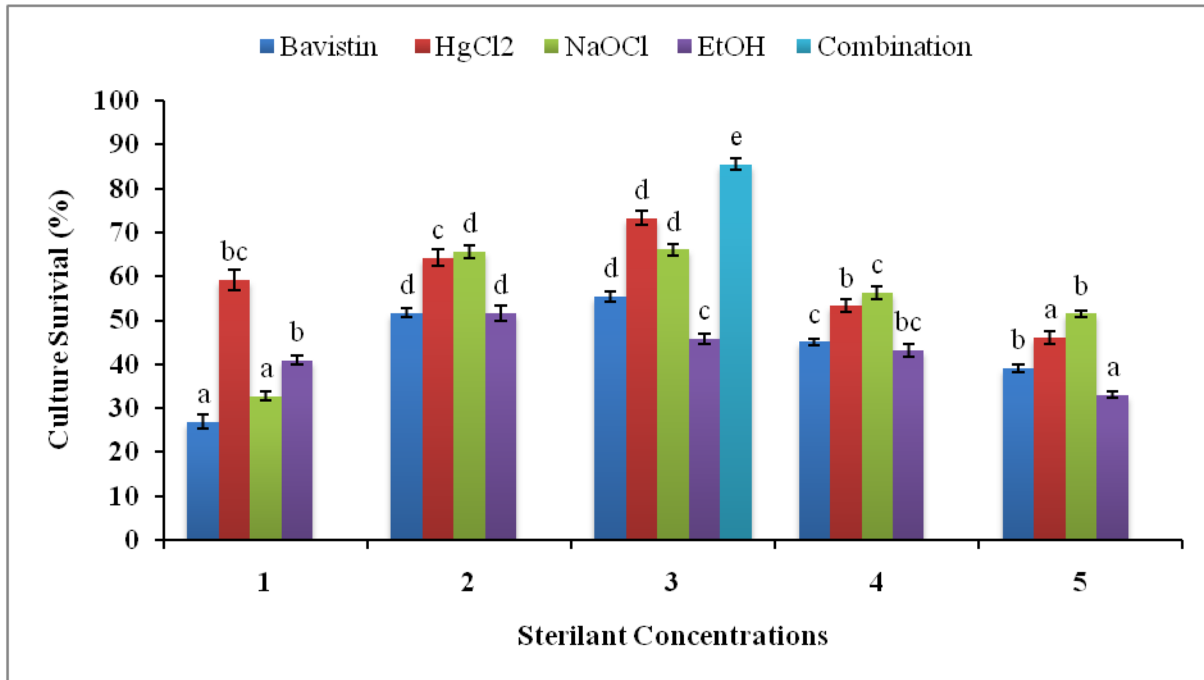
A significantly ($p<0.05$) higher culture survival percent (55.4%) and healthy uniform shoots growth as compared to other Bavistin treatments was observed in explants treated with 0.1% Bavistin. However, the culture survival percent did not differ significantly ($p>0.05$) in explants treated with 0.05 and 0.1% Bavistin. A significantly ($p<0.05$) lesser survival percent was to be noted in explants treated with 0.01% Bavistin and significantly ($p<0.05$) lesser contamination was recorded to be 0.2 % treated explants but shoot induction was found to be impaired.

Mercuric Chloride ($HgCl_2$), a corrosive chemical compound used as disinfectant and had been reported as sterilant in plant tissue culture studies by many researchers (Preethi *et al.,* 2011; Anburaj *et al.,* 2011).

Table.1 Effect of various treatments on survival and growth pattern of cultures

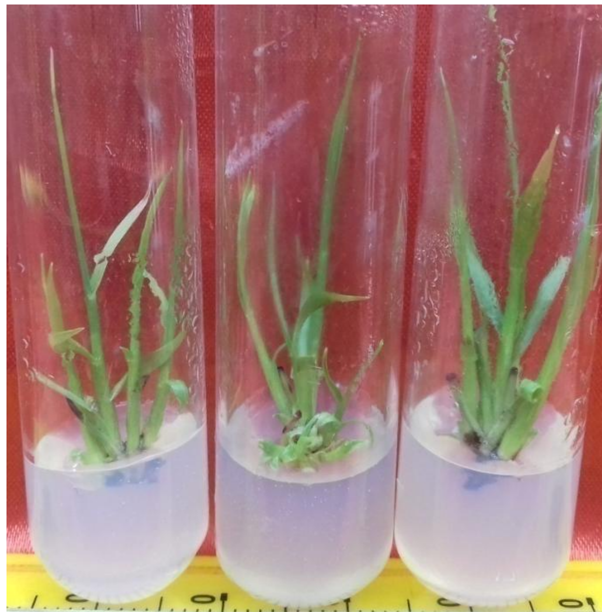
Sterilants	Concentraion (%)	Time (min)	Survival (%)	Shoot induction and growth pattern
Control	No sterilant	--	NIL	Contaminated
Bavistin	0.01	10	26.8 ± 1.56^{a}	Non-uniform, contaminated
	0.05	10	51.6 ± 1.08^{d}	Healthy, uniform
	0.1	10	55.4 ± 1.12^{d}	Healthy, uniform
	0.2	10	45 ± 0.84^{c}	Medium, non-uniform
	0.4	10	39 ± 0.84^{b}	No shoot induction
Mercuric Cholride ($HgCl_2$)	0.01	5	59.2 ± 2.29^{bc}	Non-uniform, Contaminated
	0.05	5	64.2 ± 1.83^{c}	Medium, uniform, contaminated
	0.1	5	73.2 ± 1.56^{d}	Healthy, uniform
	0.2	5	53.2 ± 1.39^{b}	Medium, non-uniform
	0.4	5	46 ± 1.34^{a}	No shoot induction
Sodium hypochlorite (NaOCl)	2	10	32.6 ± 1.03^{a}	Medium, non-uniform, contaminated
	4	10	65.6 ± 1.50^{d}	Healthy, uniform
	6	10	66 ± 1.41^{d}	Healthy, uniform
	8	10	56.2 ± 1.46^{c}	Healthy, non-uniform
	10	10	51.4 ± 0.75^{b}	Medium, non-uniform
Ethanol (EtOH)	60	1	40.8 ± 1.07^{b}	Medium, non-uniform
	70	1	51.6 ± 1.72^{d}	Healthy, uniform
	80	1	45.8 ± 1.16^{c}	Medium, non-uniform
	90	1	43.2 ± 1.46^{bc}	Medium, non uniform
	100	1	33 ± 0.71^{a}	Medium, non-uniform
Combination (Bavistin+$HgCl_2$+NaOCl+EtOH)	(0.1+0.1+6+70)	(10+5+10+1)	85.4 ± 1.29^{e}	Healthy, uniform, vigorous shoots

Fig.1 Effect of various concentrations of different sterilant on culture survival



Note: Sterilant Concentrations-
1=0.01% for Bavistin and $HgCl_2$*, 2%for NaOCl, 60% for EtOH;*
2= 0.05% for Bavistin and $HgCl_2$*, 4%for NaOCl, 70% for EtOH;*
3= 0.1% for Bavistin and $HgCl_2$*, 6%for NaOCl, 80% for EtOH;*
4= 0.2% for Bavistin and $HgCl_2$*, 8%for NaOCl, 90% for EtOH;*
5= 0.4% for Bavistin and $HgCl_2$*, 10%for NaOCl, 100% for EtOH*
Combination= 0.1% Bavistin + 0.1% $HgCl_2$ *+ 6% NaOCl + 70% EtOH*

Fig.2 Shoot establishment and multiplication



The explants treated with various concentrations of $HgCl_2$ showed culture survival percent within a range of 46 to 73.2% and found to be statistically different ($p<0.05$) (Table 1 and Fig. 1). The explants treated with 0.1% $HgCl_2$ for 5 min showed a significant ($p<0.05$) increase in culture survival percent (73.2%) with healthy and uniform shoot growth in comparison to other treatments. Similar findings were reported by Sawant and Tawar (2011). Babaei *et al.,* (2013) and Tiwari *et al.,* (2012) also reported the 0.1% $HgCl_2$ for 5 min as effective treatment for explant surface sterilization with minimum tissue necrosis. However, explants treated with 0.05% $HgCl_2$ also showed higher culture survival as compared to other $HgCl_2$ treatments but a microbial growth after one week was observed. The significantly ($p>0.05$) lesser culture survival percent sometimes no shoot induction was obtained in explants treated with 0.4% $HgCl_2$. Increase in $HgCl_2$ % found to be resulted in deleterious effect on explants survival, explants turned into brownish black and failed to proliferate during incubation as a result of tissue necrosis. It may me the results of bleaching action of two chloride atoms and ions that combine strongly with proteins and cause necrosis to plant tissues. The findings of this study are in agreement with earlier researcher reports of Johnson *et al.,* (2011) and Wesely *et al.,* (2011).

Sodium hypochlorite (NaOCl) is a compound effectively used for water purification as disinfectants also used in various concentrations to show the effect on explant sterilization. The culture survival was found to be ranged from 32.6 to 65.6% (Table 1 and Fig. 1) under studied concentrations of NaOCl. The explants treated with 6% NaOCl for 10 min showed a significantly ($p<0.05$) higher culture survival percent (66%) and uniform shoot growth as compared to other NaOCl treatments. However, culture survival percent was significantly ($p>0.05$) in explants treated with 4 and 6% NaOCl. A significant ($p<0.05$) lesser culture survival percent was shown to be in explants treated with 2% NaOCl. A significant ($p<0.05$) increase in culture survival percent was shown with increasing NaOCl concentration but shoot growth was non-uniform. Similar findings also reported by Reda *et al.,* (2004) and suggested sodium hypochlorite as an effective surface sterilant for sterilization. Wlodkowski and Rosenkranz (1975) and Danso *et al.,* (2011) reported NaOCl diluted with water lead to formation of HClO and resulted in lethal DNA damage (Dukan *et al.,* 1999).

Ethanol is used as strong phytotoxic sterilizing agent. Earlier researchers also reported that alcohols have higher bactericidal activity as compared to other bacteriostatic agents against bacterial vegetative cells but do not destroy spore cells (Bloomfield, 1978). In present study, various concentration of ethanol had been used to determine its effectiveness on explant sterilization that found to be ranged from 33 to 51.6% (Table 1 and Fig. 1). The explants treated with 70% ethanol for 1min showed a significantly ($p<0.05$) higher survival percent (51.6%) with healthy and uniform shoot growth as compared to other EtOH treatments but shoot induction was slower. However, minimum culture survival percent and non-uniform shoot growth was to be noted in explants treated with 100% of Ethanol.

A significantly ($p<0.05$) higher culture survival percent (85.4%) with vigorous shoot growth as compared to other treatments was observed in explants treated with a combination of 0.1% Bavistin for 10 min followed by 0.1% $HgCl_2$ for 5 min, 6% NaOCl for 10 min and 70% EtOH for 1 min with subsequent three washing of de-ionized water after each treatment (Table 1 and Fig. 1). The regenerated shoots were healthy and

green coloured and aseptic tissue growth was observed and media was apparent clear with no turbidity. However, treatment with single sterilant resulted in good shoot proliferation but subsequently failed to control microbial contamination in second week (Fig. 2). No survival was observed in explants inoculated without any sterilant treatment. The results under study also indicate that contamination percent was reduced when explants were treated with either of 0.2% Bavistin, 0.2% $HgCl_2$ or 8% NaOCl alone but at the same time there was adverse effect on tissue growth was to be seen due to increased necrosis leading to failure of shoot induction. The Variations in shoot induction and proliferation was the result of sterilant impact and damage to the explants during treatment.

In present study, each sterilant differed in its activity on explants surface sterilization that suggest sterilization process differs with tissue and type of explant. The sterilants combination used under study was found to me more effective for meristem shoot tip explants sterilization and culture establishment in sugarcane variety Co 0118.

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