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Experimental evaluation of surface sterilization protocol for seed explants of *Ephedra foliata* Boiss. ex C.A. Mey.

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Abstract: The most essential step for *in vitro* culture to obtain contamination free plants is surface sterilization of explants. This study investigated the sterilization procedure for seed explants from *Ephedra foliata* by varying the concentration and time of four sterilizing agents: sodium hypochlorite (NaOCl), mercuric chloride ($HgCl_2$), hydrogen peroxide (H_2O_2) and Bavistin. We found that pre sterilization using 0.4% bavistin for 25 minutes raised the number of infection free explants, followed by surface sterilization using NaOCl. Treatment with 0.1% mercuric chloride $HgCl_2$ for 2 minutes and 3% NaOCl for 10 minutes gave the best results. High concentrations of H_2O_2 and mercuric chloride were unable to control the bacterial and fungal contamination in the explants. We find out that mercuric chloride along with bavistin treatment developed a really potent sterilization method producing 60-65% infection free explants after fifteen days in culture.

Keywords: Contamination, *in vitro*, explant, sterilization, *Ephedra foliata.*

I. INTRODUCTION

The genus *Ephedra* is seed bearing non flowering, xerophytic, perennial gymnospermic shurbs belongs to family Ephedraceae (Bhtanagar and Moitra, 1996). These plants grow in dry climates mainly in northern hemisphere across Southern Europe, North Africa, and Central Asia (Ghada Abd El moneim Hegazi and Taghried Mohammed,2011). India represent the single species of *Ephedra foliata* gymnosperm in arid and semi-arid area of Thar Desert, Punjab Pakistan and Punjab Rajasthan borders (Bhandari 1990, Shekhawat at el 2012) and known as soma, somlata or unthphog locally. It is a dioecious climber have weak stem with node and internodes. The female plant bears semitransparent, nutritious edible berry like fruits (seed cones) which have pleasant sweet taste due to presence of flashy bract (Deepika lodha nd NS Shekhwat 2014). The flashy bract of fruits used as emergency food during the time of scarcity in arid regions (Bhandari 1990, Kotia 2008). Ephedra has been used for more than 5000 years to treat conditions like cold, fever, flu, headache, asthma and nasal congestion. It has also been an ingredient in many dietary supplements and used for weight loss, increased energy and enhanced athletics performance (Manjul Dhiman, sushma moitra; somatic embryogenesis and plant regeneration in *E. foliata*. 2010).

The increased demand of the plant in pharma industry has resulted in the over-exploition leading towards its slow extinction. Its conventional methods of propagation are slow and less effective, as compared to its exploited rate. Sterilization is the basic and pre requirement of any *in vitro* culture to establish aseptic cultures. So, the present study was carried out with an aim to develop the highly desirable and efficient protocol to effectively sterilize and establish healthy cultures of the seed explants in *in vitro* conditions on MS medium.

II. MATERIAL AND METHODS

The experiment was conducted in Plant Tissue culture Laboratory, Forest Department, Govt. of Haryana, India in March-April of 2017. The seeds were collected from a mature *Ephedra foliata* plant growing in the Botanical Garden of Kurukshetra University, Kurukshetra in the same season. Before sterilization, seeds were washed under tap water for 15 minutes. The explants were treated with Tween-20 to remove the dust particles for two minutes followed by rinsing twice in DDW (Double Distilled Water). Then, seeds were subjected for best sterilization protocol to treatment among four sterilizing agents Sodium Hypochlorite (NaOCl), Mercuric Chloride ($HgCl_2$), Hydrogen peroxide (H_2O_2) and Bavistin for various sterilization treatments.

After sterilization treatment, seeds were rinsed five times with sterilized DDW to remove the any trace of sterilizing agent left adhering to the seeds. Finally, seeds were inoculated in test tubes containing half strength MS medium solidified using 8g/L agar. The pH of the medium

was adjusted to 5.8 ± 0.2 before autoclaving the medium at 121 ˚C and 15 psi for 20 min. All inoculated test tubes were incubated for 20 days in a culture room under 16 h light/ 8 h dark photoperiod at 25 ± 2 ˚C and 60 % relative humidity. After 20 days the per cent, sterilized cultures, seed germination and seed health was recorded. The experiment was conducted using 30 replicates per treatment and was repeated three times.

III. RESULTS AND DISCUSSION

In this investigation among the four sterilizing agents, HgCl2 (Mercuric chloride) and NaOCl (Sodium hypochlorite) treatments tested at different concentrations reported the highest percent of seed germination. The NaOCl treatments of different concentrations at same exposure duration reported an increase in the seed germination percentage with good seedling health. 3 % NaOCl treatment give best result with 60 % germination and average seedling health (Table-1). The H2O2 and Bavistin treatments were not give satisfactory results with low germination and poor seedling health (Table-3 and Table-4). However in HgCl2 treatments, 30 % germination with excellent seedling health observed at 0.1% HgCl2 for two minutes time duration (Table-2). Observation were made higher concentrations of HgCl2 treatments with same exposure duration reduces the germination percentage and seedling health both. We find out that mercuric chloride along with bavistin treatment developed a really potent sterilization method producing 60-65% infection free explants after fifteen days in culture.

The sterilizing effects of NaOCl (N Ozalp et al., 2006) and HgCl2 has been reported by many workers (MP Das et al., 2012; SN Mahmoud, NK Al-Ani, 2016; R Amarasinghe, JH Wang et al., 2018; OA Bello, OO Obembe, 2018). The high exposure period and concentrations of sterilizing agents including NaOCl and HgCl2 has bed effects on the germination and health of seedlings (Danso et al., 2011). This may be the reason for poor germination, the sterilizing agents act as disinfectant set by impairing, remodeling, supplanting, transposition, inactivation or blocking the functioning of important cell molecules and organelles (Patra et al., 2004; Patra & Sharma, 2000).

Table 1: sterilizing agent Sodium hypochlorite (NaOCl) used with different concentrations, time of exposure, percent sterilization, seed germination and seedling health.

Sterilizing agent	Concentration %	Time of exposure	Per cent sterilization	Seed germination %	Seedling health
NaOCl	0.5	10	10	3	++++
NaOCl	1.0	10	10	3	+++++
NaOCl	2.0	10	30	7	+++
NaOCl	3.0	10	80	60	+++

Table 2: Sterilizing agent Mercuric chloride ($HgCl_2$) used with different concentrations, time of exposure, percent sterilization, seed germination and seedling health.

Sterilizing agent	Concentration %	Time of exposure	Per cent sterilization	Seed germination in %	Seedling health
$HgCl_2$	0.1	4	85	65	+++++
$HgCl_2$	0.2	4	50	10	++++
$HgCl_2$	0.3	4	50	8	++
$HgCl_2$	0.4	4	80	2	+

Table 3: Sterilizing agent Hydrogen peroxide (H_2O_2) used with different concentrations, time of exposure, percent sterilization, seed germination and seedling health.

Sterilizing agent	Concentration %	Time of exposure	Per cent sterilization	Seed germination in %	Seedling health
H_2O_2	5	5	40	15	++++
H_2O_2	10	5	20	5	+++
H_2O_2	15	5	10	1	++

Table 4: Sterilizing agent Bavistin used with different concentrations, time of exposure, percent sterilization, seed germination and seedling health.

Sterilizing agent	Concentration %	Time of exposure	Per cent sterilization	Seed germination in %	Seedling health
Bavistin	0.1	25	10	2	++++
Bavistin	0.2	25	15	2	++
Bavistin	0.3	25	20	2.5	++
Bavistin	0.4	25	25	3	+

IV. CONCLUSION

The present study reveals the NaOCl and HgCl2 were most potent sterilizing agent at lower concentrations to inhibit the infection in explant whereas at higher concentration the explants were unable to germinate. The concentration and time duration of agent played pivotal role in surface sterilization of explant. The bavist in and streptomycin were not effective alone, Hydrogen peroxide somehow inhibited the infection at higher concentration. Further investigation could be done on molecular level and will be try to find out the hidden mechanism after the inhibition of germination at higher concentration of these sterilizing agents.

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