ACTA SCIENTIFIC MICROBIOLOGY (ISSN: 2581-3226)

Volume 2 Issue 5 May 2019

Research Article

Feasible Surface Sterilization Protocol for Microbial Contamination of Some Forest Trees on *in vitro* Cultures Explants

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Received: March 13, 2019; **Published:** April 18, 2019

Abstract

In vitro germination and micropropagation techniques for mass production of plant materials face vital problems that induced by microorganism's contaminants such as; bacteria, fungi and endophytic ones. The role of these contaminants in tissue cultures techniques and laboratories was not been studied in our laboratories whereas; it was been related to explants regeneration capacity, animal and human cells transformation under *in vitro* cultivation. Therefore the paper objective is to illustrate microbial contamination in some tree seeds and explants *in vitro* cultured and; to eliminate surface contaminants by applying economically feasible sterilant. The work was conducted at the Tissue Culture Laboratory at Forest and gum Arabic Research Centre in Sudan. The method starts with washing of *Acacia tortilis*, *A. senegal* (El-Hashab) and *Dalbergia melanoxylen* seeds or young and mature explants under tap water for 30 minutes then, soaked in a solution mixture (100mg/l ascorbic +150mg/l citric acids) for 10 minutes. Separately surface pretreated seeds with 50% sulphuric acid for 45 min then subjected to various steps as explants; were immersed in 70% ethanol for 15 seconds, washed with several changes of sterilized double distilled water then; immersed in 100 ml 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 sterilize distilled water under laminar airflow cabinet and then cultured on culture tubes. The result showed that; percentages of commercial Clorox applied had significant effect ($p \leq 0.05$) in sterilization and elimination of contaminants hence, survival of *A. senegal* explants. The result showed a high number of uncontaminated and survival of field 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 plant tissue cultures by manipulate 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 rendering some plant species endangered, vulnerable, and or threatened [1]. Therefore, for continuous supply and flow of wood and non-wood products as well as sustainable utilization of forest resources; a suggested solution is application of biotechnology methods for propagation of targeted endangered and multipurpose plants species.

Generally, work on plant *in vitro* germination and micropropagation using tissue culture started since 1980's; and recently it includes medicinal plants and some socioeconomic forest trees, woody and non-woody such as; Acacia senegal (El-Hashab), A. tortilis, Grewia sp., Balanitis aegyptiaca and others [2]. *In vitro* micropropagation techniques for mass seedlings production faces vital problems induced by microorganism's contaminants such as; bacteria, fungi and endophytic ones. The identified microorganisms are apparently non pathogenic and some can survive surface sterilization in initial stages of plant tissue and organ cut wounds. Bacteria inhabit surfaces of most plants and also present inside many plant organs, hence may have positive, negative or neutral impacts on plant hosts. Positive effect may improve plant nutrition or increase resistance to biotic and abiotic stresses whereas; pathogenic ones may kill or reduce vigor of plant hosts. In addition, some bacteria inhabit plants and profit from excess metabolites or protection [3]. However endophytic contamination reduces efficiency in micropropagation as well as causes plant culture losses [4,5]. However *in vitro* plant culture contamination has negative economic impact due to its direct influence in losses during *in vitro* cultured plants. The losses due to contamination average between 3-15% at every subculture in majority of commercial and scientific plant tissue culture laboratories [6].

The establishment of sterile plant 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 [7]. 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 [8] medium; were found capable to support growth of common microorganisms.

Fungal and bacterial contamination was possibly detected after few days of explants cultured in a laboratory growth room. The sources of microbial contamination are not determined and can be explants, worker personnel, various indoor insects, laminar airflow hood, growth media and instruments. Schreiber., *et al*. [9] 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 20 min or by heating for 6–8s over a Bunsen burner. Additionally, Clavibacter may survive alcohol flaming, hence washing instruments to remove contaminants on surface and periodic autoclaving are desirable. Some Anis., *et al*. [10] work on Balanites aegyptiaca use 1% (w/v) Bavistin, a fungicide then explants were immersed in 5% (v/v) a Teepol detergent solution, then 0.1% (w/v) mercuric chloride solution as surface disinfectant. Nineteen microbial contaminants of which eleven bacteria and eight fungi were found and identified associated with tissue culture plants and laboratory environments [11]. However, autoclaving at 121°C, 15 lb/sq. in, for at least 15 minutes, is a 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 [2].

Explants sterilization may be enhanced by placing explants in 70% ethyl alcohol solution prior to treatment with other disinfectant solution. The use of a two-step sterilization procedure has proven beneficial with certain plant species. Using a wetting agent, such as Tween 20, can be added to disinfectants to reduce surface tension and allow better surface contact. Generally, less concen¬trated solution for the shortest time interval to obtain clean explants is desirable. However the use of antibiotics or fungicides in nutrient medium was reported to be unsuccessful [12] where, antibiotics need filter sterilization and added to cooled media as they are heat labile. These agents can repress growth of some microorgan¬isms and also suppress or even kill 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 [2].

Nevertheless large scale monitor methods of seedlings populations for qualitative desirable quantitative traits and; maintenance of clone germplasm for long-term evaluation of plant material have to be developed. In addition the use of biotechnology on trees has opened up new possibilities for rapid mass multiplication of existing stocks of germplasm, as well as conservation of medicinally important plants parts [13,14]. Accordingly based on plant species, media composition and culture conditions *in vitro* propagation can be achieve by direct shoot organogenesis [15,16]. Generally plant propagation by seeds does not produce plant. Seeds proved to be rich sources of some useful secondary metabolic compounds more than plants grown in-vitro and; seed raised plants were proved to be another source rich of those compounds than seeds that directed to commercial use. Therefore it is important to employ tissue culture techniques to establish in vitro germination and micropropagation protocols for threatened and endangered forest tree species [17].

Materials and Methods

- **Plant materials:** Various developmental stages of explants were obtained from aseptically germinated seedlings, from new twigs from mature trees of *A. senegal* from Elobeid, Hashab belt, and from Shambat yard at the Faculty of Forestry, University of Khartoum. *Dalbergia melanoxylen* explants were obtained from field grown mature trees and seeds were obtained from the certified National Seed Centre in Sudan. The work was carried out during the years 2011, 2015 and 2017 and currently is running at Forestry and gum Arabic research centre in Sudan.
- **Seed surface sterilization**; seeds treated with 50% (W/V) sulphuric acid for 45 min., washed in several changes of sterilized water, dipped in absolute ethanol for 3-5 seconds; washed thoroughly by distilled water then after immersed in 20% (v/v) sodium hypochlorite solution containing 1.5% available chlorine for 20 min. and washed in four changes sterilized distill water.
- **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 150 mg/l citric acid for 10 minutes. The explants were immersed in 70% ethanol for 15 seconds, washed by several changes of sterilized distilled water; and then immersed in 100 ml Clorox solution of 20%, 30% and 60% 20% (v/v) sodium hypochlorite (NaOCl) solution containing 1.5% available chlorine and solution found in laundry bleach, is approximately 5.25% (v/v); mixed with 3 drops of Tween 20 for 20 minutes for both mature explants [2]. After continuous shaking the explants were rinsed three times with sterilized distilled water under laminar airflow cabinet. Regenerated explants excised from *in vitro* germinated seedlings of *Acacia senegal* and *A. tortilis* were cultured without sterilization.
- **Statistical analysis**: In all experiments carried out Completely Randomized design (CRD) was used. Each treatment also had 3 replicates of culture vessel with different number of vessels for different plant species. All data presented, contaminated seed germination and

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 for sterilization processes in plant tissues for *in vitro* cultures but; some showed toxicity to plant tissues, hence proper concentration of sterilant, period of time exposing explants to various sterilants, all has to be standardized to minimize explants loss and achieve high survival rates (Table 1). In essence sterilant concentration and time of exposure differ from one plant species to another one, and even for different parts of plants. Clorox of various percentages was found to be effective in sterilization and survival of A. senegal explants collected from Khartoum city yard. The 30% concentration resulted in high number of uncontaminated and survived explants from Shambat yard compared to 20% ones. 30% and 60% concentrations had no significant effect ($p \leq 0.05$) on sterilization of explants obtained from Elobeid Hashab gum belt gardens 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) on and A. tortilis and Ali., *et al.* [2] on A. senegal. Similarly minimum contamination have been achieved by Fatema., *et al.* [18] work on Althaea officinalis using 4% NaOCl for 10 min followed by 0.3% HgCl2 for 7 min. Nevertheless cultures exhibiting visible contamination are discarded.

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

Table 1: Effectiveness of some chemical compounds used for sterilization for *in vitro* micropropagation (Forest and gum Arabic Tissue culture laboratory).

Clorox concentration 5.25% (v/v)	Contaminated explants%		Survival%	
	Field yard	Elobeid	Field yard	Elobeid
0	95	100	5	0
20	50	100	50	0
30	4	100	96	0
60	0	100	0	0

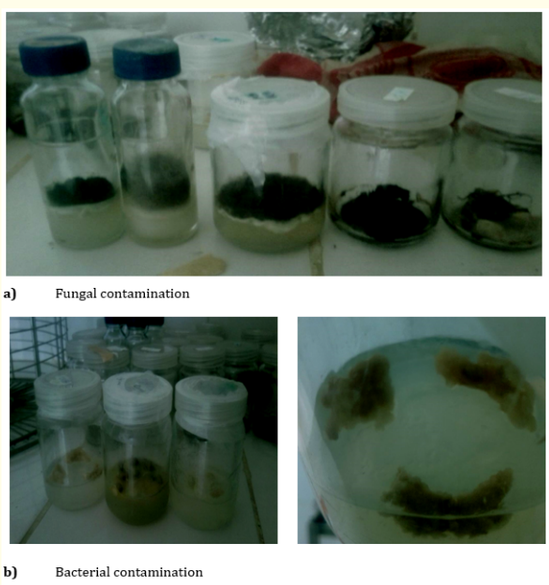
Table 2: Effect of Clorox concentrations on contamination level of cultured explants from mature trees of *A. senegal* from two sources.

Source of Variation	SS	df	MS	F	P. value	F crit.*
Between Groups	17116	3	5705.333	16.17769	0.003	4.757063
Within Groups	2116	6	352.6667			

Table 3: ANOVA Effect of Clorox concentrations on explants sterilization.

Null hypothesis was rejected $p < 0.05$ (Different Means) *F crit. F Criteria.

The observed fungal infection was showed by white, dark black coloured mycelia and Chlamydia spores whereas; yellowish to brownish growths indicate bacterial infection. The contamination showed by explants obtained from Elobeid gum belt gardens probably may be attributed to effects exerted by season of year; location on where plant material is being grown and origin of explants on mother plant; all are reported as sig¬nificant factors in establish of clean *in vitro* cultures. The obtained low percentages also may be associated with location and ecological habitats that probably had their effects. Plant materials grown in yards and *in vitro* ones probably may be in an active state of growth and development that is generally found to be cleaner as compared to dormant shoot tissue from the field that is often more contaminated. The results were in line 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 of A. tortilis and A. senegal (Figure 3 A and B) trees showed high positive response to *in vitro* manipulation comparable to mature ones of Dalbergia melanoxylen (Figure 4).

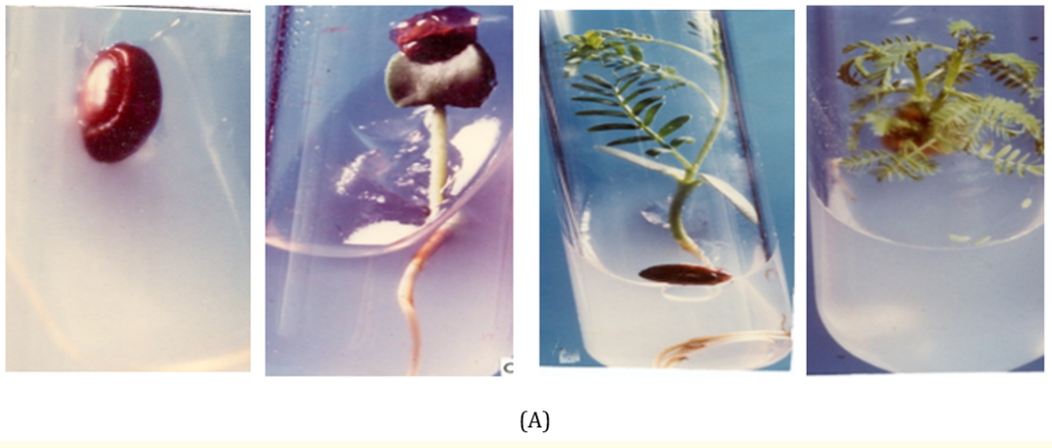


a) Fungal contamination

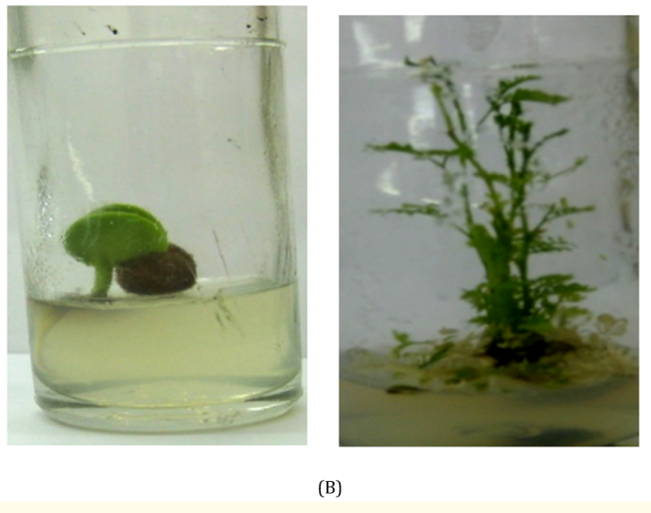
b) Bacterial contamination

Figure 1 a and b: Infectious Acacia senegal cultured jars in culture transfer room (Forest research tissue culture laboratory 2017).

Figure 2: Infectious cultures by A) Bacteria and B) Fungi (Forest research tissue culture laboratory 2017.



(A)



(B)

Figure 3 A and B: Clean in vitro seed and seedlings cultures from in vitro germinated A. tortilis seeds and A. senegal seed and seedlings (Forest research tissue culture laboratory 2012).

Figure 4: Infection of mature Dalbergia melanoxylen explants (Forest research tissue culture laboratory 2018).

The longer life span of trees probably adds problems of contamination *in vitro* by symbiotic association of microorganisms. Evident that, few successful micropropagation protocols were obtained from mature tree explants and; many woody plant species and particularly tree legumes are known for their recalcitrant nature of regeneration [13]. Routine laboratory investigation and research studies are required to determine doses of various sterilizers and efficacy in eliminating of contaminants from *in vitro* explants cultures. Also, phytotoxicity studies should be conducted to determine the effect of antibiotic and antifungal agents on *in vitro* plantlets and seedling growth. Microplants as a consequence of a sub-optimal production process, from autotrophic tissue cultures may be physiologically mature and show greater constitutive disease-resistance than those from heterotrophic culture. Autotrophic cultures probably do not support high level of contamination with microorganisms and may be used to rescue contaminated cultures. 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 and A. tortilis add to; in vitro seed germination and seedling growth.

Conclusion

The best strategy to control tissue culture contamination is to establish aseptic cultures, maintain good laboratory practice, including routine testing for contamination by cultivable microorganisms. The establishment of sterile cultures can be a challenge specifically with field obtained plant materials. The initial process of cleaning and disinfecting plant material especially if parent plant is rare and the supply is limited can be time-consuming and expensive. However recently demand for seeds and special plantation drives and greening activities have substantially increased demand for tree seedlings. Innovative and cost-effective technologies are needed to develop and, select and test desirable genotypes, to rapidly propagate selected genotypes, and produce genetically modified varieties of commercially important plants.

Contamination of epiphytic microorganisms due to poor aseptic technique or improper sterilization of equipment can be eliminated or minimized by; improvements in training technical staff, handling of equipment and media manipulation. Eliminating of endophytic contaminants is a challenge and more problematic that facing tissue culture techniques application for explants of field grown trees. Indexing cultures at initiation stages and throughout culture cycle significantly assist in reducing number of explants having contaminants escaping detection. Identification and classification of persistent endophytic contaminants, testing plant material possibly with antibiotics and sterilant to determine appropriate concentrations possibly for effective sterilization treatment and minimal phytotoxicity and losses.

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