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EFFICIENT STERILIZATION PROTOCOLS FOR DIFFERENT EXPLANTS OF AN ENDANGERED MEDICINAL HERB *SWERTIA CHIRAYITA*

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ABSTRACT
Swertia chirayita is a very important medicinal plant indigenous to temperate Himalayas. This specie is now identified as an endangered species due to its immense medicinal properties. To conserve it tissue culture methods are being developed for its mass multiplication. Sterilization is most important step of the tissue culture; therefore a protocol has been standardized for sterilization of nodal segments and seeds of *Swertia chirayita* for its micropropagation intended for its mass propagation and conservation. Three sterilizing agents *viz.*, $HgCl_2$, NaOCl and CH_3CH_2OH were tested for sterilization by varying their concentration and time of exposure. Maximum healthy shoots were obtained when explants were sterilized with 0.1% $HgCl_2$ for 10 minutes, inoculated on MS basal media with appropriate hormones and observing them for 30 days, while 1% NaOCl for 5 minutes exposure provided best of aseptic seed germination. Results showed that out of three sterilizing agents $HgCl_2$ was significantly reducing the contamination of explants and NaOCl for seeds.

KEYWORDS: conservation, contamination, micropropagation, sterilization, *Swertia chirayita*

INTRODUCTION

Out of rich biodiversity of Uttarakhand, *Swertia chirayita* is a medicinal plant indigenous to temperate Himalayas. Its medicinal usage is reported in Indian pharmaceutical keycodex, the British and the American pharmacopoeias and in different traditional systems of medicines such as the Ayurveda, Unani and Siddha. *Swertia chirayita* has an established domestic (Indian) and international market. Due to its high demand in the local, national and international drug manufacturers, illegal, unscientific and indiscriminate extraction of *Swertia chirayita* from its wild habitat has increased. This has led to the low population of *Swertia chirayita* and hence it's endangered status.

Conservation through vegetative propagation is slow and time consuming but tissue culture offers an alternative tool for rapid multiplication and conservation of disease free propagules in a short period, which will further enable uninterrupted supply of raw material, *Swertia chirayita* for drug preparation. Tissue culture comprises of various stages: selection of explants; aseptic culture establishment; multiplication of propagules; rooting and acclimatization of plantlets. But the most important and challenging step is sterilization of explant for aseptic culture establishment. Sterilization is the process of making explants contamination free before establishment of culture.

Explant contamination depends on the several plant and environmental related factors such as species, age, explant source and prevailing weather condition. In fact according to losses due to contamination under *in-vitro* conditions average between 3-15% at every subculture in the majority of commercial and scientific plant tissue culture laboratories (Leifert *et al.*,1989), the majority of which is caused by fungal, yeast and bacterial contaminant (Leifert *et al.*,1994). Consequently leading to the waste of time, effort and material which if not mitigated can have serious economic problems. *Swertia chirayita* is an important and endangered medicinal plant belonging to the family Gentinaceae. As *Swertia chirayita* is an endangered medicinal herb, optimum conditions like type of sterilizing agent, its concentration and time of exposure to that sterilizing agent are mandatory for asepsis of *Swertia chirayita*. These sterilants are toxic to the plant tissue, hence the type, concentration, time of exposure and removal of traces of sterilizing agent becomes important in standardizing sterilization protocol.
Therefore, the present study has been done to standardize the sterilization method for explant and seeds of *Swertia chirayita* for *in-vitro* propagation intended for its conservation by using different types of sterilizing agents by varying their concentration and duration of exposure.

MATERIALS AND METHODS

Sample Collection

The plants and seeds of *Swertia chirayita* were procured from Hitech Forest Nursery, Deoban (Chakrata), and Munsiyari, Uttarakhand respectively. The flowering plants were dried and prepared herbarium was submitted to Botanical Survey of India, Northern Regional Centre, Dehradun (BSI) for identification upto species level and plants were given the accession no.113342. Young, juvenile and healthy plants were selected for further experimentation. Seed samples were sent to NBPGR, Pusa Campus, New Delhi, for its germplasm conservation and the accession No. IC-567642 was obtained. Seeds were washed and air dried at room temperature and sealed in sample bag till further use. Potted plants procured from the nursery were maintained in the polyhouse till further use. All the glassware and instruments to be used were thoroughly cleaned and autoclaved at 15 psi for 40 minutes after drying them at 90ºC in oven.

Explant Sterilization

Nodal segments of *Swertia chirayita* were excised from plants of *Swertia chirayita*. These nodal segments were trimmed to approx 4 cm. in size and large fleshy leaves were removed. All the brown skins were cleaned thoroughly. Procedure of sterilization for *Swertia chirayita* had been divided into two phases: Phase I (outside Laminar Air Flow) and Phase II (inside Laminar Air Flow). In phase I detergent washing was performed and phase II comprised of exposure to sterilizing agent and then washing to remove the traces of sterilizing agent. Three different kinds of sterilizing agents *viz.*, Mercuric Chloride ($HgCl_2$) (.05%-.15%), Sodium Hypochlorite (NaOCl) (0.5%-1.5%) and Ethyl alcohol (CH_3CH_2OH) (70%-90%) were tested for explant sterilization by varying their concentration and time of exposure (1-15 minutes).

Seed Sterilization

Seeds of *Swertia chirayita* were small in size; this made its washing and sterilization a little bit uneasy. To avoid this situation the autoclaved surgical gauge was used as a sieving device and hence their handling was made easy. Varying concentrations of different sterilizing agents *viz.* Mercuric Chloride ($HgCl_2$) (.05%-.15%), Sodium Hypochlorite (NaOCl) (0.5%-1.5%) and Ethyl alcohol (CH_3CH_2OH) (70%-90%) and time of exposure (1-15 minutes) were used for decontaminating the seeds.

Inoculation

Murashige and Skoog basal medium supplemented with appropriate cytokinins and auxin was used for inoculation. Medium was checked for the contamination before inoculation. Sterilized explants were trimmed suitably to remove sterilizing agent affected parts/brown parts. Explants and seeds were then inoculated on the appropriate medium and labeled properly. Regular and proper record for contamination, browning and growth/bud break/germination (seeds) were taken for 30 days.

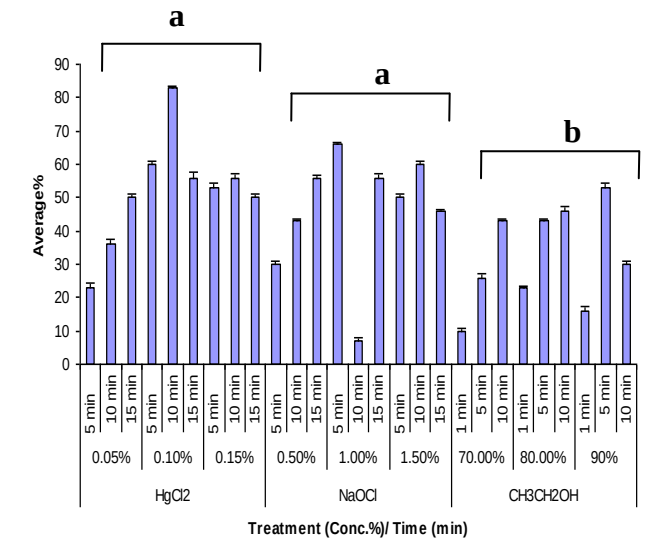
Statistical Analysis

Statistical analysis was done to find out the effect of different sterilizing agents its concentration and time of exposure on the asepsis of the said plant species. For each experiment, ten nodal segments and 20 seeds each in three replicates were used. The mean infected plant, healthy plant and dead plant percentage and mean germination percentage and their standard error was calculated. Data collected was subjected to two-way ANOVA to find out the significance level of effect of varying concentrations and time of exposure of different sterilizing agent on growth and asepsis of plants of *Swertia chirayita.*

RESULTS

Explant Sterilization

After observing the inoculated explants for 30 days for growth and contamination, it was found that increasing time and concentration significantly reduced contamination but showed adverse effect on explants (Figure 1A). Among all the three sterilizing agents *viz.*, $HgCl_2$, NaOCl and CH_3CH_2OH, treatment with 0.1% (w/v) $HgCl_2$ for 10 minutes gave maximum 83% healthy shoots ($p<1.0\%$). Increasing concentration and time of exposure to $HgCl_2$ provided more population of dead shoots.



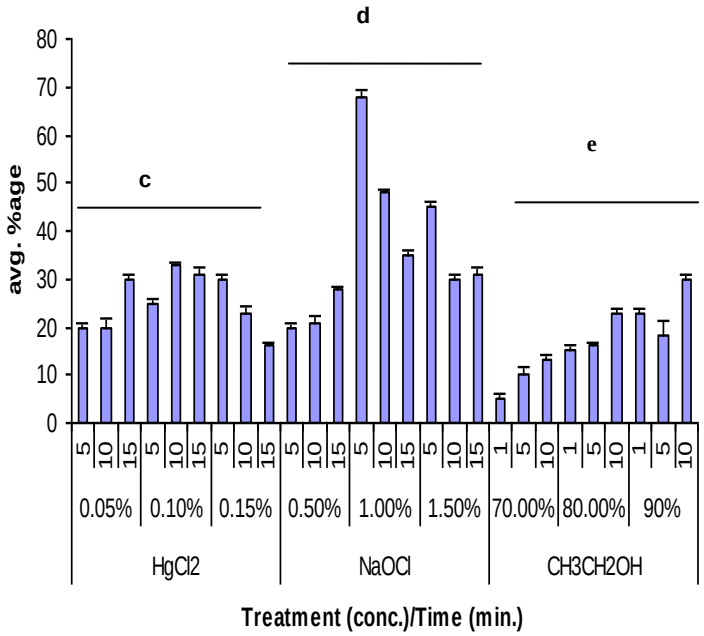


Figure 1. Average % of sterilization of ; A- Explants- avg.% of healthy shoots obtained after sterilization [a= concentration (p<1%) and interaction b/w concentration and time (p<5%); b= time (p<1%)and interaction b/w concentration and time (p<5%)] B- Average aseptic seed germination [c= concentration and interaction b/w concentration and time (p<5%); d= concentration, time and interaction b/w concentration and time (p<1%); e= concentration (p<1%), time (p<5%) and interaction b/w concentration and time non-significant;]

A minimum 10% of healthy shoots were observed in 70% CH_3CH_2OH for 1 minute. NaOCl being mild sterilizing agents provided more percentage of infection. Increasing concentration and time of sterilization with NaOCl, showed almost negligible reduction in contamination. Same was the case with CH_3CH_2OH where infected explants were more even on increasing concentration.

Seed Sterilization

In-vitro propagation through seeds also provides a useful technique for conservation as *in-vitro* condition make the seeds of *Swertia chirayita* to germinate. So sterilization of seeds before inoculation in the media is necessary. Various sterilizing agent with different concentration and time of exposure (Figure 1B) were tested, out of which 1% NaOCl for 5 minutes gave the maximum (68%, p<1%) germinated and healthy seedlings while less germination and more percentage of contamination was observed with other two sterilizing agents. Here also increasing time and concentration significantly reduced the contamination, but on the other hand it also effected the germination of seeds.

DISCUSSION

Tissue culture provides a best tool for large scale production of propagules especially in case of endangered medicinal herbs. *Swertia chirayita* has been declared as an endangered medicinal herb by new International Union for Conservation of Nature and Natural Resources (IUCN) criteria (Dhar *et al.,* 2002). *Swertia chirayita* is reputed for its various medicinal and pharmaceutical properties. Micropropagation provides a best tool for large scale production of propagules and its conservation especially in case of endangered medicinal herbs, where explant material is available in a very small quantity. Viability of seeds, age of explant and the tissue source from which the explant is excised are very important for high frequency of regeneration. The most important treatment prior to culture initiation is essentially surface sterilization of plants. Since in-vitro propagation provide suitable environment for growth of fungus and bacteria, unsuccessful sterilization hinders the progress of micropropagation studies. Many of the organisms that are residents on mammalian skin can survive in in-vitro cultures and therefore faulty aseptic techniques can also result in contamination. Therefore, reduction of contamination requires efficient aseptic techniques in tandem with effective sterilization methods (Faikiner, 1990). Sterilization of a material (explant/seeds) before subjecting them for in-vitro propagation is essential for the production of 'clean' in-vitro plantlets that ensures the reduction of the contaminants as well as high survival rate of explants.

Requirements may differ for different parts of plants depending on their morphological characters like softness and hardness of the tissue and plant parts. Therefore, in the present study, three sterilizing agents in different concentration with varying time of exposure were tested for sterilization of explants as well as for seeds of *Swertia chirayita*. In case of nodal segments taken as explant, 83% healthy plants were obtained with 0.1% (w/v) HgCl2 at 10 minutes showing significant reduction in both the bacterial as well as fungal contamination, while other two sterilizing agent did not give acceptable sterilization percentage even on increasing time and concentration. The results are very much in conformity with other previous studies on various medicinal plants medicinal plants viz., *Podophyllum hexandrum* (Sultan *et al.,* 2006), *Asparagus densiflorous* (Dasgupta *et al.,* 2007), *Balanites aegyptiaca* (Gour et al., 2007), *Cinnamomum camphora* (Soulange *et al.,* 2007) and C. verum, *Plumbago zeylanica* Linn. (Sivanesan, 2007), *Basilium polystachyon* (Amutha et al., 2008). HgCl2 was effective in case of *Inula racemosa* Hook.f.(Jabeen *et al.,* 2007) and *Picrorhiza kurroa* (Sood *et al.,* 2009) but the time of exposure was comparatively less, 2 minutes and 30 sec respectively. Also mercuric chloride has been studied to have promotory effect on the regeneration capability of groundnuts (*Arachis hypoglea* L) (Muthoni *et al.,* 2010).68% of aseptic seed germination was obtained when sterilized for 5 minutes with 1% NaOCl. This difference shows that time and concentration of sterilizing agent may vary with the type of tissue used for sterilization. The other two sterilizing agents HgCl2 and CH3CH2OH did not give acceptable sterilization even on increasing concentration. The detailed review of the earlier studies reveals that there is only scanty published data on sterilization of *Swertia chirayita*. As sterilization is the initial and vital step of micropropagation, minute error can lead to loss of whole culture with waste of time and labor. So, much attention is needed while sterilizing specially when dealing with such a valuable and endangered medicinal herb. Rate of propagation of *Swertia chirayita* is far less as compared to its exploitation. Results of the study reveal that the protocol developed for the sterilization of *Swertia chirayita* has the potential to be reproduced and utilized for the large scale multiplication of disease free plants of *Swertia chirayita* for its uninterrupted supply to herbal drug industries and simultaneously conserving this medicinal herb, an indigenous endangered medicinal plant.

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