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Research Article

Effect of Various Sterilizing agents on the Micropropagation of Endangered Alpine Medicinal Plant Trillium govanianum Wall. ex-Royle.

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Abstract
The use of higher altitude grown medicinal plants as a direct source of explant material to produce 'clean' *in vitro* plantlets, presents a major challenge. The microorganisms (fungi or bacteria or endophytes) that usually live within plant tissues without showing symptoms of disease. In most cases, these endophytes exhibit a symbiotic relationship with the host plant, but in tissue culture procedures, these endophytes overgrow in established cultures, thus hindering the practice of *in vitro* establishment. In our research we use the plant *Trillium govanianum* from the family melantheiaceae. Surface sterilization trials were conducted on the various plants including leaf, rhizome stem and roots using as the explants for the *in vitro* propagation. This study aims to obtain an optimal explant surface sterilization protocol for *Trillium govanianum* tissue culture that is free from contamination, browning, and explant death. Initial sterilization steps i.e., explants under running tap water time duration should be at least one hour for underground plant parts and 40- 45 minutes for other plants parts. Nine types of chemicals are used as a sterilizing agent along with different concentration with different exposure of the time. The only part of the plant that can grow roots & shoots is rhizome bud; the other plant parts show no response. Therefore, sterilizing agents such as streptomycin (antibiotic), Bavistin (Fungicide), Mercuric chloride, 70% ethanol, sodium hypochlorite shows the best response. Therefore, this study helps in maintaining healthy *in vitro* cultures of this valuable and endangered medicinal herb for its mass propagation and conservation aspects.

Keywords: Sterilizing agents, *Trillium govanianum*, Endangered medicinal plant, Surface sterilization, Contamination, Micropropagation.

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Introduction

Trillium species are found in temperate parts of North America and Asia, but the southern mountainous regions of the southeast United States have the greatest diversity. Trillium govanianum Wall. ex D. Don (Melanthiaceae family), sometimes called as 'Himalayan Trillium', is native to the Himalayan Region. Trillium govanianum is an allotetraploid intergeneric hybrid of Trillium and Daiswa (4x=20) (Chauhan and Bisht 2020). It is seen in Himalayan provinces such as India, Bhutan, Nepal, China, and Pakistan (Rahman et al. 2017).

As a gloom-adoring perennial, it prefers dark, chilly, wet locations beneath rocks and dense canopies. It is a trifoliate, single-stem herbaceous plant with a solitary purple bloom in the centre and a rhizome at the basal end bearing many adventitious roots (Chauhan 1990). Trillarin is found in the rhizomes and roots, and when hydrolysed, it yields 2.5% diosgenin (corticosteroid hormone). Corticosteroid hormone is mostly used to balance sex hormones, treat arthritis, regulate menstrual flow, and treat gastrointestinal disorders (Ur Rahman et al. 2015).

Trillium govanianum rhizomes are collected from natural populations and traded in national and international markets. Conventionally, propagating this medicinal plant is challenging due to its low multiplication rate under natural settings, as well as very poor seed setting and germination. Studies on its reproductive biology revealed that the species pollinates in two ways: self-pollination and cross-pollination. As a result, it has been discovered that this species is primarily self-pollinated, but because to its ambophilous character, it also prefers cross-pollination (Chandola et al. 2023; Rashid et al. 2023).

The plant tissue culture technology has been successfully utilized to mass multiply a variety of uncommon, endangered, and threatened medicinal plants. Most importantly, micropropagation is an effective strategy for ex situ conservation of plant biodiversity and rapid plant multiplication from a limited amount of available plant material (Chaudhary et al.,2024). Endophytic microbial contamination is a severe concern in several plant tissue culture experiments because it frequently hinders growth, reproduction, root formation, and, in some circumstances, plant death (Quambusch et al. 2016).

Roots, rhizomes, stems, leaves, seeds, fruits, tubers, ovules, and nodules are among the various above- and below-ground plant elements from which endophytes have been separated. Physiological, molecular, biochemical, and morphological methods can all be used to identify the isolated bacterium. According to Srinivasan et al. (2015), 16S rRNA is the most used of these to identify bacteria and ascertain their phylogenetic relatedness. Bacillus, Burkholderia, Microbacterium, Micrococcus, Pantoea, Pseudomonas, and Stenotrophomonas are the most isolated bacterial genera, with Bacillus and Pseudomonas being the most common genera (Chaturvedi et al. 2016).

A significant obstacle to the establishment and maintenance of viable in vitro cultures is endophytic contamination. When they are plant pathogens, these pollutants are especially harmful. Direct sourcing of explant material from Himalayan regions increases the issue. In this research, contamination is defined as the presence of bacteria or fungus on the surface and natural pores on the explant material that appear after commencement and can be either overt or covert. In contrast to covert contamination, which involves the use of specialized indexing and/or assaying techniques for identification, overt contamination is one that may be detected by observation. Plant species, age, explant source, and current weather conditions are some of the environmental and plant-related factors that affect the risk of contamination. Eliminating contamination from in vitro-grown plants is nearly challenging, even with the finest timing and selection efforts. Leifert et al. (1994) found that in most commercial and scientific plant tissue culture laboratories, losses from contamination in vitro average between 3 and 15% at each subculture, with bacterial, yeast, and fungal contaminants responsible for most of these losses.

For several reasons, micropropagation plants can get infected. When surface sterilization is not done properly, bacteria that were concealed in explants, added during sub-culturing, or infected simultaneously in cultures after a long period of development can suddenly show up (Hesami et al. 2021). Significant financial losses have been caused by contaminants containing microorganisms such bacteria, fungus, yeasts, viruses, and endophytes in plant tissue culture labs (Al-Dasary et al. 2011). Thus, the establishment of an effective sterilizing process is essential. Antibiotics have a limited use in controlling plant pollutants since they impact plastids, mitochondria, and chlorophyll synthesis (Verhaegen et al. 2023). The base of the explants started to exhibit indications of the growth of microorganisms (fungi and/or bacteria) within a week of being transferred to an MS media. These microorganisms often cause higher culture mortality, but they can also cause variable growth, tissue necrosis, decreased roots, and shoot proliferation (Oyebanji et al. 2009). This problem might have been caused by the bacteria in the explants, inadequate surface sterilization of the explants, and subpar aseptic techniques used during operation.

It was later shown that the in vitro cultivation of the medicinal plant Trillium govanianum led to the formation of endophytic bacteria, which was harmful to several micropropagation techniques (Chaudhary et al. 2022). Because it is challenging to prevent bacterial and fungal contamination of medicinal plants from field sources, surface sterilization is the most crucial step in preparing explants for micropropagation (Pranjic et al. 2013). According to Rezadost et al. (2013), no single sterilizing technique would be enough for all species. A single formula might not work at different periods, even for the same species or variety. This is because the load and type of microorganisms on explants are dependent

on the season. Therefore, nine best surface sterilizing treatments, including control were selected to perform the sterilization process on the explants for micropropagation. This study was carried out with the aim of standardizing, Optimizing, Characterizing and in vitro establishment of the medicinal plant Trillium govanianum.

Materials and Methods

Experimental material

The Trillium govanianum planting material was obtained from the natural habitat in various high-altitude geographical regions of the districts of Chamba (Suppa, Bharmour), Kangra (Kareri lake, Raj gunda), and Mandi (Barot) in Himachal Pradesh. The plant was kept in a controlled environment at the Plant Tissue Culture Laboratory, CSIR-IHBT, Palampur, Kangra (H.P) India (figure 3). Whole plants were employed as micropropagation explants. In order to guarantee that the experimental materials are free of other environmental contaminants, suitable aseptic conditions were used, beginning with the removal of mother plants from their natural habitat and proceeding to the most crucial stage, which is the sterilization of the explants for use using a variety of sterilizing agents at varying concentrations and exposure times to obtain the best response and each experiment was reported in three replicates and control.

Establishment of aseptic environment

Several techniques are employed to provide a protective environment to preserve our culture when employing the plant tissue culture technique for in-vitro establishment. One such technique is dry heat sterilization, which involves sterilizing glassware, metal tools, and other equipment in a hot air oven. The autoclaving process is used to sterilize liquids, paper goods, media, etc. The materials are sterilized for 15 to 30 minutes at 121°C and 15 PSI of pressure. The volume of the liquid to be autoclaved determines how long the sterilization process takes, the larger the volume, the higher the temperature. Chemical sterilization is used to sterilize the tools, the workspace, and the surfaces of the explants that will be employed.

Explant preparation for sterilization and in vitro culture establishment

Healthy & Young plant material having one-leaf stage (Vegetative stage) & three-leaf stage (Reproductive stage) were collected in the month of early May-June & October-November from different geographical sites of Himachal Pradesh (Figure 2). Their leaves, stem, rhizome was separated and washed for further sterilization process.

Explant Surface sterilization

Sterilization step was done into different steps to get rid from the contamination which was the major challenges during the in-vitro culture establishment. Firstly, proper explant was taken and washed under running tap water & then gentle brushing with detergent to remove the extra contaminants from the surface of the explants. Then using the antibiotics & fungicide is the second step for the sterilization process. The last final step was done under aseptic condition inside the laminae air flow cabinet using chemical and sterilized instruments.

Statistical analysis A completely randomized experimental (CRD) design was employed for all conducted experiments. In the explant's sterilization, each treatment level was replicated three times with two bud or rhizome part and three leaves, stem as explants in each replicate. The experiments were repeated thrice to ensure data reliability. Data collection for all parameters took place after 1-2 weeks of duration. The contamination percentage and the survival percentage were calculated as the total number of in vitro propagated that exhibited contamination divided by the total number of replicates and multiplied by 100. Data analysis was performed using OPSTAT software (version 1.0.2). The normality of the data was accessed by using one-way ANOVA. In cases where the normality test yielded a non-significant result ($p \geq 0.05$), a parametric test, such as one-way ANOVA at a significance level of $\alpha=0.05$, was utilized to compare the means. All results were expressed as mean values ± standard error.

Results & Discussion

Surface sterilization in this present investigation was influence by using different sterilizing agents with different exposure of time (Figure 1). Using different sterilizing agents with different time taken shows was observed. Best sterilizing agents was observed by mercuric chloride, sodium hypochlorite, ethanol, Bavistin, Streptomycin & hydrogen peroxide shows best response within 10-15 days after inoculation. Generally, the growth of contamination starts within 5 -8 days after inoculation. Sterilizing agents are blended with different concentration in double distilled water. Best response was observed by using sliver nitrate (1%) for 30 minutes, Calcium hypochlorite (10%) for 20 minutes, Mercuric chloride (0.15%) for 15 minutes, Streptomycin (0.15%) + Bavistin (1.5%) for 40 minutes, Hydrogen peroxide (10%) for 7 minutes, Sodium hypochlorite (1%) for 10 minutes, Ethanol (70%) for 55 seconds (Table 1). Least no. of contamination was observed using these sterilizing agents along with time duration we used (Figure 5). Data were analysed by using Opstat software and the analysis of the variance confirmed the significant differences ($p < 0.005$) among all the treatments (Figure 3).

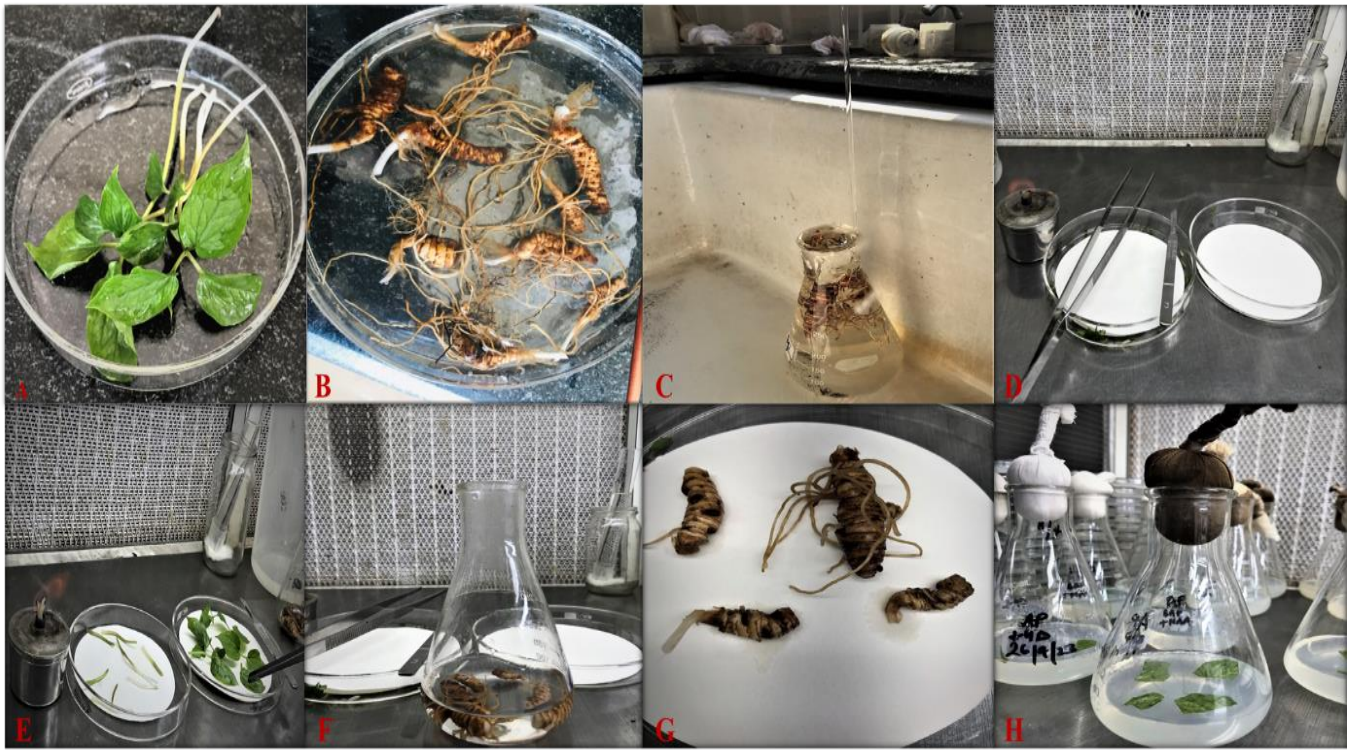

Figure 1 (A & B) The explant separated into different petri plates for the further sterilization process. (C) Explants under running Tap water for 1 hour to remove all the dirt. (D) Types of equipment used for Inoculation. (E & F) Sterilization of explant with chemical sterilant. (G) Drying of the explant by dapping with filter paper before inoculation to remove the extra water. (H) Inoculation of the explant on media having different concentrations of Hormones.

Figure 2 (a & b) Mother plant in the vegetative stage of Trillium govanianum in different geographical regions of Himachal Pradesh (c & d) Fruiting on the mother plant i.e. reproductive stage of Trillium govanianum. (e) Mother plant in laboratory conditions. (f) Rhizome for sterilization process & in-vitro propagation (g & h) Mature plant seeds.

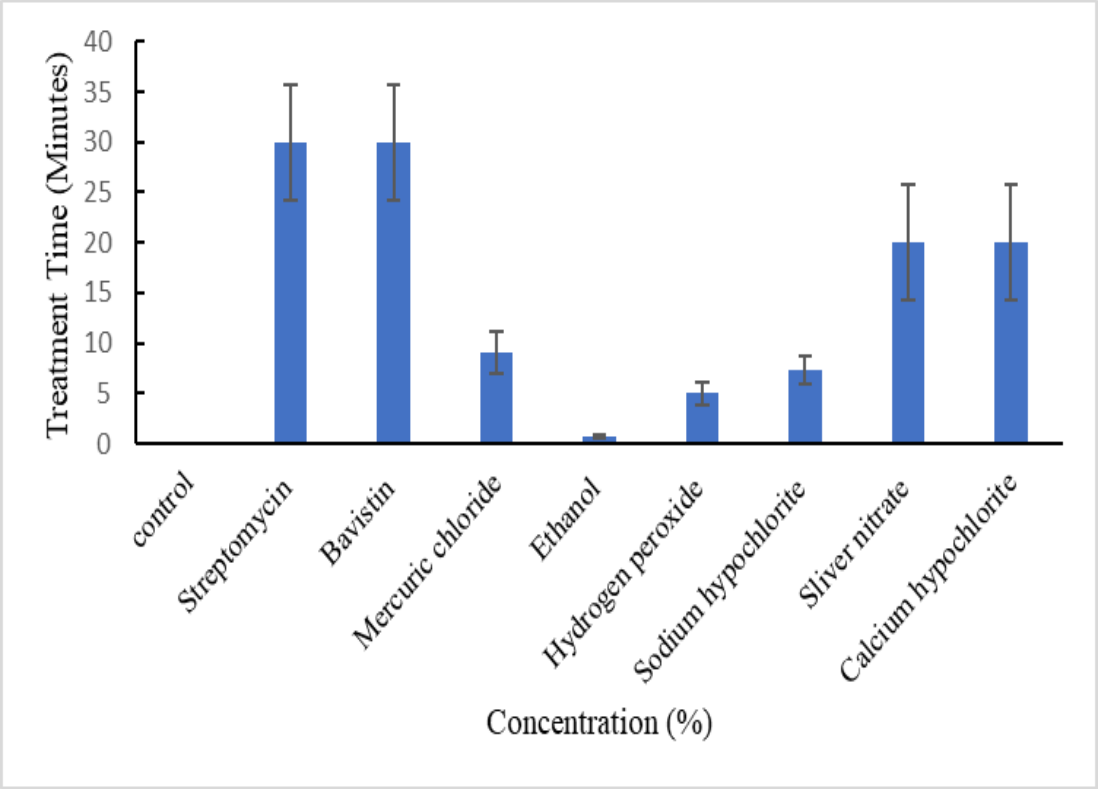

Figure 3 Standardization of optimum concentration of sterilizing agents on Trillium govanianum rhiz ome as explant for in-vitro propagation supplemented on MS media with different concentrations of PGRs along with different treatment time. Bars indicates the mean ± standard error. Significant differences by CRD test at p<0.005.

Table 1 Use of various sterilizing agents/ disinfectant with different concentrations and different exposure of time.

Disinfectant	Concentrations	Treatment time
Tween-20	2-3 drops maximum	5-10 minutes
Streptomycin	0.5%to 0.1%	15-20 minutes,30-40 minutes
Bavistin	0.05% to 0.15%	15-20minutes, 30-40 minutes
Mercuric chloride	0.01 to1.15%	2 -12 minutes
Ethyl alcohol	70% to 95%	30-45seconds
Hydrogen peroxide	3% to 10%	5-7 minutes
Sodium hypochlorite	0.5% to 1%	5-10 minutes
Silver nitrate	1%	5-30 minutes
Calcium hypochlorite	9-10%	5-30 minutes

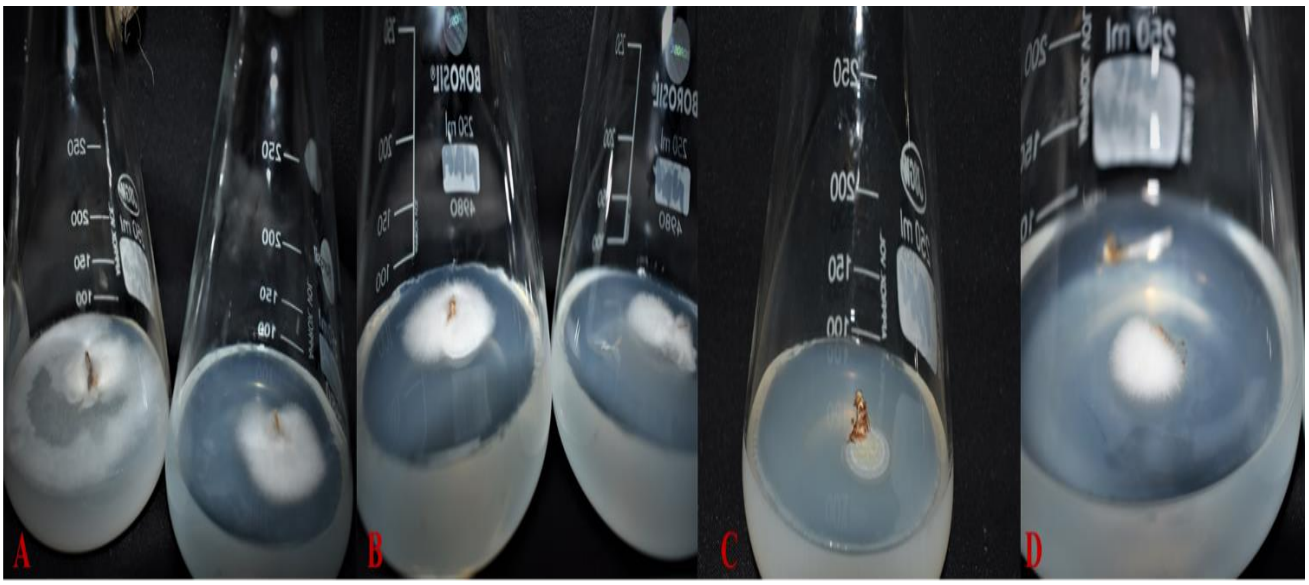

Figure 4 (A-D) Various types of contamination observed before the standardization of sterilizing agents for Trillium govanianum.

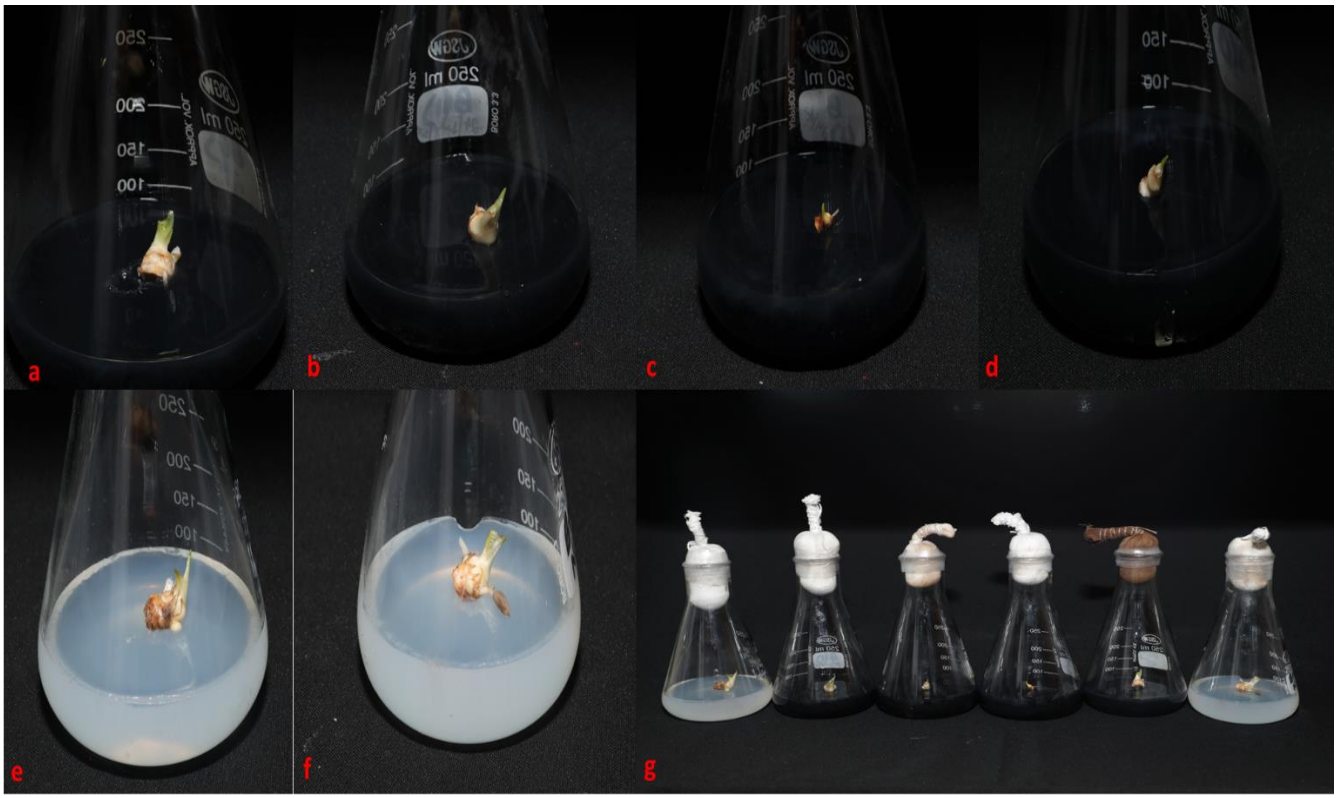

Figure 5 (a-g) Least no. of contamination or contamination free explants were observed after standardization of the different sterilizing agents with different exposure of time.

Conclusion

In conclusion, the study provides an optimal surface sterilization protocol for Trillium govanianum tissue culture that is free from contamination, browning, and explant death. This study shows the 90% survival rate of in-vitro propagated plants after the standardized protocol of surface sterilization. The most effect sterilizing agents are 0.15%- 0.30% Mercuric chloride, 70% ethanol, 1 % Bavistin, 0.20% streptomycin, 1% sodium hypochlorite, 10% hydrogen peroxide and 1% silver nitrate are the most effect and shows the great response. The future scope of this research includes further investigation of the effectiveness of different sterilization agents and concentrations, as well as exploring the potential for in-vitro propagation of this plant species.

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