Standardization of Sterilization Protocol for Micropropagation of *Aconitum heterophyllum*- An Endangered Medicinal Herb

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Abstract: A protocol has been standardized for sterilization of nodal segments and seeds of *Aconitum heterophyllum* for its micropropagation intended for its mass propagation and conservation. Three sterilizing agents *viz.*, $HgCl_2$, NaOCl and H_2O_2 were tested for sterilization by varying their concentration and time of exposure. 100% healthy shoots were obtained when explants were sterilized with 0.1% $HgCl_2$ for 5 minutes, inoculated on MS basal media with appropriate hormones and observing them for 30 days, while at 7.5% concentration of H_2O_2, 5 minutes exposure provided 90% of aseptic seed germination. Results showed that out of three sterilizing agents $HgCl_2$ was significantly reducing the contamination of explants and H_2O_2 of seeds in *in-vitro*, which shows that requirement of sterilization, may vary with the type tissue used for micropropagation. [Academia Arena, 2010;2(6):62-66] (ISSN 1553-992X).

Keywords: *Aconitum heterophyllum*, sterilization, micropropagation, conservation, contamination

1. Introduction

In-vitro propagation 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. *Aconitum heterophyllum* Wall is an important and endangered medicinal plant belonging to the family Ranunculacea. 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, *Aconitum heterophyllum* for drug preparation. As *Aconitum heterophyllum* 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 *Aconitum heterophyllum*. 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 *Aconitum heterophyllum* for *in-vitro* propagation intended for its conservation using different types of sterilizing agents by varying their concentration and duration of exposure.

2. Materials and Methods

2.1 Sample Collection

The plants and seeds of *Aconitum heterophyllum* were procured from Forest Nursery, Deoban, Chakrata, Uttarakhand. Seed samples were sent to NBPGR, Pusa Campus, New Delhi, for its germplasm conservation and the Accession No. IC-567646 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.

***2.3* Seed Sterilization**

Seeds of *Aconitum heterophyllum* were subjected to float test for determining the viability. Seeds of *Aconitum heterophyllum* are small in size; this makes its washing and sterilization little bit uneasy.

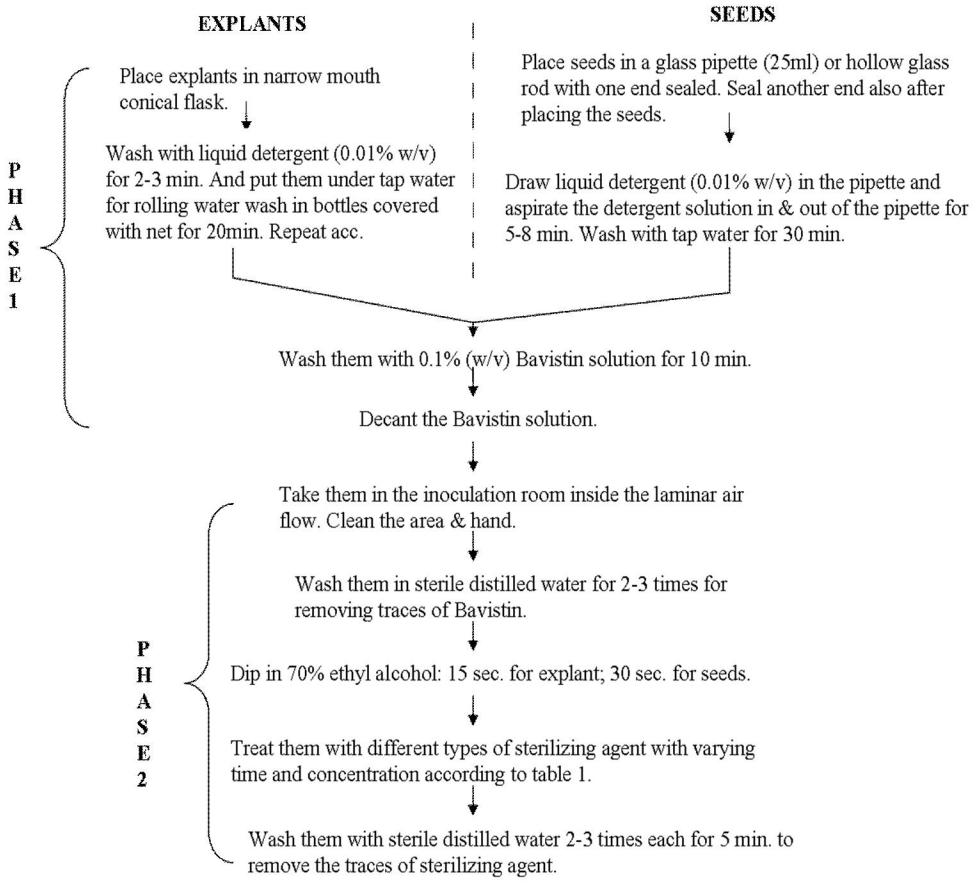

Figure 1. Procedure of sterilization of explants and seeds of *Aconitum heterophyllum*

2.2 Explant Sterilization

Nodal segments of *Aconitum heterophyllum* were excised from the pot grown plants. These nodal segments were trimmed to approx 2 cm. in size and large fleshy leaves were removed. All the brown skins were cleaned thoroughly. Procedure of sterilization (Figure 1.) for *Aconitum heterophyllum* had been divided into two phases: Phase I (outside Laminar Air Flow) and Phase II (inside Laminar Air Flow). Three different kinds of sterilizing agents' *viz.*, Mercuric Chloride ($HgCl_2$), Sodium Hypochlorite (NaOCl) and Hydrogen Peroxide (H_2O_2) are tested for explant sterilization by varying their concentration and time of exposure (Table 1).

As these seeds run out from the flask while washing, using glass pipette (20-25ml) or hollow glass rod with one end sealed can avoid this situation. Process of seed sterilization has been shown through a flow chart (Figure 1). Table 1 shows the concentration and time of exposure to different sterilizing agents used for decontaminating the seeds.

Table 1. Types of sterilizing agents used in a different concentration with varying time of exposure for sterilizing explants and seeds of *Aconitum heterophyllum.*

STERILIZING AGENT	CONC. (%)	TIME OF EXPOSURE (minutes)
Mercuric Chloride ($HgCl_2$ w/v)	**0.05**	**2, 5, 8.**
	0.1	**2, 5, 8.**
	0.15	**2, 5, 8.**
Sodium Hypochlorite (NaOCl w/v)	0.5	2, 5, 8.
	1.0	2, 5, 8.
	1.5	2, 5, 8.
Hydrogen Peroxide (H_2O_2 v/v)	**5.0**	**2, 5, 8.**
	7.5	**2, 5, 8.**
	10	**2, 5, 8.**

2.4 Inoculation

Murashige and Skoog basal medium supplemented with appropriate cytokinins and auxins were 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.

3. 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 (SPSS 15.0) 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 *Aconitum heterophyllum.*

4. Results

4.1 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 2). Among all the three sterilizing agents *viz.*, $HgCl_2$, NaOCl and H_2O_2, treatment with 0.1% (w/v) $HgCl_2$ for 5 min. gave the 100% healthy shoots ($p<1.0\%$). Increasing concentration and time of exposure to $HgCl_2$ provided more population of dead shoots.

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 H_2O_2 where infected explants were more even on increasing concentration up to 10% and time to 8 minutes.

4.2 Seed Sterilization

In-vitro propagation through seeds also provides a useful technique for conservation as *in-vitro* condition make the seeds of *Aconitum heterophyllum* to germinate dormant seeds. So sterilization of seeds before inoculation in the media is obligatory.100% seed viability was obtained. Various sterilizing agent with different concentration and time of exposure (Table 1) were tested, out of which 7.5% (v/v) H_2O_2 for 5 min. gave the maximum (90%, $p<1\%$) germinated and healthy seedlings while less germination and more percentage of contamination was observed with NaOCl, while

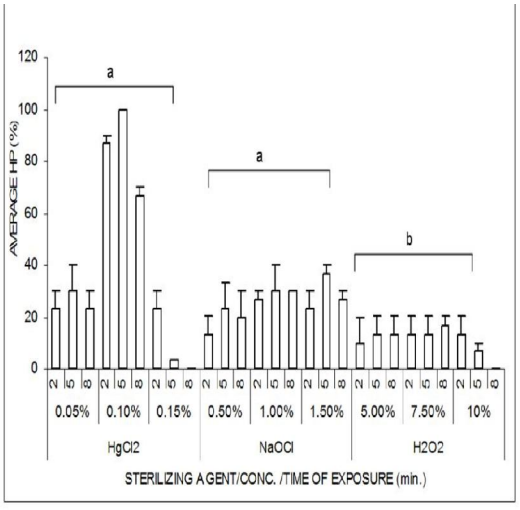

Figure 2. Percentage of Healthy Plants (HP) obtained after sterilization of explants of *Aconitum heterophyllum.* (a= significant at p<1%, b= interaction b/w concentration & time non significant)

$HgCl_2$ showed adverse effect on germination. Here also increasing time and concentration significantly reduced the contamination, but on the other hand it also effected the germination of seeds (Figure 3).

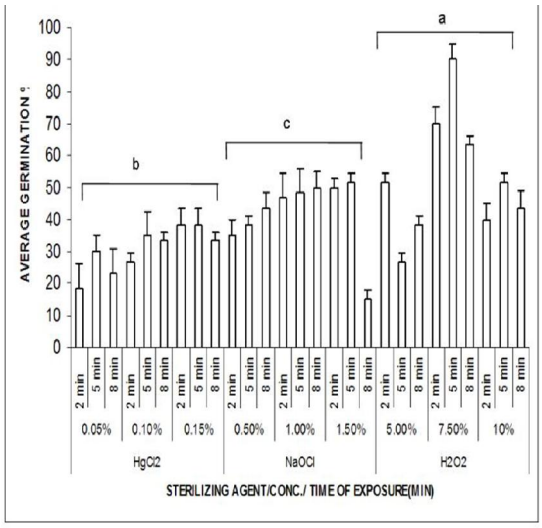

Figure 3. Average % of aseptic seed germinated after sterilization of seeds of *Aconitum heterophyllum*. (a= significant at p<1%, b= interaction b/w concentration & time non significant & c= time variation and interaction b/w concentration & time non significant)

5. Discussion

Tissue culture provides a best tool for large scale production of propagules especially in case of endangered medicinal herb. *Aconitum heterophyllum* has been declared as an endangered medicinal herb of Uttarakhand by the CAMP Workshop, 1998 (Dhar *et al.,* 2002). *Aconitum heterophyllum* is reputed for its various medicinal and pharmaceutical properties. Due to its high demand in the local, national and international drug manufacturers, illegal, unscientific and indiscriminate extraction of *Aconitum heterophyllum* Wall from its wild habitat has increased. Micropropagation provides a best tool for large scale production of propagules and its conservation especially in case of endangered medicinal herb, 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 perhaps 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 (Falkiner, 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 /hardness of the tissue etc. 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 *Aconitum heterophyllum.*

In case of nodal segments taken as explant, 100% healthy plants were obtained with 0.1% (w/v) $HgCl_2$ at 5 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, Asparagus densiflorous, Balanites aegyptiaca* (L) Del., *Cinnamomum camphora and C. verum, Plumbago zeylanica* Linn., *Basilium polystachyon* etc. (Sultan *et al.*, 2006; Dasgupta *et al.,* 2007; Gour *et al.,* 2007; Soulange *et al.,* 2007; Sivanesan, 2007; Amutha *et al.,* 2008). Same concentration of $HgCl_2$ was effective in case of *Inula racemosa* Hook.f. (Jabeen *et al.,* 2007) and *Picrorhiza kurroa* (Sood & Chauhan, 2009) but the time of exposure was comparatively less, 2 min. and 30 sec respectively.

90% of aseptic seed germination was obtained when sterilized for 5 minutes with 7.5% (v/v) H_2O_2. The other two sterilizing agents NaOCl and $HgCl_2$ did not give acceptable sterilization even on increasing concentration. The statistical interaction between time and concentration with $HgCl_2$ was found to be significant, while it was insignificant in case of NaOCl. This difference shows that time and concentration of sterilizing agent may vary with the type of tissue used for sterilization. As compare to the hilum/radicle of the seeds, the nodal explants are more open to the external environmental elements, so it requires comparatively strong sterilizing agent which is observed in the present study.

The detailed review of the earlier studies reveal that there is only scanty published data on sterilization of *Aconitum heterophyllum*, 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 *Aconitum heterophyllum* is far less as compared to its exploitation. Results of the study reveal that the protocol developed for the sterilization of *Aconitum heterophyllum* has the

potential to be reproduced and utilized for the large scale multiplication of disease free plants of *Aconitum heterophyllum* for its uninterrupted supply to herbal drug industries and simultaneously conserving this medicinal herb, an indigenous endangered medicinal plant.

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