Effective and economical explants surface sterilization protocol for microbial contamination of field grown explants in vitro cultures of some forest trees; Acacia senegal as a model

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ABSTRACT

In vitro micropropagation technique for mass production of plant materials faces vital problems induced by microorganism's contaminants such as: bacteria, fungi and endophytic ones, this research study aimed to manifest and manipulate the microbial contamination in forest tree tissue culture laboratory at the Forestry and gum Arabic Research Centre in Sudan in vitro cultures, and to eliminate surface contaminants by economically feasible sterilant. The tissue culture work was conducted on young and mature explants of Acacia senegal (El-Hashab). Explants; were washed under running tap water for 30 minutes, then soaked in a solution mix of 100mg/l ascorbic and 150 mg/l citric acid for 10 minutes. The explants were immersed in 70% ethanol for 15 seconds, washed by several changes of sterilized double distilled water; then immersed in 100ml Clorox solution of 0.0%, 20%, 30% and 60% mixed with 3 drops of Tween 20 for 20 minutes. After continuous shaking explants were rinsed three times with sterilized distilled water under laminar airflow cabinet, and then cultured on culture tubes. In this study percentages of commercial Clorox were found to be effective in sterilization, elimination of contaminants and survival of A. senegal explants. The result showed a high number of uncontaminated and survival of Shambat yard explants with 20% Clorox. The concentrations 30% and 60% were found not effective for sterilization of explants from Elobeid Hashab belt that showed 100% contamination. The conclusion is that; the package of contamination control of tissue cultures by manipulating well aseptic cultures and to maintain good laboratory practice, with carrying routine testing for contamination.

Keywords: Plant tissue culture, microorganisms, contaminants, sterilization.

INTRODUCTION

The rapid increasing of world population is resulting in extreme pressure on existing trees and forests that is rendering some plant species endangered, vulnerable, and or threatened (IUCN, 1998). Therefore, for continuous supply and flow of wood and non-wood products as well as sustainable utilization of forest resources; the suggested solution is on application of biotechnological methods for propagation of targeted...
Generally, work on propagation tissue culture started since 1980’s; and recently it includes medicinal plants and some socioeconomic forest trees, woody and non-woody ones such as; Acacia senegal (El-Hashab), A. tortilis, Grewia sp. and others (Ali et al., 2012). In vitro micropropagation technique for mass production of plant materials faces vital problems induced by microorganism’s contaminants such as; bacteria, fungi and endophytic ones. However in vitro plant culture contamination has economic impact due to its direct influence in losses during in vitro culture of plants. Consequently, the losses due to contamination average is between 3-15% at every subculture in the majority of commercial and scientific plant tissue culture laboratories (Leifert et al., 1989).

Therefore the establishment of sterile cultures was reported by many workers as a major challenge with some plant materials as well as; the process of cleaning and disinfecting explants material. The subsequent failure will results in loss of rare plus parent plant, time and cost, as well as the loss of nutrient culture media due to contamination. However, tissue culture media such as; Murashige and Skoog (1962) medium; were found capable to support growth of common microorganisms.

Fungal and bacterial contamination was detected after few days of explants cultured in the laboratory growth room. The sources of microbial contamination are not determined and can be explants, worker personnel, various in door insects, laminar airflow hood, media and instruments. Schreiber et al. (1996) reported that Bacillus macerans, a bacterial contaminant, could be viable on forceps even after being stored in 95% ethanol for several weeks, and remained viable after flaming. The bacteria are eliminated only by autoclaving at 121°C for 20min or by heating for 6–8s over a Bunsen burner. Additionally, Clavibacter may survive alcohol flaming, hence washing the instruments to remove contaminants on surface and periodic autoclaving are desirable. Nineteen microbial contaminants of which eleven bacteria and eight fungi were found and identified associated with tissue culture plants and the laboratory environments (Odutayo et al., 2007). However, autoclaving at 121°C, 15 lb/sq. in, for at least 15 minutes, is the method commonly used for sterilizing; glassware, handling and surgical instruments and media. Moreover to reduce risks of contamination from air settings in cultures, therefore all working places and Laminar-flow Cabinet should be kept clean (Ali et al., 2012).

Explants sterilization may be enhanced by placing explants in a 70% ethyl alcohol solution prior to treatment with another disinfectant solution. The use of a two-step sterilization procedure has proven beneficial with certain species. Using a wetting agent, such as Tween 20, can be added to the disinfectants to reduce surface tension and allow better surface contact. Generally, the less concentrated solution for the shortest time interval to obtain clean explants is desirable. The use of antibiotics or fungicides in nutrient medium is generally not successful where antibiotics need filter sterilization and added to cooled media as they are heat labile. These agents can repress the growth of some microorganisms and can also suppress or even kill the plant tissue. Moreover conducting sterilization process under vacuum may result in removal of air bubbles and provides more efficient sterilization process. However few mature tree species have been propagated by tissue culture methods (Ali, 1997).

Nevertheless, large scale monitor methods of seedlings populations for qualitative desirable quantitative traits and; maintenance of clone germplasm for long-term evaluation of planted material, have to be developed. Accordingly, based on the plant species, media composition and culture conditions in vitro propagation could be achieved by direct shoot organogenesis (Seeni and Latha, 2000; Ndakidemi, 2013). It is therefore important to employ tissue culture techniques to establish in vitro micropropagation protocols for threatened and endangered forest tree species (Ali, 2009).

**MATERIALS AND METHODS**

**Plant materials**

Various developmental stages of explants were obtained from aseptically germinated seedlings, from new twigs, mature trees of A. senegal, Elobeid, Hashab belt, and Shambat yard at the Faculty of Forestry, University of Khartoum.

**Explants sterilization**

The mature explants were washed under running tap water for 30 minutes to remove surface dust and then soaked in a solution mixture of 100mg/l ascorbic and 150mg/l citric acid for 10 minutes. The explants were then immersed in 70% ethanol for 15 seconds, washed by several changes of sterilized distilled water; and then immersed in 100ml Clorox solution of 20%, 30% and 60% (Sodium hypochlorite (NaOCl) solution found in laundry bleach, is approximately a 5.25% (v/v); mixed with 3 drops of Tween 20 for 20 minutes for both mature explants (Ali, 1997). After continuous shaking the explants were rinsed three times with sterilized distilled water under laminar airflow cabinet. Explants excised from in vitro germinated seedlings were cultured without sterilization.
Table 1. Effectiveness of some chemical compounds used for sterilization for in vitro micropropagation (Forest and gum Arabic Tissue culture laboratory)

<table>
<thead>
<tr>
<th>Disinfectant</th>
<th>Concentration (%)</th>
<th>Exposure (min)</th>
<th>Effectiveness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium hypochlorite</td>
<td>9-10</td>
<td>5-30</td>
<td>V. good</td>
</tr>
<tr>
<td>Sodium hypochlorite*</td>
<td>0.5-5</td>
<td>5-30</td>
<td>V. good</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>3-12</td>
<td>5-15</td>
<td>Good</td>
</tr>
<tr>
<td>Ethyl alcohol</td>
<td>70-95</td>
<td>0.1-5.0</td>
<td>V. good</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>1</td>
<td>5-30</td>
<td>Good</td>
</tr>
<tr>
<td>Mercuric chloride</td>
<td>0.1-1.0</td>
<td>2-10</td>
<td>Satisfactory</td>
</tr>
</tbody>
</table>

Table 2. Effect of Clorox concentrations on contamination level of cultured explants from mature trees of A. senegal from two sources.

<table>
<thead>
<tr>
<th>Clorox concentration</th>
<th>Contaminated explants%</th>
<th>Survival%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shambat</td>
<td>Elobeid</td>
</tr>
<tr>
<td></td>
<td>Shambat</td>
<td>Elobeid</td>
</tr>
<tr>
<td>0</td>
<td>95</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>60</td>
<td>-</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 3. ANOVA Effect of Clorox concentrations on explants sterilization

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P. value</th>
<th>F crit.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>17116</td>
<td>3</td>
<td>5705.333</td>
<td>16.17769</td>
<td>0.003</td>
<td>4.757063</td>
</tr>
<tr>
<td>Within Groups</td>
<td>2116</td>
<td>6</td>
<td>352.6667</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Null hypothesis was rejected p < 0.05 (Means are Different)
F crit. F Critical Value

Statistical analysis

All data presented as contaminated explants percentages, the level of significance was p<0.05, Microsoft QI Macros 2016 programme (ANOVA) was used to accept or reject the null hypothesis exploring the effectiveness of Clorox.

RESULTS AND DISCUSSION

Various sterilant agents are used to for sterilization processes in plant tissues for in vitro cultures, but some show toxicity to the plant tissues, hence proper concentration of sterilants duration of exposing explants to various sterilants, all has to be standardized to minimize explants losses and achieve high survival rates. In essence, requirements of concentration and time of exposure differ from one plant species to another and for different parts of plants depending on morphological characters and formulation of the growth medium (Table 1). Clorox of various percentages was found to be effective in sterilization and survival of A. senegal explants collected from Shambat yard. The 30% concentration resulted in high number of uncontaminated and survived explants from Shambat yard compared to 20% once. However, 30% and 60% concentrations were found had no effect in sterilization of explants obtained from Elobeid Hashab garden, where 100% contamination was obtained (Table 2). Similar result was obtained by El-tayeb, (2004) work on in vitro propagationon A. seyal and Ali (2009) work on A. senegal and A. tortilis. (Figure 1 below).

The observed fungal infection showed white, dark mycelia and chlamydospores, whereas; yellowish to brownish growths pointed to bacterial infection. (Figure 2 below). Nevertheless cultures exhibiting visible contamination are discarded. The contamination showed by explants obtained from Elobeid may be due to the season of the year, where plant material is being grown and location of the explants on the source plant are reported to be significant factors in the establishing clean cultures. The low percentage also can be associated with the location and ecological habitats that probably had their effect. Plant material grown in yards may be in an active state of growth that is generally found to be cleaner as compared to dormant shoot tissue from the field that is often more contaminated.

The result was inline with many scientists' findings that few forest tree species with explants from mature trees have been propagated by tissue culture techniques. On the other hand juvenile tissues from forest trees are more
Figure 1. A and B Infectious *A. senegal* cultured jars in culture transfer room. (a) Fungal contamination (b) Bacterial contamination
responsive to in vitro manipulation comparable with mature ones. The longer life span of trees may add to the problem of contamination in vitro by the symbiotic association of microorganisms. It is evident that there are few micropropagation protocols from mature tree explants. Also, many woody plant species and particularly tree legumes are known for their recalcitrant nature of regeneration (Anis et al., 2005; Jha et al., 2004; Chang et al. (2001).

CONCLUSION

Routine laboratory investigation and research studies are required for various sterilizers’ doses to their effectiveness in eliminating contaminants from in vitro explants cultures. Also, phytotoxicity studies should be conducted to determine the effect of the antibiotic and antifungal agents on the in vitro plantlets growth. Microplants as a consequence of a sub-optimal production process, from autotrophic tissue cultures may be physiologically more mature and show greater constitutive disease-resistance than those from heterotrophic culture. Autotrophic cultures do not support high levels of contamination with micro-organisms and may be used to rescue contaminated cultures.

The best strategy to control tissue culture contamination is to establish aseptic cultures and to maintain good laboratory practice, including routine testing for contamination by cultivable micro-organisms. The establishment of sterile cultures can be a major challenge specifically with field obtained plant materials. The initial process of cleaning and disinfecting plant material especially if the parent plant is rare and the supply is limited can be time-consuming and expensive. However recently demand for seeds and special

**Figure 2.** Infectious cultures by Fungi and Bacteris

**Figure 3.** Clean in vitro seed and seedlings cultures from in vitro germinated A. tortilis seeds and A. senegal seed and seedlings
plantation drives and greening activities have substantially increased the demand for tree seedlings. Innovative and cost-effective technologies are needed to develop and to select and test desirable genotypes, to rapidly propagate selected genotypes, and to produce genetically modified varieties of commercially important plants. According to this research finding the authors were indebted to recommend the use of 20% Colrex to sterilize explants obtained from plantation habitat for simple feasible in vitro propagation protocol of A. senegal tree.

REFERENCES


