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STERILIZATION PROTOCOL OF NODAL EXPLANTS FOR *IN VITRO* PROPAGATION OF LAVENDER (*LAVANDULA ANGUSTIFOLIA*)

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ABSTRACT

The present research was carried out in Centre for Tissue Culture Technology, JIBB, SHUATS, Prayagraj during August 2023 to May 2024. The experiment was laid out in Completely Randomized Design. Present experiment investigates the optimization of sterilant concentration on contamination and survival percentage of inoculated explants and along with optimization of *in vitro* regeneration protocol for lavender. Lavender is an economically important floriculture crop in terms of both as cut flower and with its medicinal and therapeutic property in terms of essential oil. Vegetative shoot serves as a good starting material for *in vitro* culture of plants. However, the heavy microbial contamination loads they harbor, especially the axillary nodes, make in vitro culture establishment problematic. This problem of microbial contamination is usually overcome through effective surface sterilization of explants among other aseptic techniques. Surface sterilization of explant is a process which involves the immersion of explants into appropriate concentration of chemical sterilant for a specified time resulting in the establishment of a contamination-free culture.

For sterilization procedure treatment T_{13}(0.1% $HgCl_2$ and 70% ethanol for 60seconds) was proved to be the best at it has highest survival percentage.

Key words : Sterilization protocol, Explant, Lavender, Propagation.

Introduction

Lavender (*Lavandula angustifolia*), a member of the Lamiaceae family, is a perennial shrub native to the Mediterranean region (Sharma *et al*., 2020). Renowned for its aromatic flowers, lavender has been cultivated for centuries for its ornamental, medicinal, and culinary properties. The conventional method of propagating lavender involves seed germination or vegetative propagation through cuttings. While effective, these methods often exhibit limitations in terms of propagation efficiency, uniformity, and genetic stability of the resulting plants. Moreover, the propagation of lavender through seeds often leads to genetic variability, resulting in differences in plant characteristics such as growth habit, flower color, and essential oil composition. This variability can pose challenges for commercial lavender growers seeking uniformity in their crops and consistent product quality.

Micropropagation, also known as tissue culture, offers an alternative approach for the mass production of high-quality lavender planting material. This technique involves the growth of plant cells, tissues or organs in aseptic conditions on a nutrient medium supplemented with growth regulators. Micropropagation offers several advantages over traditional propagation methods, including rapid multiplication, production of disease-free plants and preservation of genetic uniformity.

Field grown plants are invariably contaminated with microbes. Dusts that are confined on the external surface of the explants are removed by exposing them under running tap water. Developing a woody species through micropropagation and to maintain contaminant free

cultures remains a tough task (Garg *et al*., 2014). Bacteria or fungi that occurs on the surface of the explants or on the natural openings of the explants will be revealed only after initiation which may be macroscopic or microscopic (Jan *et al*., 2013).

As the initiation of contaminant free plant cultures remains a challenging task, the prime concern is to focus on the selection of the sterilizing agents. The time required for the exposure of these agents on the explants is also necessary to obtain healthy shoots. The sterilization protocol is the first step in determining the success of micropropagation.

Disinfection of plant material is important step of micropropagation protocol. In sterilization, an attempt is made to eliminate microbial contamination from plant material providing explant competition free environment to thrive and multiply *in vitro*. For establishing aseptic plant tissue culture, surface disinfection is one of the most crucial steps (da Silva *et al*., 2015). Establishing aseptic cultures from field-grown plants is a challenge, as there is always a high risk of internal and external contamination (Hennerty *et al*., 1987). Selection of surface disinfectant, concentration and duration depends on the explant used because sterilization should only eliminate contaminants and not the viability of plant material. Explant were swabbed with 80% ethanol, washed in 1% NaOCl (containing two drops of Tween 20) for 30 min and then washed for three 30min periods in sterile distilled water, resulted in minimum contamination (Finnie and Staden, 1986). Literature showed that various types of disinfectants are used, they include ethanol (or isopropyl alcohol), sodium hypochlorite (NaOCl)/calcium ($Ca(ClO)_2$), hydrogen peroxide (H_2O_2), mercuric chloride ($HgCl_2$), silver nitrate ($AgNO_3$) and bromine water (Singh *et al*., 2011; da Silva *et al*., 2016).

Materials and Methods

Materials

The nodal segments were excised using a sharp cutter just before starting sterilization procedure and kept into ice cold water to decrease stress on excised section of the plant. These nodal segments were used as explant for the *in vitro* plant regeneration.

Chemicals required

i. Carbendazim - 0.1%

ii. Tween-20

iii. Sodium hypochlorite - 4%

iv. Mercuric chloride - 0.001%, 0.01%, 0.1%, 1%

v. Ethanol - 70%

Methods

Preparation of sterilant

i. Carbendazim 0.1%(w/v) - To prepare this sterilant solution 0.1g of Bavistin powder was dissolved in distilled water to make 100 ml of the solution.

ii. Sodium hypochlorite 4% (v/v) - To prepare this sterilant solution 4ml of sodium hypochlorite was dissolved in distilled water to make 100 ml of the solution.

iii. Ethanol 70% (v/v) - 70 ml of ethanol was mixed with 30 ml of distilled water to make 100 ml of solution.

iv. Mercuric chloride (w/v) - To prepare 1% mercuric chloride solution, 1g of mercuric chloride was mixed with distilled water to make 100 ml of solution. This solution was diluted to make different concentration *i.e.*, 0.001%, 0.01% and 0.1% solution.

Surface sterilization of explant outside laminar air flow

The first phase of sterilization was completed outside of laminar air flow, whereas the second phase was carried out in laminar air flow. The explants were washed

Table 1 : Treatment details for nodal explant sterilization.

S. no.	Treatment	Treatment details
1	T_0	70% ethanol for 0sec
2	T_1	70% ethanol for 30sec
3	T_2	70% ethanol for 45sec
4	T_3	70% ethanol for 60sec
5	T_4	70% ethanol for 75sec
6	T_5	0.01% $HgCl_2$ and 70% ethanol for 0sec
7	T_6	0.01% $HgCl_2$ and 70% ethanol for 30sec
8	T_7	0.01% $HgCl_2$ and 70% ethanol for 45sec
9	T_8	0.01% $HgCl_2$ and 70% ethanol for 60sec
10	T_9	0.01% $HgCl_2$ and 70% ethanol for 75sec
11	T_{10}	0.1% $HgCl_2$ and 70% ethanol for 0sec
12	T_{11}	0.1% $HgCl_2$ and 70% ethanol for 30sec
13	T_{12}	0.1% $HgCl_2$ and 70% ethanol for 45sec
14	T_{13}	0.1% $HgCl_2$ and 70% ethanol for 60sec
15	T_{14}	0.1% $HgCl_2$ and 70% ethanol for 75sec
16	T_{15}	1% $HgCl_2$ and 70% ethanol for 0sec
17	T_{16}	1% $HgCl_2$ and 70% ethanol for 30sec
18	T_{17}	1% $HgCl_2$ and 70% ethanol for 45sec
19	T_{18}	1% $HgCl_2$ and 70% ethanol for 60sec
20	T_{19}	1% $HgCl_2$ and 70% ethanol for 75sec

thoroughly in running tap water for 30 min followed by washing with liquid detergent Tween 20 for 15 minutes. The explants were again kept under running tap water to remove any traces of detergent for 30 minutes. Further explants were treated with 0.1% fungicide solution (Bavistin) for 30 minutes followed by keeping explant in running tap water again to remove any trace of chemical sterilants used.

Surface sterilization of explant inside laminar air flow

The second phase of sterilization comprised disinfection and it was done in three steps. In the first step the explants were dipped in 70% ethyl alcohol for different time interval *i.e.*, 0s, 30s, 45s, 60s and 75s followed by three times washing with sterilized distilled water for 5 minutes each. Further the explants previously sterilized with ethanol were dipped in freshly prepared 0.001%, 0.01%, 0.1% and 1% (w/v) aqueous solution of mercuric chloride for 5.0 min with continuous shaking. Further the explants were thoroughly washed for 3-4 times with sterilized distilled water to remove all the traces of mercuric chloride. In the final step of sterilization, the explants were treated with 4% (v/v) aqueous solution of sodium hypochlorite for 10.0 min and the explants were washed 3-5 times with sterilized distilled water to remove all the traces of sodium hypochlorite.

Results and Discussion

In sterilization procedure, among all the treatments significantly least amount of contamination (0%) is observed when nodal explants are treated with treatment T_{13}(0.1% $HgCl_2$ and 70% ethanol for 60seconds) and T_{14}(0.1% $HgCl_2$, 70% ethanol for 75 seconds) along with treatments T_{17} (1% $HgCl_2$ and 70% ethanol for 45 seconds), T_{18} (1%$HgCl_2$ and 70% ethanol for 45 seconds) and T_{19} (1%$HgCl_2$ and 70% ethanol for 45 seconds), followed by treatment T_9 (0.01%$HgCl_2$ and 70% Ethanol for 60 seconds), while higher contamination was reported in T_0 (Control). The best treatment of sterilizing nodal explant is T_{13} (0.1% $HgCl_2$ and 70% ethanol for 60 seconds), as it has highest survival percentage which is 92.567% as mentioned in Table 1.

Despite of no contamination in treatments T_{17} (1%$HgCl_2$ and 70% ethanol for 45 seconds), T_{18} (1%$HgCl_2$ and 70% ethanol for 45 seconds) and T_{19} (1%$HgCl_2$ and 70% ethanol for 45 seconds), survival of explants in T_{17}(1%$HgCl_2$ and 70% ethanol for 45 seconds) were minimum (1.9%) and none of the explants survived in treatment), T_{18} (1%$HgCl_2$ and 70% ethanol for 45 seconds) and T_{19} (1%$HgCl_2$ and 70% ethanol for 45 seconds). Non-viable explants showed desiccated surface along with browning.

Mahmoud and Al-Ani (2016) reported similar findings

Table 2 : Sterilant concentration and treatment time along with contamination and survival percentage.

S. no.	Treatments	Contamination %	Survival%
1	70% ethanol for 0sec and 4%NaOCl for 10 minutes	100.0	0.0
2	70% ethanol for 30sec and 4%NaOCl for 10 minutes	79.7	20.3
3	70% ethanol for 45sec and 4%NaOCl for 10 minutes	70.4	29.6
4	70% ethanol for 60sec and 4%NaOCl for 10 minutes	44.5	48.2
5	70% ethanol for 75sec and 4%NaOCl for 10 minutes	14.6	42.6
6	0.01% $HgCl_2$, 70% ethanol for 0sec and 4%NaOCl for 10 minutes	68.6	29.7
7	0.01% $HgCl_2$,70% ethanol for 30sec and 4%NaOCl for 10 minutes	57.4	42.6
8	0.01% $HgCl_2$, 70% ethanol for 45sec and 4%NaOCl for 10 minutes	48.1	51.9
9	0.01% $HgCl_2$, 70% ethanol for 60sec and 4%NaOCl for 10 minutes	22.4	62.6
10	0.01% $HgCl_2$, 70% ethanol for 75sec and 4%NaOCl for 10 minutes	11.2	42.6
11	0.1% $HgCl_2$, 70% ethanol for 0sec and 4%NaOCl for 10 minutes	40.7	59.3
12	0.1% $HgCl_2$, 70% ethanol for 30sec and 4%NaOCl for 10 minutes	29.8	68.6
13	0.1% $HgCl_2$, 70% ethanol for 45sec and 4%NaOCl for 10 minutes	16.8	83.4
14	0.1% $HgCl_2$, 70% ethanol for 60sec and 4%NaOCl for 10 minutes	0.0	92.6
15	0.1% $HgCl_2$, 70% ethanol for 75sec and 4%NaOCl for 10 minutes	0.0	46.3
16	1% $HgCl_2$, 70% ethanol for 0sec and 4%NaOCl for 10 minutes	20.7	7.5
17	1% $HgCl_2$, 70% ethanol for 30sec and 4%NaOCl for 10 minutes	13.0	5.6
18	1% $HgCl_2$, 70% ethanol for 45sec and 4%NaOCl for 10 minutes	0.0	1.9
19	1% $HgCl_2$, 70% ethanol for 60sec and 4%NaOCl for 10 minutes	0.0	0.0
20	1% $HgCl_2$, 70% ethanol for 75sec and 4%NaOCl for 10 minutes	0.0	0.0

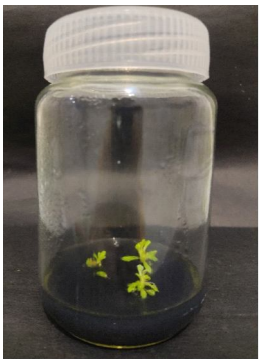


Fig. 1 : Shoot initiation without contamination.

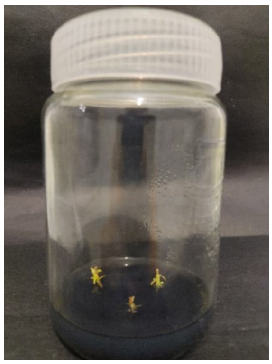


Fig. 2 : Inoculated explants without shoot initiation and contamination.

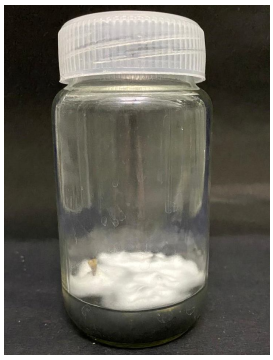


Fig. 3 : Contaminated explants.

Fig. 4 : Callus induced without contamination when explants sterilized in treatment T_{13}.

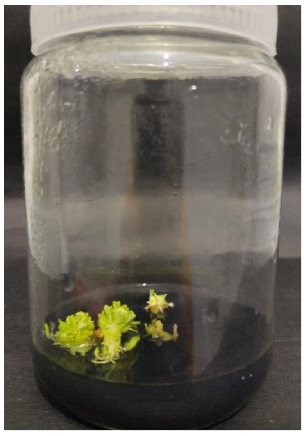
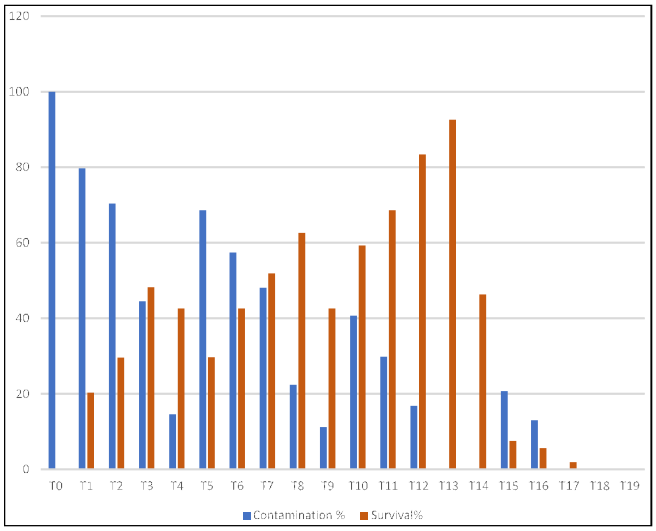


Fig. 5 : Multiple shoots induced without contamination when explants sterilized in treatment.

Fig. 6 : Roots induced without contamination when explants sterilized in treatment T_{13}.



Graph 1 : Graphical representation of sterilant concentration and treatment time along with contamination and survival percentage.

with use of $HgCl_2$ at concentration of 0.1% for 5 min along with NaOCl and 70% ethanol resulted in minimal phytotoxicity to shoot tip explants and presented acceptable control of contamination and maximum survival of explants.

Sodium hypochlorite as a bleaching substance is usually the main choice for surface disinfection. Because of toxic effects on explants, balance between concentrations and exposure durations should be determined experimentally for any explants (Putri *et al.*, 2019).

Pratiwi *et al.* (2021), mentioned that the treatment of 70% alcohol for 5 minutes and 10% NaOCl for ten minutes in young leaf explants of oil palm were sufficiently effective in reducing contamination with the lowest percentage of browning in explants and high growth response rate. They have also reported that treatment with higher concentration of alcohol (80% and 90%) caused death of explants, while higher concentration of sodium hypochlorite increased browning explants.

Discussion

Disinfectants such as ethanol and sodium hypochlorite (NaOCl) hamper the growth rate of fungi and bacteria in the growth medium by interfering with cell metabolism of microbes (Eliwa *et al.*, 2024).

Mathew and Goyal (2023) reported that 70% ethanol treatment causes slow coagulation of protein present in

cell wall of microbes facilitating its penetration to the cell wall before protein coagulation can block it from entering inside cell hence, effectively killing the microbes.

The effect of NaOCl sterilization is related to chlorine ions, which trigger oxidative reactions responsible for enzymatic inactivation and lipid and fatty acid degeneration, hence killing microbes causing contamination (Saran *et al*., 1998; Estrela *et al*., 2002).

$HgCl_2$ is responsible for oxidative stress which negatively affects culture viability by causing browning, irradicating contamination but inhibiting growth and ultimately impacting the success of *in vitro* cultures. The findings from the study highlight the importance of managing browning through effective sterilization techniques. Lower concentration of $HgCl_2$ (below 0.1%) for 10 -20 minutes results in lower contamination without affecting explants vitality while higher concentration cause browning and lower survival percentage (Fauzan *et al*., 2017).

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