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Optimizing the sterilization methods for initiation of the five different clones of the *Eucalyptus* hybrid species

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ABSTRACT

In this study, five improved clones of the *Eucalyptus* hybrid species namely, *E. pellita* X *E. camaldulensis* (Clone No. 9), *E. camaldulensis* X *E. pellita* (Clone No. 21), *E. camaldulensis* X *E. tereticornis* (Clone No. 4), *E. camaldulensis* X *E. pellitta* (Clone No. 16) and *E. tereticornis* X *E. urophylla* (Clone No. 41) were selected. The sterilization protocol were optimized and studied thoroughly for its contamination and survival rates. This aids in the micro-propagation process of the five different clones of the *Eucalyptus* hybrid species. The nodal explants collected and stored were surface sterilized with different sterilizing agents namely, 70% Ethanol, 1% Sodium hypochlorite, 0.1% Mercuric chloride ($HgCl_2$) and rifampicin (1 mg/mL) at different exposure period. Among the various sterilization methods tested, it was concluded that the best one was found to be with 0.1% $HgCl_2$ for 3 min and rifampicin (1 mg/mL) for 5 min along with 1% sodium hypochlorite and 70% ethanol. The explants treated with this method showed higher survival rates in the plant cultures for all the five clones of the *Eucalyptus* hybrid species tested.

1. Introduction

Contamination is the major problem in plant tissue culture. It remains to be a serious obstacle for the culture initiation. Hence the efficiency of multiplication and rooting gets reduced (Compton and Koch, 2001). For the maintenance of aseptic cultures, the essential factors are aseptic techniques involved during handling of the cultures, appropriate growth conditions, suitable explants as well as nutrient medium (Brondani et al., 2013). Henceforth these well maintained tissue cultures are free of microbes. Some microbes that are not macroscopically visible to the naked eye have been observed in the plant tissue culture vessels without affecting the growth of the plants (Holland and Polacco, 1994). Contamination is an accidental event that may occur at any point of time during the tissue culture process. It is not possible to avoid contamination absolutely. Care should be taken to follow the aseptic conditions to minimize the contamination (Khafagi et al., 2001).

Bacterial contamination occurring in plant tissue culture is of wide range that includes plant pathogens, epiphytes, endophytes, and microbes from air or humans during handling etc., (Williamson et al., 1997). Exogenous as well as endogenous bacterial contamination remains a major challenge in plant tissue culture. The explants are initially surface sterilized to remove most of the exogenous contaminants. Some organisms remain internally in the living tissues (Wendling et al., 2014b). These organisms in most of the cases appear immediately after initiation of the explants. In some cases there may be delayed bacterial growth wherein the growth of the plantlets may be retarded. However the bacteria may appear only when there is a change in the culture medium composition or increase in temperature or due to several other factors (Liu et al., 2005). The contaminants are introduced into the plant tissue cultures during the stage of initiation, though the explants are

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Table 1
List of *Eucalyptus* hybrid clones selected for optimization of the sterilization protocol for the micropropagation studies.

Reference Number	Clone name (Male)	Species name	Clone name (Female)	Species name	Type of hybrid
9 (*E. pellita* X *E. camaldulensis*)	E29	*E. pellita*	IFTGB EC1	*E. camaldulensis*	Inter
21 (*E. camaldulensis* X *E. pellita*)	TNPL 103	*E. camaldulensis*	EP29	*E. pellita*	Inter
4 (*E. camaldulensis* X *E. tereticornis*)	53	*E. camaldulensis*	186	*E. tereticornis*	Inter
16 (*E. camaldulensis* X *E. pellitta*)	44	*E. camaldulensis*	EP29	*E. pellitta*	Inter
41 (*E. tereticornis* X *E. urophylla*)	186	*E. tereticornis*	EU8	*E. urophylla*	Inter

surface sterilized. This still remains to be a major hurdle for the initiation of the explants, efficient shooting and rooting processes (Singh and Chand, 2003).

Surface sterilization process is required to eliminate or kill the contaminants but definitely not to affect the biological activity of the explants (Felek et al., 2015). The explant sterilization is the most crucial step in the plant tissue culture and an optimized protocol gives a higher success rates (Wendling et al., 2014a). As there lies a competition between the contaminants and the explants for nutrients, the growth of the explants is found in a lesser number as a result of microbes (Tiwari et al., 2012). Explants must be treated with the disinfectants for a specified period and at a concentration suitable for them (Oyebanji et al., 2009) because higher concentration can lead to the death of the plant tissues. Surface sterilization of the explants beyond 15 min with 0.5% chlorinated local bleach leads to phytotoxicity (Ervin and Wetzel, 2002). Optimizing the concentration as well as the time of exposure is important for the metal surface sterilizing agents like 0.1% $HgCl_2$ as it is toxic to plants as well as humans (Tiwari et al., 2012).

Field grown plants are invariably contaminated with microbes. Dusts that are confined on the external surface of the explants are removed by exposing them under running tap water (Sharuti et al., 2011). Developing a woody species through micropropagation and to maintain contaminant free cultures remains a tough task (Garg et al., 2014). Bacteria or fungi that occurs on the surface of the explants or on the natural openings of the explants will be revealed only after initiation which may be macroscopic or microscopic (Jan et al., 2013).

As the initiation of contaminant free plant cultures remains a challenging task, our prime concern is to focus on the selection of the sterilizing agents. We also need to look upon the time required for the exposure of these agents on the explants to obtain healthy shoots. The sterilization protocol is the first step in determining the success of micropropagation. The successful micropropagation can yield numerous plantlets within a shorter span of time. Present study was aimed to optimize the surface sterilization protocol for the five different *Eucalyptus* hybrid species.

2. Materials and methods

2.1. Preparation of the explants

In this study, five improved clones of *Eucalyptus* hybrid species were used and their details are given in Table No 1. Good and actively growing coppice shoots were selected from the healthy mother plant. For direct regeneration, nodes with axillary buds were used as explants. Sprouted branches (approximately 100 mM) of *Eucalyptus* species with performed axillary buds were harvested and cut into explants of about 1 cm, bearing nodal segments with axillary buds. The explants were washed initially with liquid detergent and then vigorously under the running tap water for 60 min to remove the dust particles and the microbial load. Then they were washed with distilled water for 3–5 times followed with Bavsitin (100 mg/100 mL), a fungicide for 1 h, (a systemic fungicide for effective control of wide range of diseases and other fungi in explants). Explants were washed in distilled water for 2–3 times and stored in bottles.

Table 2
Different surface sterilization protocols followed for the *Eucalyptus* hybrid clones.

S. No.	Treatment Methods
1.	Control
2.	Method 1–70% ethanol
3.	Method 2–70% ethanol +1% sodium hypochlorite (12 min) + 0.1% mercuric chloride at different time scale of 1, 2, 3 min respectively.
4.	Method 3–70% ethanol +1% sodium hypochlorite (12 min) + (1 mg/mL) rifampicin at different scale of 1, 3, 5 min respectively.
5.	Method 4–70% ethanol +1% sodium hypochlorite (12 min)+ 0.1% mercuric chloride at different exposure period and (1 mg/mL) rifampicin at different exposure period

2.2. Media preparation

Murashige and Skoog basal medium was gelled with 0.3% gelrite and 30 g/L of sucrose was used in all the experiments as a carbohydrate source (Murashige and Skoog, 1962). The pH of the medium was adjusted to 5.8 using 1N NaOH or 1N HCl and finally autoclaved at 121 °C for 20 min at 1.05 kg/cm^2.

2.3. Sterilization of the glassware's, equipment's and laminar air hood

The surfaces of the laminar air flow hood was wiped using absolute alcohol and was sterilized with UV light for 30 min prior working. The materials like forceps, needles and the glasswares necessary for the inoculation experiments were sterilized by autoclaving them at 121 °C for 20 min at 1.05 kg/cm^2. The stored explants and the sterilized materials were taken to the laminar air hood, and kept under sterile conditions until use.

2.4. Sterilization of the explants of Eucalyptus species

The explants were surface sterilized with different surface sterilizing agents at various period of exposure. Each sterilization treatment was given to five different clones of *Eucalyptus* hybrids used in this study. Four different sterilization protocol were followed for each clones. The protocols involved in the different sterilization methods are given in Table No 2.

2.5. Inoculation and culture conditions

All the explants were washed twice with distilled water. The medium was transferred to the culture vessels. Under aseptic conditions the sterilized explants with different treatments were cut at both the ends to remove the damaged cells by the surface sterilizing agents used and were inoculated into the culture tubes. The cultures were maintained at a photoperiod of 16 h light and 8 h dark at a temperature of 25–27 °C for 2–4 weeks and checked for its contamination visually.

2.6. Data interpretation

The data recorded included the survived, contaminated and dried

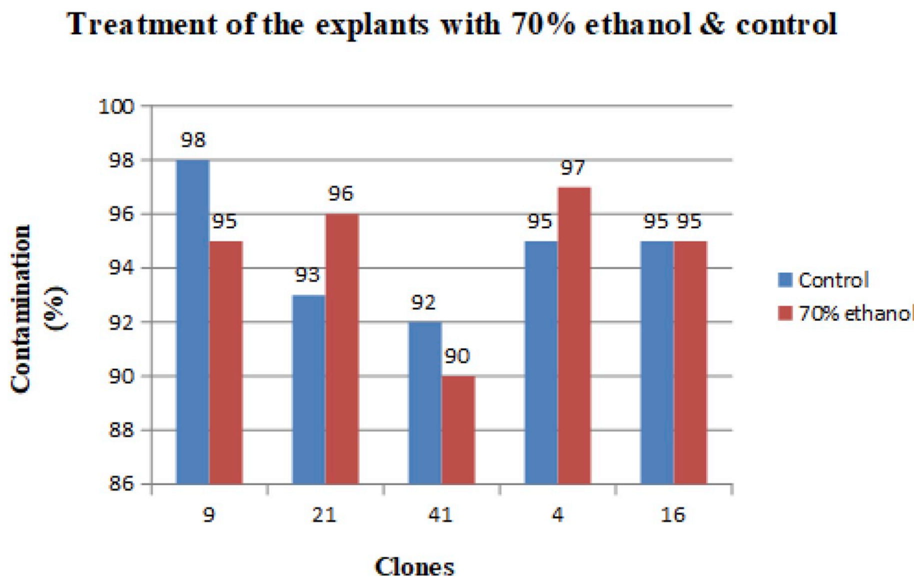

Fig. 1. Percentage of contamination of the five different clones of the *Eucalyptus* hybrid species when their explants were surface sterilized with 70% ethanol and their control group.

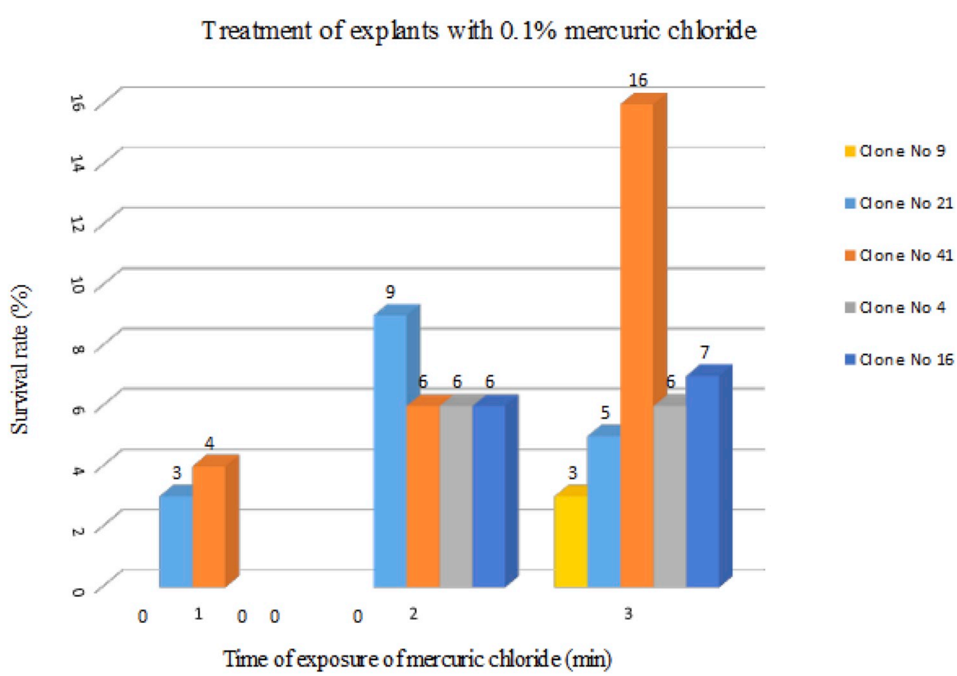

Fig. 2. Percentage of the rate of survival of the five different clones of the *Eucalyptus* hybrid species when their explants were surface sterilized with 0.1% mercuric chloride at different exposure period along with 70% ethanol and 1% sodium hypochlorite.

cultures after 10 days of culturing. The data were converted into percentage. The effective sterilization protocol was found by observing the level of contamination formed during the different sterilization treatments.

3. Results and discussion

3.1. Effect of sterilization with 70% ethanol

Explants when treated with only 70% ethanol as a sterilizing agent exhibited a very high contamination rate of above 90% in all of the clones of the *Eucalyptus* hybrid species. The control tubes also showed a higher level of contamination which was also above 90% in all of the clones. Contamination was on its extreme for control as well as when 70% ethanol was used (Fig. 1).

3.2. Effect of sterilization with 70% ethanol, 1% sodium hypochlorite and 0.1% $HgCl_2$ at different exposure period

When the method 2 was used, the survival rate for clone No.41 was found to be 16%, while 60% of the explants got contaminated and 24% were dried when 0.1% $HgCl_2$ was used for 3 min. The level of contamination was 82%, the survival rate was minimum of 4%, while 14% of the explants dried when 0.1% $HgCl_2$ was used for 1 min. Almost 47% of the explants dried while only 6% survived and the remaining got contaminated when $HgCl_2$ was used for 2 min.

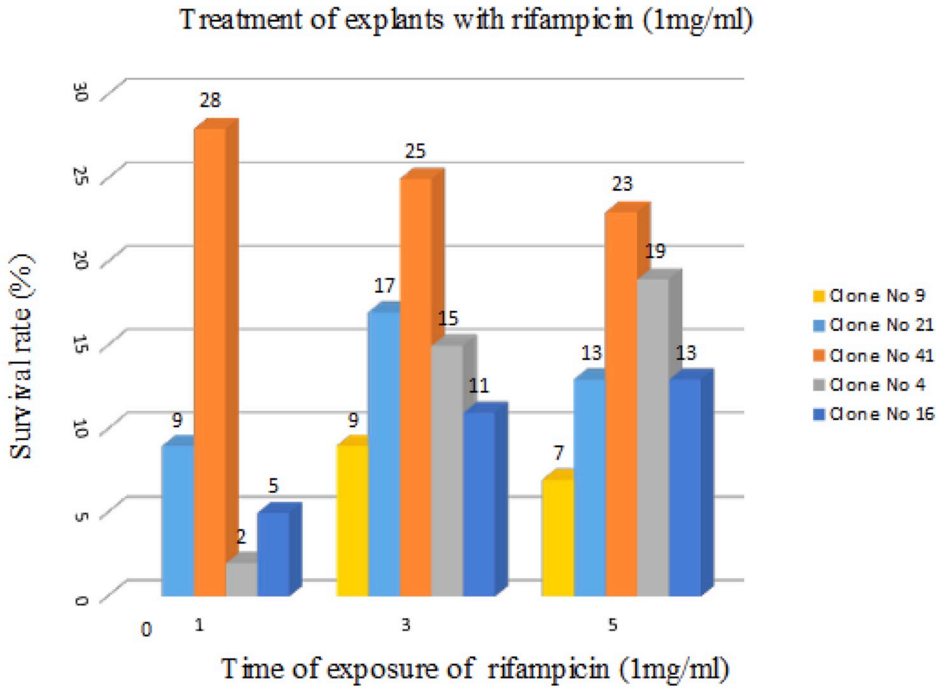

Fig. 3. Percentage of the rate of survival of the five different clones of the *Eucalyptus* hybrid species when their explants were surface sterilized with 1 mg/mL rifampicin at different exposure period along with 70% ethanol and 1% sodium hypochlorite.

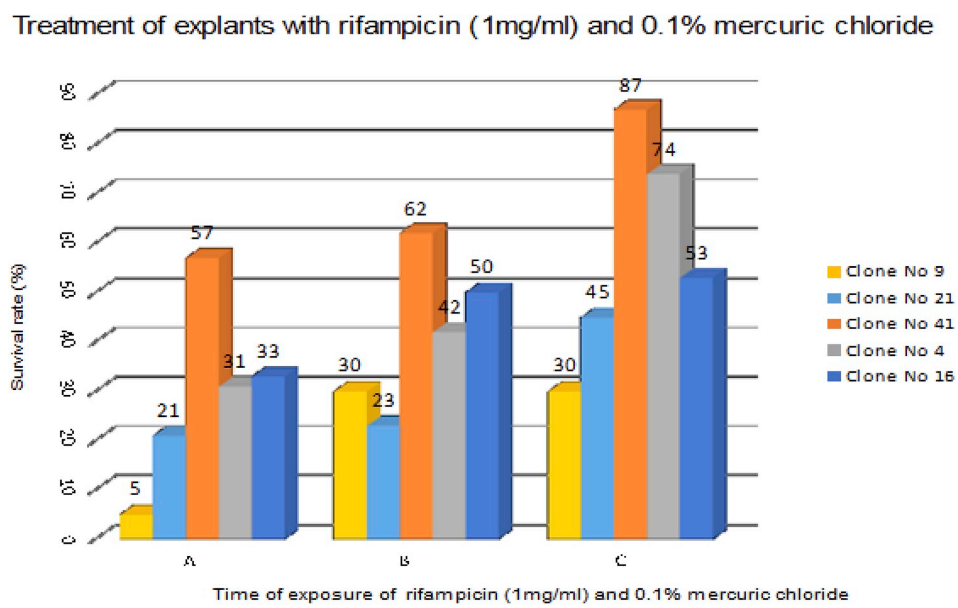

Fig. 4. Percentage of the rate of survival of the five different clones of the *Eucalyptus* hybrid species when their explants were surface sterilized with 0.1% mercuric chloride and 1 mg/mL rifampicin at different exposure period along with 70% ethanol, 1% sodium hypochlorite. A - 0.1% $HgCl_2$ for 1 min & Rifampicin (1 mg/mL) for 1 min; B - 0.1% $HgCl_2$ for 2 min & Rifampicin (1 mg/mL) for 3 min; C - 0.1% $HgCl_2$ for 3 min & Rifampicin (1 mg/mL) for 5 min.

With regard to clone No. 4, the contamination was higher than 80%. The survival rate was found to be 6% when 0.1% $HgCl_2$ was used for 2 and 3 min. Whereas the cultures did not survive at 1 min exposure of 0.1% $HgCl_2$. The survival rates of clone No.21 was found to be below 10%. The level of contamination was found to vary from 65% to 75%, when method 2 was used in clone No.16. None of the cultures survived in clone No.9 when the explants were exposed to 0.1% $HgCl_2$ for 1 min and 2 min (Fig. 2).

3.3. *Effect of sterilization with 70% ethanol, 1% sodium hypochlorite and rifampicin (1 mg/mL) at different exposure period*

In clone No.41, the survival rates of the cultures were above 20% when method 3 was used. The level of contamination was nearly 80% for clone No.4. The level of contamination was found to be above 50%, while the percentage of the cultures dried was also found to be nearly 35% with regard to clone No. 21. The survival rate was found to be above 10% in clone No.16 when the cultures where exposed to rifampicin for 3 min and 5 min. The survival rate was found to be below 10% when method 3 was used in clone No.9 (Fig. 3).

3.4. Effect of sterilization with 70% ethanol, 1% sodium chloride, rifampicin and 0.1% $HgCl_2$ at different exposure period

All the clones showed very less contamination rate when rifampicin (1 mg/mL) and 0.1% $HgCl_2$ was used together (method 4). But still the survival rates of the clones were found to be on the higher side when the explants were exposed to 0.1% $HgCl_2$ for 3 min and 1 mg/mL rifampicin for 5 min (Fig. 4). Even the smallest concentration of $HgCl_2$ serves to be an effective sterilizing agent (Sen et al., 2013). Explants treated with 0.1% $HgCl_2$ and rifampicin (1 mg/mL) at different time exposure showed significant differences in the survival rate of the plant tissues. When 0.1% $HgCl_2$ and rifampicin (1 mg/mL) were used together, for 3 min and 5 min respectively, the survival rates of the nodal explants were found to be improved.

$HgCl_2$ is said to have deleterious effect on explants (Danso et al., 2011) and many plant tissue culture experiments use them as surface sterilizing agents (Naika and Krishna, 2008). Browning and death of the explants occur if the explants are exposed to $HgCl_2$ for a very longer period (Johnson et al., 2005). So we have used 0.1% $HgCl_2$ only for a very shorter period. At lower concentrations it is the most effective disinfective agent against soil borne microbes (Ansar and Iqbal, 2015). Even when ethanol was used at a concentration of 100%, contamination was found to be at its peak (100%) and there was no proliferation of the explants which supports our results (Fig. 1). To achieve a better productivity, antibiotics that are less harmful to the plants were used as the sterilizing agents. Rifampicin had no toxic effect on plants. Plants grew well in the presence of rifampicin during the surface sterilization process (Phillips et al., 1981).

$HgCl_2$ exhibits bactericidal action which is more powerful and serves to be a good sterilizing agent. Gautam et al. reveals that treating the explants with 0.1% $HgCl_2$ gave maximum survival rates of cultures with minimal contamination which is in accordance with our results (Gautam et al., 2001).

$HgCl_2$ and rifampicin which when used individually at a different period of exposure the contamination rates were found to be very high, but when they were used together it lowered the contamination rates. It was also noticed that the time of exposure of 1% $NaOCl_2$ varied between 13 and 15 min. When the explants were exposed to $NaOCl_2$ for longer periods it resulted in the browning of the explants leading to death of the explants. Explants must be handled with care so as to avoid the over-exposure of the explants to $NaOCl_2$, but when used between 13 and 15 min resulted in reduced contamination rates.

All the five *Eucalyptus* hybrid clones showed uniform sterilization protocol. Clone No.41 *E.tereticornis X E.urophylla*) showed fruitful results; almost 87% of the explants remained uncontaminated and green. Bud break was found in the MS media in all of the five clones. Clone No.4 showed 74% of healthy cultures while 53% of the cultures remained green in clone No. 16. Clone No. 21 had 45% of uncontaminated cultures while it was found to be 30% in clone No. 9. On the basis of these findings 0.1% $HgCl_2$ was used for 3 min and rifampicin (1 mg/mL) was used for 5 min while sodium hypochlorite was used for about 13–15 min in the subsequent experiments. All these surface sterilizing agents will provide better results for the micropropagation of the clones.

4. Conclusions

Proper sterilization protocol results in efficient shoot regeneration in the initiation stage of the micropropagation. The choice of the disinfectants plays a key role in producing uncontaminated cultures. The success of the micropropagation is the outcome of the properly standardized sterilization procedures that were followed. However properly sterilized equipment's and aseptic conditions are also necessary. These sterilization guidelines can be followed for the micropropagation of the *Eucalyptus* hybrid clones used in the present study.

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