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Standardization of Protocol for the Surface Sterilization and Callus Induction of *Saussurea lappa:* An Endangered Medicinal plant

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Abstract

Saussurea lappa is an endangered medicinal plant, used against lot of diseases like fever, headache, pain, arthritis, ulcers, cough, flatulence, asthma, colic, diarrhea, dyspepsia, vomiting, tenesmus, and dysentery. The plant is endangered due to over exploitation for commercial and medicinal purposes. The study was carried out to develop a protocol for the surface sterilization and callus induction of the seeds of *S. lappa*. Seeds of the plant were used as explants for the sterilization and callus induction. The explants were surface sterilized with 5% solution of tween-20 for 20 minutes followed by three different concentrations (0.05%, 0.1% and 0.2%) of mercuric chloride for 8minutes in three different experiments and rinsed 5 times thoroughly with autoclaved distilled water. Results were shown in percentage. The concentration of 0.2% solution of mercuric chloride showed best sterilization (96%) compared to 0.1% (48%) and 0.05% (0.2%) solution of mercuric chloride. Solid Murashige and Skoog (MS) medium fortified with 1mgL^{-1}Dichlorophenoxyacetic acid (2-4 D) alone and in combination with 0.25 mgL^{-1} kinetin (Kn) were used for the callus induction. Relative Growth Rate (RGR) of the callus was determined after 15 days of inoculation and found that the combination of 2-4 D and Kn produced maximum (0.141week^{-1}) callus compared to 2-4D (0.083 week^{-1}) used alone. The results showed that 0.2% $HgCl_2$ solution is the best seed sterilizing agent while 2-4 D (1mgL^{-1}) and Kn (0.25mgL^{-1}) are the best combination for the callus induction and fresh weight bio mass.

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Introduction

Saussurea lappa C.B. Clarke, Belongs to Family Asteraceae, is commonly known as costus (Kirtikar and Basu, 2001). It is an endangered medicinal plant (Gupta and Ray, 1967) used against a lot of diseases like fever, head ache, pain, arthritis, skin diseases, ulcers, cough, flatulence, asthma, colic, general weakness etc (Arora *et al.,* 1989), diarrhea (Negi *et al.,*2013) and also warms the center area, and regulate the stomach hormones, dyspepsia , vomiting, tenesmus, and dysentery (Xiao, 2006) and treat migraine (Backer *et al.,* 2012) cardiovascular disorders, as well as its anti-ulcer, anticancer, anti-inflammatory and antimicrobial properties (Pandey, 2007). The over exploitation of *Saussurea lappa* for diverse commercial and medicinal purposes is decreasing the availability of the plant in wild gradually. The critically endangered medicinal plant, *S. lappa*, is enlisted in Endangered plant Species of Wild Fauna and Flora (CITES) and is one of the 37 endangered Himalayan therapeutic plants so as to prioritized for conservation both in situ and ex situ (Kuniyal *et al.,* 2005).

Plant tissue culture techniques have been become compulsory for conservation of endangered medicinally important plants in order to save them from extinction (Murch *et al.,* 2000). Callus formation is the most important step for research based on tissue culture and consequent regeneration of plant (Visarada *et al.,* 2002). Contamination is a serious feature while developing new cultures from explants collected from field grown plant materials due to their exposure to a variety of microbes that are though not a trouble in*in vivo*, but a great problem in *in vitro* culture as it contaminates the rich nutritive medium of culture (Perez-Tonero *et al.,* 1999). Thus proper sterilization of explants is compulsory proceeding to inoculation on to controlled nutritional medium. Although aseptic conditions are usually provided but *in vitro* cultures may not be remain contamination free. Cultures can be invaded by microorganisms from explants, improper aseptic conduct, deprived hygienic settings of laboratory or laboratory instruments. These microbes contest with plant tissue cultures for nutrients and frequently cause contamination, tissues motility, and uneven growth, tissue necrosis as well as reduced root and shoot production (Oyebanji *et al.,* 2009). For sterilization of explants a variety of chemical agents are used but some are toxic for plant tissues. Hence proper exposure time, sterilizing agent and the sequences of using them has to be optimized to minimize explants damage and achieve better survival (Central Potato Research Institute, 1992). Mercuric chloride is a an extremely strong and significantly reduce contamination by applying 0.1% or 0.2% aqueous solution of $HgCl_2$ used for 3 minutes in barley seeds (Ramakrishna, 1991). $AsHgCl_2$ is highly toxic thus its concentration and exposure time to explants need to be optimized to minimize mortality rate of the explants tissues. Contamination with high percentage is decrease when treat with long time but also bring about reasonable reduction in germination of seeds (Anolles, 1990). In this context our study was focused to optimize a best protocol for the surface sterilization and Relative growth rate of callus.

Material and methods

The present research work was carried out in Plant Tissue Culture Lab. Institute of Biotechnology and Genetic Engineering Agriculture University Peshawar.

Plant material

Seeds of *S. lappa* were collected from Pakistan Forest Institute Peshawar. Healthy seeds were selected for culture on the basis of their physical appearance.

The medium and surface sterilization

Murashige and Skoog, (1962) medium supplemented with 0.8% agar having a PH of 5.8 was used. For callus induction2-4D (1mg/l) alone and also in combination with Kn (0.25mg/l) was used. Sucrose (3%) was used as a source of carbon and energy. In plant tissue culture technique, sucrose is the most frequently used carbohydrate source due to its high water solubility and high prevalence as a molecule carrier. A number of *in vitro* researchers reported that sucrose sustains near optimum growth rates and

carry out multiple functions in the provision of carbon and energy while promoting growth and division of cell (Swedlund and locy, 1993).

The culture medium and all used glass wares were autoclaved at 121 ^{o}C for 20 minutes and directly transferred to laminar flow cabinet, where subjected for 20 min to UV radiation. The whole culture was carried out in sterilized and aseptic environment in laminar flow cabinet.

S. lappa seeds were washed with tape water for 30 minutes to remove foreign contaminations, followed by 5% solution of surfactant (tween-20) along with detergent for 20 minutes to make contact of water deep in material and thoroughly washed with sterilized distilled water. The seeds were soaked in sterilized distilled water for 6hours and subsequently the explants were taken to laminar flow cabinet for further sterilization. Now the seeds were treated with three concentrations 0.05%, 0.1% and 0.2% of mercuric chloride for 8 minutes simultaneously in three different experiments and washed 5 times with autoclaved distilled water to remove any trace of disinfectant under aseptic conditions. Mercuric chloride ($HgCl_2$) is a very strong sterilizing agent because it decreases contamination significantly when used in solution forms of 0.05%, 0.1% or 0.2% for 3 minutes (Ramakrishna, 1991). The seeds were de husked before inoculation.

Inoculation

The de husked seeds were picked up with the help of sterilized forceps and inoculated in 100 ml flasks containing MS medium fortified with 2-4 D + Kn(1mg L^{-1}+ 0.25mg L^{-1}) and 2-4 D (1mg L^{-1}) alone and also a medium without growth regulators used for callus induction. Single seed was inoculated per flask. In order to get maximum sterilization, forceps were kept in ethanol and then burned with flame after every inoculation. The whole process of inoculation was carried in a closed proximity to the flame so as to diminish the chance of contamination. The cultured flasks were sealed tightly with aluminum foil and para film and labeled after inoculation. Then the 100 ml flasks were placed in growth room, where the cultured flasks were kept under dark conditions. The flasks were monitored for contamination. Callus initiation of *S. lappa* seeds were observed daily. The effect of sterilization was observed as percentage of contamination free flasks containing medium and the days of callus induction were noted (Ahmad *et al.,* 2016).

Measurement of Relative Growth Rate(RGR)

The callus relative growth was determined by the method of Shah *et al.,* (1990) as: Initial and final fresh weight of the explant (callus) was recorded.

Growth of the callus was expressed as RGR $week^{-1}$ with the following formula:

RGR=ln (final weight) – In (initial weight) / 2 $week^{-1}$

Statistical analysis

Analysis of variance (ANOVA) was performed using SPSS. Results were presented as means of three replicates ± standard deviation (SD) for Relative Growth Rate (RGR) while for sterilization means percentage of 50 replicates were taken.

Results and discussion

In vitro culture establishment

Culture organization is the primary and most important stage for plant variety and development using *in vitro* techniques. Contamination is a critical characteristic while developing new cultures from field grown plant materials due to their exposure to a variety of microbes which is a great problem in *in vitro* culture as it contaminate the rich nutritive medium of culture (Perez-Tonero *et al.,* 1999). Thus, it is compulsory to eradicate foreign contaminants including microbes (bacteria, fungi etc) before the establishment of *in vitro* culture (Zulfiqar *et al.,* 2009).

In the present study, the combination of two surfactant (tween 20 and detergent) and different concentrations of $HgCl_2$ (0.05%, 0.1%, 0.2% for 8 minutes) were used for sterilization in three different experiments. The percentage of sterilization was calculated on the basis of percentage of

contamination free flasks after 15 days of inoculation. The data presented in Table1 indicates the percentage of *S. lappa* seeds sterilization after treatment with three different (0.05%, 0.1%, 0.2%) strengths of aqueous $HgCl_2$ solutions. The data indicates that increasing concentration of $HgCl_2$ increased the percentage of sterilization. Maximum sterilization percentage (96%) was recorded with 0.2% $HgCl_2$ followed by 0.1% (48%) while minimum sterilization percentage (16%) was found with 0.05% $HgCl_2$ solution. However 0.2% $HgCl_2$ for 8 minutes showed significant reduction in microbial contamination while others were ineffective to control contamination (Rao, 2008).Thus it is observed that (0.2%) $HgCl_2$ used for 8 minutes can control contamination in seeds of *S. lappa* effectively. It was reported that $HgCl_2$ at the 0.2% concentration is the best agent of sterilization for seeds. $HgCl_2$ has also been reported as the best sterilizing agent for alfalfa and white clover seeds (Anolles, 1990).Maximum sterilization (90 %) has been noticed by using 0.2% $HgCl_2$ for 12 minutes (Daud, 2012). Combination of ethanol and Clorox instead of $HgCl_2$ reduce the sterilization percentage (73.6%) for rice seeds (Hidayatullah, 2007). Some researchers used 0.1% $HgCl_2$ to reduce the percentage of contamination (Singh, 2009).

Table 1. Sterilization at different strengths of $HgCl_2$ solution as sterilizing agent.

Treatments	Concentration of $HgCl_2$(mg/100ml)	Time (min)	Total No. of flasks	100 ml flasks free of contamination	Sterilization (%)
1	0.05	8	50	10	20
2	0.1	8	50	24	48
3	0.2	8	50	48	96

Callus induction

Sterilized seeds were cultured on MS medium supplemented with combination of 1mgL^{-1} 2-4 D and0.25mg L^{-1}kinetin and on the medium supplemented only with 1mg L^{-1}2-4D as well as on growth regulators free MS medium. Abdel-Abdel-Rahim *et al.* (1998**)** reported that Kinetin and 2-4 D are the best combination of growth regulators for callus production in date palm. Induction of callus occurred after 3-5 days of inoculation in medium supplemented with 2-4 D and Kn whereas medium supplemented with only2-4 D taken 4-7 days in callus initiation (Amiri *et al.,* 2011).

The calli data of RGR $week^{-1}$ is illustrated in the Fig.1.The RGR $week^{-1}$ of callus was calculated on the basis of callus fresh weight. The figure presents the RGR of two treatments and of control. Relative growth rate of the calli was determined after 15 days of inoculation (Shah *et al.,* 1990).The MS medium supplemented with both 2-4 D and kinetin showed better results of callus induction compared to the medium fortified with 2-4 D(1mg L^{-1}) alone. While no callus induction was found in medium without growth regulators (Control). Maximum RGR (0.1417$week^{-1}$) was found in medium containing combination of 2-4 D and Kn whereas less RGR (0.083 $week^{-1}$) $week^{-1}$ was shown by medium containing 2-4 D alone.

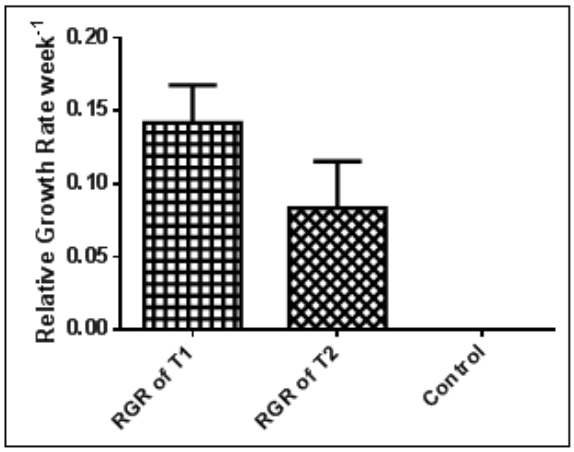

Fig. 1. The effect of 2-4 D and Kn on Relative Growth Rate of calli of two treatments. The data presented in the graph are the means of three replicates ± SD.

As compared to natural auxins (IAA) and cytokinin synthetic auxin and cytokine produces better percentage of calli even at low concentration (Amiri *et al.,* 2011; Tamil, 2012). Synthetic plant growth regulators are more potential to survive physical and

enzymatic degradation as compared to naturally occurring growth hormones (Pierik, 1990). Our results presented that MS basal medium supplemented with 2-4 D and Kn were taken 3-5 days in callus induction whereas the medium fortified with only 2-4 D was taken 4-7 days to initiate callusing in seeds of *S. lappa*.

These findings indicate that combination of 2-4 D with Kn and their concentration are suitable for callus formation (Amiri*et al.,* 2011). After two weeks of inoculation the RGR was calculated on fresh weight bases. From the figure it is clear that combination of 2-4 D and kinetin show better RGR of callus than the medium containing only 2-4 D after two weeks of explants
inoculation (Gupta *et al.,* 2010).

Conclusion

It is concluded from our findings that better seeds surface sterilization was achieved by using 0.2% mercuric chloride aqueous solution for 8 minutes. No damage was observed to the explants (Seeds) by using 0.2% $HgCl_2$ as a sterilization agent. It was noticed that the combination and concentration of 2-4 D ($1mgL^{-1}$) and Kn ($0.25mgL^{-1}$) are the best growth regulators for calli formation and can be used to induce calli in other explants.

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