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RESEARCH ARTICLE

Optimization of *in vitro* sterilization protocol for Hydrangea (*Hydrangea macrophylla* Thumb. Ser)

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Abstract

Hydrangea, a widely used ornamental plant in landscaping, is routinely featured but has recently gained popularity as a potted plant and for use in both fresh and dried cut flower products. The research established an appropriate sterilization method for six distinct types of plant explants. The pretreatment consisted of Carbendazim and Mancozeb fungicides applied alone and in combination with various concentrations and timings to evaluate their performance in front of surface sterilization treatments with 0.1 % $HgCl_2$ and 0.5 % NaOCl utilized at different exposure durations and concentrations. The combined fungicide treatment of explants with 0.2 % for 30 min followed by $HgCl_2$ and NaOCl sterilization proved effective at controlling both fungal (11.67 %), bacterial (1.67 %) infection, total contamination levels (13.33 %) and achieved the highest survival percentage increase (86.67 %). Surface sterilization with $HgCl_2$ for 6 min followed by NaOCl at 2 min applied to *H. macrophylla* nodes and internodes and shoot tip explants led to the highest survival (85.54 %) while generating cultures with minimal overall contamination (3.97 % bacterial and 96.03 % aseptic), accompanied by low mortality (10.49 %). The combination of $HgCl_2$ for 5 min with NaOCl for 2 min produced the most aseptic leaf cultures (85.10 %) with (68.33 %) leaf explant survival while minimizing total infections (15.00 %) between microorganisms.

Keywords: aseptic; explants; fungicides; hydrangea; *in vitro*; micropropagation; sterilization

Introduction

In vitro plant tissue culture is an important biotechnological technique for rapid large-scale propagation, particularly of fruit trees and ornamental plants (1). However, optimization of the sterilization process is highly significant for the establishment of these protocols. Explant contamination is attributed to the presence of different microorganisms (endogenic and exogenic) in the explant, limiting the development of successful regeneration protocols (2, 3). Therefore, the sterilization process requires careful attention and optimized techniques to eliminate contamination while preserving the viability for *in vitro* regeneration (4). The optimization of the whole sterilization process is dependent on variable factors ranging from plant age, size, location, physiological and environmental factors and explant (2). These factors later on regulate the *in vitro* regeneration process and selection of sterilizing agent and exposure time are significant, with focus on obtaining contamination-free explants without substantial damage (5). The most commonly used sterilizing agents are NaOCl (sodium hypochlorite), H_2O_2 (hydrogen peroxide), $Ca(ClO)_2$ (calcium hypochlorite), $HgCl_2$ (mercury II chloride), silver-based chemicals like $AgNO_3$ (silver nitrate) or NS (Nano-silver) (1). Despite its known toxicity, mercuric chloride remains a commonly employed surface sterilant in tissue culture due to its high efficacy in minimizing microbial contamination. Additionally, commercial chemicals, antibiotics and fungicides have been used in sterilization protocols (6, 7). Therefore, it is highly important to select a sterilizing agent with low phytotoxicity and high inhibition rate of contaminants (2).

Hydrangea macrophylla, also known as 'Hortensia,' is a commercially important ornamental species, widely cultivated and traded across global horticultural markets due to its aesthetic appeal, adaptability and consistent consumer demand in both potted and landscape applications for garden decoration in various settings, including group plantings, shrub borders and containers and now it is being commercially cultivated for cut flowers. Its economic significance is underscored by its ranking as the eighth most traded pot plant globally, reflecting its prominent role in the international floriculture industry (8). In commercial cultivation, hydrangeas are propagated using either seeds or stem cuttings, each with its own limitations. Seed plants show variability and do not consistently reproduce desirable traits. While cuttings take up considerable space, are slow to establish, often lacking good basal branching, moreover mother plants requirement is high. Hence, micropropagation provides a solution for rapid propagation of hydrangea propagules and

offers an efficient way to maintain stock material in sterile *in vitro* conditions. The main challenge is ensuring the availability of contamination-free explants in the culture medium, as this affects the successful regeneration from sterilized explants.

In this context, we aimed to establish an effective sterilization protocol for establishing a rapid and economical *in vitro* regeneration protocol for hydrangea. With this in mind, the study was designed to optimize the production of contamination -free plantlets in hydrangea by employing effective chemical sterilization methods for *in vitro* propagation, with lower contamination and higher survival percentage of explants.

Materials and Methods

Hydrangea macrophylla, a significant potted flowering plant, was investigated under the current study for developing a successful sterilization procedure for *in vitro* establishment of contamination-free plantlets at Biotechnology-cum-Tissue Culture Centre, OUAT, Baramunda, Bhubaneswar. The mother plants were naturally grown in pots in the greenhouse at the Department of Floriculture and Landscaping, College of Agriculture, OUAT, Bhubaneswar, for the initial establishment of cultures. Various explants, viz. nodes, internodes, shoot tips, leaf blades, petiole and midrib were utilized. Healthy and uninfected plants were selected for explant collection. The researcher cleaned explants by removing external contaminants before beginning the treatment procedure. The investigator cut the exposed explant ends to access fresh conducting tissue so antibiotics could pass deeply into the tissue for enhanced disinfection outcome. The explants were immersed for 10 min in running tap water to eliminate surface contaminants from their surfaces. A 0.1 % Tween 20 solution and a glass rod were used to wash the explants for 15 min before a tap water rinse lasting 10-15 min to eliminate remaining detergent traces.

Standardization of pre-treatments for different explants of *Hydrangea macrophylla*

Following the preparation of explants collected from potted mother plants grown in the greenhouse, specific sterilization protocols were applied to establish contamination-free cultures of *Hydrangea*. Different explants were subjected to various pre-treatments with individual and different combinations in various concentrations (0.1 % and 0.2 %) and durations (15 and 30 min) for lowering the contamination in the cultures. The pre-treated explants were surface sterilized using a standard dose of sterilant (0.1 % $HgCl_2$ for 5 min and 0.5 % NaOCl for 2 min) and subsequently inoculated onto culture medium.

Effect of surface steriliant and timing of surface sterilization of nodes, internodes and shoot tip explants of *Hydrangea macrophylla*

After determining the standard pre-treatment, surface sterilization experiment was carried out in a mix of nodes, internodes and shoot tip explants (Fig. 1) of >2 cm in length. Using 0.1 % $HgCl_2$ and 0.5 % NaOCl of different exposure periods (2, 5, 6 and 7 min.) to determine the suitable duration and type of sterilant for lowering the contamination and enhancing the survival percentage.

Effect of surface steriliant and timing of surface sterilization of leaf blade, petiole and midrib explants of *Hydrangea macrophylla*

After giving the standard pretreatment, a mix of the leaf blade, petiole and midrib explants (Fig. 2) of 5 mm square pieces of leaf blades and midribs and petioles < 3 cm long were sterilized using 0.1 % $HgCl_2$ and 0.5 % NaOCl as surface sterilizing agents for different exposure periods (2, 5 and 6 min). Observations on various parameters in all three experiments were recorded after 3 weeks of inoculation. All sterilization experiments were performed in a laminar flow chamber. To eliminate toxic sterilizing agent remnants, the explants received four to five rinses with sterilized double-distilled water. The culture bottles with nutrient media, glassware, equipment and distilled water were autoclaved at 15 lbs/in^2 pressure and 121 °C for 20 min. Under aseptic conditions, researchers utilized a laminar air flow chamber that received 30 min Ultraviolet sterilization before explant sterilization. The chamber received 70 % ethanol disinfection. The inoculated MS (Murashige and Skoog) nutrient medium bottles received immediate capping before moving to the culture room, which used fluorescent light at 3000-3200 lux under controlled conditions with 25 ± 10°C temperatures, 70 % relative humidity during their 16-08-hour (day-night) photoperiod. Research included twenty explants for each replication. Each experiment followed the Completely Randomized Design (CRD). The data assessment utilized OPSTAT software (Operational Statistical Software), Version [1.0.2], while ANOVA needed angular transformed percentage results (9). In all experiments, twenty explants were cultured per replication and replicated three times.

Fig. 1. **a.** Node; **b.** Internode; **c.** Shoot tip explants of *Hydrangea macrophylla.*

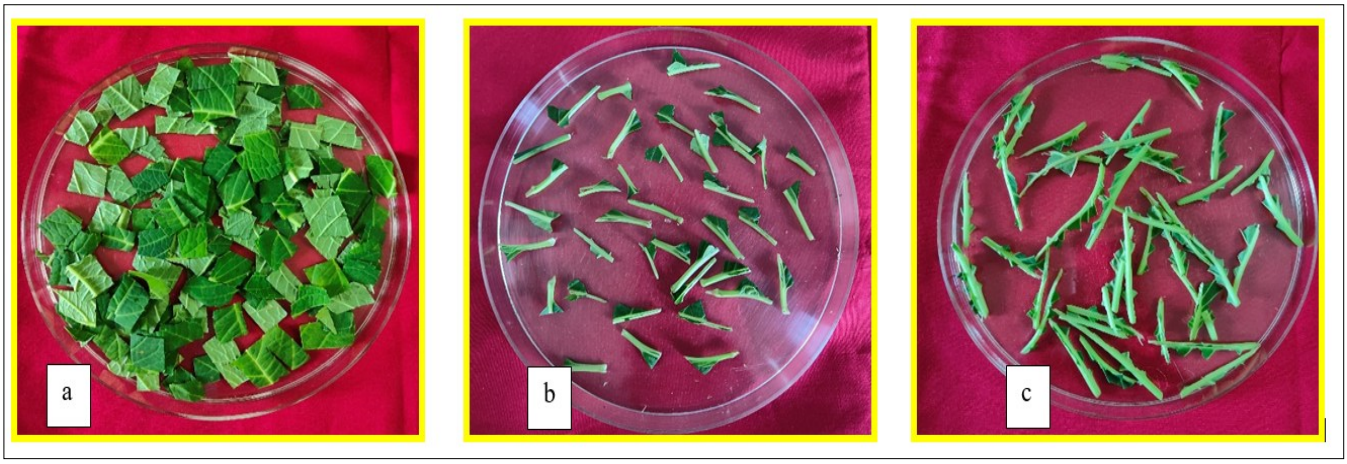

Fig. 2. **a.** Leaf lade; **b.** Petiole; **c.** Mid-rib explants of *Hydrangea macrophylla*.

Results

Contamination assessment (bacterial or fungal) was conducted through visual inspection of the plant material and the culture medium under fluorescent lighting. Bacteria typically do not proliferate effectively in plant tissue culture media and their presence may instead be indicated by a slight darkening of the explant. Fungal growth was observed as cottony growth, while bacterial growth was observed by a slimy oozy fluid (Fig. 3-4) (10). Explants that exhibit no signs of microbial growth are considered aseptic. Only aseptic explants showing regeneration were counted toward the survival rate. The percentage of contamination, aseptic and survival percent of explants was tabulated in Table 1.

Standardization of pre-treatments for different explants of *Hydrangea macrophylla*

The effect of different pre-treatments on explants such as nodes, internodes, shoot tips, leaf blades, petioles and midrib of Hydrangea is presented in Table 1. When Carbendazim (0.2 %) + Mancozeb (0.2 %) treatment was applied to explants over 30 min, it resulted in the lowest level of fungal infection (11.67 %) alongside bacterial infection (1.67 %) and total contamination (13.33 %) when compared to control treatment (T_1) which had a complete (100.00 %) microbial infection rate with 6.67 % bacteria and 93.33 % fungus. The combination of Carbendazim (0.1 %) + Mancozeb (0.1 %) for 15 min exposure led to maximum bacterial infection (13.33) among all treatment groups. Explant survival

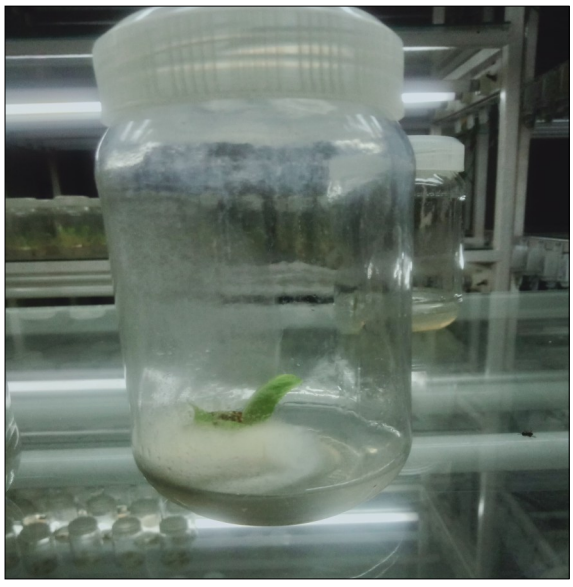

Fig. 3. Fungal infection.

Fig. 4. Bacterial infection.

was maximum (86.67 %) in treatment (T_9), which combined Carbendazim (0.2 %) with Mancozeb (0.2 %) for 30 min compared to control T_1, which showed no survival (0.00 %) with distilled water treatment. All statistical results showed that none of the treatments matched any other group (Table 1).

Effect of surface steriliant and timing of surface sterilization of nodes, internodes and shoot tip explants of *Hydrangea macrophylla*

After pre-treatment, nodes, internodes and shoot tip explants were subjected to different sterilization durations with $HgCl_2$ and NaOCl. The outcome of different exposure periods (4, 5, 6 and 7 min.) was recorded on different parameters and is presented in Table 2. Treatments T_9 demonstrated optimal fungal reduction (0.00 %) by combining 0.5 % NaOCl with 0.1 % $HgCl_2$ application for 7 min, followed by T_8, which achieved 3.97 % fungal contamination using the same solution for 6 min. Spread of fungal infection achieved its highest level at 100.00 % among treated samples in distilled water (control). The control treatment T_1 (control) produced 100 % explant mortality because all explants became infected with fungus. Bacterial infection tests showed that the combination of 0.1 % $HgCl_2$ exposure for 5, 6, or 7 min and 0.5 % NaOCl treatment for 2 min (T_7, T_8 and T_9) resulted in identical bacterial infection outcomes as the control (T_1) with zero bacterial infection. The combination of 0.1 % $HgCl_2$ with a 4 minute exposure time in T_2 resulted in the highest

Table 1. Effect of pre-treatments and timing on surface sterilization of explants of *Hydrangea macrophylla*

Pre-treatments		Duration (min)	Contamination (%)			Survival (%)
Code	Sterilants		Bacterial	Fungal	Total contamination	
T_1	Control (Distilled water)	30	6.67	93.33	100.00	0.00
T_2	Carbendazim (0.1 %)	15	8.33	75.00	83.33	16.67
T_3	Carbendazim (0.1 %)	30	5.00	61.67	66.67	33.33
T_4	Mancozeb(0.1 %)	15	11.67	68.33	80.00	20.00
T_5	Mancozeb (0.1 %)	30	8.33	61.67	70.00	30.00
T_6	Carbendazim (0.1 %) + Mancozeb (0.1 %)	15	13.33	40.00	48.33	46.67
T_7	Carbendazim (0.1 %) + Mancozeb (0.1 %)	30	8.33	28.33	41.67	63.33
T_8	Carbendazim (0.2 %) + Mancozeb (0.2 %)	15	6.67	18.33	25.00	75.00
T_9	Carbendazim (0.2 %) + Mancozeb (0.2 %)	30	1.67	11.67	13.33	86.67
	SE(m) ±		0.17	1.02	0.79	0.79
	CD (0.05)		0.52	3.04	2.35	2.35

bacterial infection rates, as 20.00 % of explants got infected. Node and internode and shoot tip explants sterilized by T_9 protocol achieved 0 % contamination of total microbes, while T_1 containing steamed distilled water (control) resulted in 100 % contamination, followed by T_2 (81.67 %) with $HgCl_2$ (0.1 %) exposure duration of 4 min.

All explants treated with $HgCl_2$ (0.1 %) for 7 min followed by 0.5 % NaOCl for 2 min in Treatment T_9 displayed a completely aseptic culture status, but with a higher mortality rate. The explants in treatment T_2 exhibited the aseptic culture (18.33 %) through $HgCl_2$ (0.1 %) exposure for 4 min. However, all explants in the control (T_1) remained infected with fungus (0.00 %).

Explant survival rates in control (T_1) were joined by T_2 (4 min), T_3 (5 min) and T_6 (4 min + 2 min) when treated with 0.1 % $HgCl_2$ + 0.5 % NaOCl in T_6. Results from all four treatments showed similar mortality rates to T_1. Explant mortality percentage reached its peak at 36.67 % in T_9after $HgCl_2$ (0.1 %) solution exposure for 7 min followed by 0.5 % NaOCl treatment for two min. Each subsequent increase in concentration and usage of the sterilant produced additional mortality.

Treatment combination T_8 with 0.1 % $HgCl_2$ for 6 min followed by 0.5 % NaOCl for 2 min generated the greatest survival rate (85.54 %) while T_7 proved somewhat less effective at 76.67 % survival using $HgCl_2$ (0.1 %) for 5 min before adding NaOCl for 2 min. Control T_1 displayed no survival due to complete fungal contamination (100 %).

Analysis of the data in Table 2 revealed that both the type and duration of surface sterilization significantly influenced the survival and contamination rates of nodal, internodal and shoot tip explants of *Hydrangea macrophylla*. Among the different treatments, T_8 involving 0.1 % mercuric chloride ($HgCl_2$) for 6 min followed by 0.5 % sodium hypochlorite (NaOCl) for 2 min proved to be the most effective. This protocol achieved the highest explant survival rate (85.54 %), along with a low overall contamination rate (3.97 %), comprising zero bacterial contamination and 3.97 % fungal contamination. It also resulted in 96.03 % aseptic cultures, with a comparatively low mortality rate of 10.49 %. These findings indicate that T_8 is the most suitable surface sterilization treatment for achieving clean and viable explants in *H. macrophylla*. However, T_9, where the explants were sterilized with 0.1 % $HgC1_2$ (7 min) then 0.5 % NaOCl (2 min) reported the highest number of dead explants (36.67 %) with the lowest total contamination percentage (bacterial and fungal), 0.00 % and 100 % aseptic cultures, but survival percentage (63.33 %) is low compared to T_8.

Effect of surface steriliant and timing of surface sterilization of leaf blade, petiole and midrib explants of *Hydrangea macrophylla*

From the perusal of data furnished in Table 3, it was observed that surface sterilizing of leaf blade, petiole and mid rib explants with 0.1 % $HgCl_2$(6 min) then 0.5 % NaOCl (2 min), i.e. T_7, most effectively reduced the percentage of fungal infection (6.67 %). However, the leaf blade, petiole and midrib explants surface sterilized with distilled water devoid of steriliants (T_1- Control) recorded maximum fungal infection (72.50 %). Prolonged exposure (5 and 6 min) of leaf explants to $HgCl_2$(0.1 %) and NaOCl (0.5 %) recorded no bacterial contamination (0.00 %), whereas the maximum contamination (27.50 %) was recorded in the cultures treated with distilled water (T_1). Total contamination percentage is maximum 100 % in T_1 (control), where no survival was reported and the least contamination percentage with $HgCl_2$ (0.1 %) and NaOCl (0.5 %) for 6 min in treatment T_7 (6.67 %).

Leaf blade, petiole and mid rib explants surface sterilized with 0.1 % $HgCl_2$ (6 min) then 0.5 % NaOCl (2 min) in T_7, which recorded the significantly maximum rate of aseptic culture (93.38 %). However, there was no aseptic culture as all the explants were infected by fungus in treatment T_1 (control). The percentage of aseptic culture was significantly higher in T_7 (93.38 %) than in T_6 (85.10 %), but $T_6$was considered the most suitable treatment. Further increase in the treatment duration of sterilizing agents over the highest aseptic treatment resulted in T_7 recorded increase in the death of explants and reduced final survival of explants.

Table 2. Effect of surface steriliant and timing of surface sterilization on nodes, internodes and shoot tip explants of *Hydrangea macrophylla*

Treatment details								
Code	$HgCl_2$ 0.1 %	NaOCl 0.5 %	Bacterial	Fungal	Total contamination	Aseptic culture (%)	Mortality (%)	Survival (%)
T_1	Control (Distilled water)		0.00	100.00	100.00	0.00	0.00	0.00
T_2	4 min	-	20.00	61.67	81.67	18.33	0.00	18.33
T_3	5 min	-	16.67	48.33	65.00	35.00	0.00	35.00
T_4	6 min	-	13.33	50.00	63.33	36.67	6.67	30.00
T_5	7 min	-	5.00	33.33	38.33	61.67	26.67	35.00
T_6	4 min	2 min	6.67	43.33	50.00	50.00	0.00	50.00
T_7	5 min	2 min	0.00	15.00	15.00	83.33	8.33	76.67
T_8	6 min	2 min	0.00	3.97	3.97	96.03	10.49	85.54
T_9	7 min	2 min	0.00	0.00	0.00	100.00	36.67	63.33
	SE (m) ±		0.16	0.45	0.62	0.69	0.20	0.69
	CD (0.05)		0.47	1.33	1.85	2.06	0.58	2.05

The percentage of mortality of the leaf explants was significantly low (0.00 %) in treatments T_1, $T_2$and T_5. The highest number of dead explants (38.33 %) was reported in T_7 (0.1 % $HgC1_2$ for 6 min followed by 0.5 % NaOCl for 2 min). Further increase in the treatment duration of sterilizing chemicals over the best treatment recorded increase in mortality rate. The effect of different durations of explant sterilization with $HgCl_2$ was found significant in influencing the explant survival and maximum survival (68.33 %) was recorded with 6 min with 0.1 % $HgCl_2$ and 2 min with 0.5 % NaOCl in treatment T_6 followed by T_7 (55.00 %), while least survival (0.00 %) was found with control T_1 (distilled water) as depicted in Table 3. Although the percentage of death of the leaf explants was significantly low (0.00 %) in treatments T_1, $T_2$and T_5 but contamination is high so, T_6(5 min with 0.1 % $HgCl_2$ and 2 min with 0.5 % NaOCl) was considered as optimal treatment because it recorded minimum percentage of total contamination (15.00 %) (bacterial and fungal infection) and maximum percentage of aseptic culture (85.10 %) and final survival (68.33 %) of leaf explants.

Discussion

Standardization of pre-treatments for different explants

Fungal and bacterial contamination is the most common problem encountered in cell cultures. While complete elimination of microbial contamination is challenging, effective pre-treatment strategies can significantly reduce its frequency and severity. The outcome of micro-propagation depends heavily on aseptic culture initiation, so the explants received preliminary fungicide treatment before culture start-up to reduce microbial contamination during aseptic culture establishment. Pretreatment can be given by using different fungicides. Here, two different types of fungicides, Carbendazim and Mancozeb, alone and in combination, were used to study their efficacy. In this study, pretreatment of all explants with Carbendazim (0.2 %) + Mancozeb (0.2 %) for 30 min effectively controlled both bacterial and fungal contaminations in all the explants. Increasing the concentration of both Carbendazim and Mancozeb from 0.1 % to 0.2 % led to a notable reduction in fungal contamination. Survival observation for explant tissue showed an increase at higher concentrations of fungicides Carbendazim and Mancozeb when used in combination from 0.1 % to 0.2 %. The survival percentage alongside contamination decreased when the treatment duration extended from 15 min to 30 min.

Mancozeb disrupts fungal cells by interacting with sulfhydryl groups, leading to the inactivation of essential enzymes. This interference affects key biochemical processes within the cytoplasm and mitochondria, including amino acid metabolism, enzyme activity, cellular respiration and ATP synthesis. As a benzimidazole compound, Carbendazim disrupts fungal DNA biosynthesis during cell division while simultaneously protecting plant microtubules from disruption. Both fungicides prove effective because Carbendazim functions as a systemic substance and Mancozeb exhibits contact properties with systemic benefits that treat multiple fungal diseases in agricultural settings and tissue culture conditions. The combination of Carbendazim and mercuric chloride has been used effectively to achieve a required measure of *in vitro* culture asepsis (11-13).

Standardization of surface sterilization treatments of different explants

Contamination generally originates from two sources: either through carryover of microorganisms on the surface of the explant or in the tissue itself (endophytic microorganisms). Most of the microorganisms are eliminated in meristem culture (depending on meristem size), however, using leaf, petiole and stem explants, infection is carried over to the cultures (14). During the current investigation, shoot and leaf explants of *Hydrangea macrophylla* were surface sterilized using various sterilants to enhance the culture asepsis in Hydrangea, like mercuric chloride and sodium hypochlorite, individually or in combination. In contrast to single chemical sterilization, combined treatments of explants with two or even more sterilants are very useful for sterilization (15).

The combination of $HgCl_2$(0.1 %) for 6 min followed by NaOCl (0.5 %) for 2 min gave the most effective results in culture sterilization in all the three explants i.e. nodes, internodes and shoot tip with maximum survival percentage (85.54 %) and minimum total contamination percentage (3.97 %), bacterial (0.00 %) and fungal (3.97 %) and 96.03 % aseptic cultures are obtained with comparatively lower mortality rate (10.49 %) as best treatment. For leaf explants, i.e. leaf blade, petiole, mid rib, treatment with 5 min 0.1 % $HgCl_2$ and 0.5 % NaOCl for 2 min was considered as the most effective treatment as it recorded the minimum total contamination percentage (15.00 %) (bacterial and fungal infection) with 85.10 % aseptic culture and 68.33 % of explants survival.

Nodal segments remained viable to a greater extent compared to leaf segments because node sections feature a compact design that includes sclerenchymatous tissues with toughened surface fibers. The data showed that shoot tips demonstrated better survival rates than leaves, possibly because their protected position results from leaf tier separation following sterilization. Sterilant treatments were proven to have toxic effects on leaf explants, which may be due to their thin epidermis, thus providing less protection against the lethal effect of sterilants and due to the toxicity caused by increased duration of sterilants exposure on explants (12). $HgCl_2$ demonstrates incredible antimicrobial properties, which destroy both fungi and bacteria as well as plants within its vicinity. $HgCl_2$ functions as an effective disinfectant, which breaks down proteins to destroy cells while damaging membranes (16). Regardless of its profound health effects, mercuric chloride is frequently utilized for surface sterilization

Table 3. Effect of surface steriliant and timing of surface sterilization on leaf blade, petiole and midrib explants of *Hydrangea macrophylla*

Treatment details			Contamination (%)			Aseptic culture (%)	Mortality (%)	Survival (%)
Code	$HgCl_2$ 0.1 %	NaOCl 0.5 %	Bacterial	Fungal	Total contamination			
T_1	Control (Distilled water)		27.50	72.50	100.00	0.00	0.00	0.00
T_2	4 min	-	21.67	63.33	85.00	15.57	0.00	15.00
T_3	5 min	-	10.00	56.67	66.67	33.78	11.67	21.67
T_4	6 min	-	11.67	51.67	63.33	37.09	15.00	21.67
T_5	4 min	2 min	8.33	53.33	61.67	38.74	0.00	38.33
T_6	5 min	2 min	0.00	15.00	15.00	85.10	16.67	68.33
T_7	6 min	2 min	0.00	6.67	6.67	93.38	38.33	55.00
	SE (m) ±		0.21	0.54	0.77	0.86	0.21	0.90
	CD (0.05)		0.64	1.64	2.32	2.59	0.63	2.72

to reduce microbial contamination in a *H. macrohylla* tissue culture. $HgCl_2$ is highly toxic to both plants and animals and as it is difficult to dispose of the chemicals, proper measures should be taken before its discard. While several adverse side effects have been reported, the use of it in plant sterilization is still significant and highlighted (2). NaOCl is advantageous due to its broad-spectrum action and lower risk of microbial resistance development. It is well known that hypochlorite kills bacteria very effectively; even micromolar doses are sufficient to reduce bacterial populations significantly (1).

It is reported that surface sterilization with $HgCl_2$ followed by Clorox resulted in the highest decontamination and survival percentage of *Trachleium caeruleum* and *Hydrangea macrophylla* (17, 18). The leaf explant of *Anthurium andreanum* cv. Fire were surface sterilized using $HgCl_2$ 0.1 % (19). Surface sterilization of nodal explants of *Tagetes erecta* L with 0.1 % $HgCl_2$ for 10 min + 1 % NaOCl for 2 min proved to be effective (20). It was reported that the results of Contamination generally originate from two sources, either through carryover of microorganisms on the surface of the explant or in the tissue itself (endophytic microorganisms). Most of the microorganisms are eliminated in meristem culture (depending on meristem size), however, using leaf, petiole and stem explants, infection is carried over to the cultures (14). During the current investigation, shoot and leaf explants of *Hydrangea macrophylla*, were surface sterilized using various sterilants to enhance the culture asepsis in Hydrangea, like mercuric chloride and sodium hypochlorite, individually or in combination. In contrast to single chemical sterilization, combined treatments of explants with two or even more sterilants are very useful for sterilization (15).

The combination of $HgCl_2$ (0.1 %) for 6 min followed by NaOCl (0.5 %) for 2 min gave the most effective protocol for results in culture sterilization in all the three explants i.e. nodes, internodes and shoot tip with maximum survival percentage (85.54 %) and minimum total contamination percentage (3.97 %), bacterial (0.00 %) and fungal (3.97 %) and 96.03 % aseptic cultures are obtained with comparatively lower mortality rate (10.49 %) as best treatment. For leaf explants, i.e. leaf blade, petiole, mid rib, treatment with 5 min 0.1 % $HgCl_2$ and 0.5 % NaOCl for 2 min was considered as the most effective treatment as it recorded the minimum total contamination percentage (15.00 %) (bacterial and fungal infection) with 85.10 % aseptic culture and 68.33 % of explants survival.

Nodal segments remained viable to a greater extent compared to leaf segments because node sections feature a compact design that includes sclerenchymatous tissues with toughened surface fibers. The data showed that shoot tips demonstrated better survival rates than leaves, possibly because their protected position results from leaf tier separation following sterilization. Sterilant treatments had toxic effects on leaf explants, likely due to their thin epidermis, which offers limited protection and increased duration of exposure to sterilants on explants (12). $HgCl_2$ demonstrates strong antimicrobial properties, which destroy both fungi and bacteria as well as plants within its vicinity. $HgCl_2$ functions as an effective disinfectant, which breaks down proteins to destroy cells while damaging membranes (16). The main benefit of NaOCl over other disinfectants is its inability to allow microorganisms to develop resistance.

It is reported that surface sterilization with $HgCl_2$ followed by Clorox resulted in the highest decontamination and survival percentage of *Trachleium caeruleum* and *Hydrangea macrophylla* (17, 18). The leaf explant of *Anthurium andreanum* cv. Fire were surface sterilized using $HgCl_2$ 0.1 % (19). Surface sterilization of nodal explants of *Tagetes erecta* L with 0.1 % $HgCl_2$ for 10 min + 1 % NaOCl for 2 min proved to be the most effective treatment (20). It was reported that 100 % contamination-free explants were obtained using 50 % Clorox combined with 0.2 % $HgCl_2$ (18). No uncontaminated explants of *H. involucrata* 'Yoraku Tama' (YT) and *H. aspera macrophylla* (AM) were achieved in any sterilization tests with NaOCl (21). It was reported that 100 % sterilized explants with the combination of 2.5 % active chlorine and 0.2-0.4 % mercuric chloride in *H. quercifolia* Snow Queen (22).

Conclusion

Selection of appropriate sterilizing agents, along with their optimal concentrations and exposure durations, plays a crucial role in achieving contamination-free and viable explants for *in vitro* regeneration. Based on the present study, the optimal protocol for successful *in vitro* sterilization of Hydrangea explants involves pretreatment with 0.2 % Carbendazim and 0.2 % Mancozeb for 30 min, followed by surface sterilization with 0.1 % $HgCl_2$ for 6 min and 0.5 % NaOCl for 2 min. The reproducibility and defined parameters of this protocol make it well-suited for scale-up in commercial plant production systems, allowing for integration into automated workflows to support large-scale, contamination-free propagation.

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Authors' contributions

NAP wrote the original draft and conducted the experiment and analysed the data with support from AD. SKP facilitated in conducting the experiment. KM facilitated the acquisition of plant samples and guided the experiment. SB, PNJ and PT conceptualized and contributed by giving essential suggestions. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The Authors do not have any conflict of interest to declare.

Ethical issues: None

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