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In vitro Sterilisation of Different Explants of Chrysanthemum *(Dendranthemum morifolium L.)* cvs. "Candid" and "Flirt"

Ambreena Din[1*], Z. A. Qadri[1], Z. A. Rather[1], M. Saleem. Mir[2], Imtiyaz Murtaza[3], F. A. Khan[4], Neelofar[1] and Muneeb Ahmad Wani[1]

[1]Division of Floriculture and Landscape Architecture, Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Jammu and Kashmir, India.
[2]Associate Directorate of Research, Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Jammu and Kashmir, India.
[3]Divison of Biochemistry, Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Jammu and Kashmir, India.
[4]Division of Basic Science and Humanities, Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Jammu and Kashmir, India.

Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

The study was conducted to standardise protocol for sterilisation of different explants of chrysanthemum cvs. "Candid and "Flirt". Different explants tried were shoot tips, petals, nodal segments and leaf. Carbendazim 200 ppm for 30 minutes + mercuric chloride (0.1%) dip for 2 minutes and final treatment with ethyl alcohol (70%) for 10 seconds proved best for culture asepsis of shoot tips followed by petals, nodal segments and leaf segments in both the cultivar. Carbendazim 200 ppm for 30 minutes + mercuric chloride (0.1%) dip for 2 minutes and final

**Corresponding author: E-mail: dinambreena@gmail.com;*

treatment with ethyl alcohol (70%) for 10 seconds proved better sterilisation for culture survival of shoot tips followed by petals, nodal segments and leaf segments in cv. “Candid” whereas, petals followed by leaf segments in case of cv. “Flirt”. This study leads to the standardisation of the shoot tip and petal explants for culture asepsis and survival in chrysanthemum cvs. “Candid” and “Flirt” and among two cultivars "candid" proved better in sterilisation than "Flirt".

Keywords: Tissue culture; in vitro sterilisation; asepsis; survival; chrysanthemum; Candid and Flirt.

ABBREVIATIONS

BAP : 6-Benzyl amino purine;
BA : 6-Benzyladenine, 2;
4-D : 2,4dichloro-phenoxyacetic acid;
GA3 : Gibberelic acid:
IAA : Indole-3-acetic acid;
IBA : Indole-3-butyric acid;
MS : Murashige and Skoog’s medium;
NAA : Naphthalene acetic acid and
Mm : Micro molar

1. INTRODUCTION

Commercial chrysanthemum cultivars are globally important cut flower and pot plant species. The name “chrysanthemum” comes from the Greek words – ‘Chrysos’ meaning golden and ‘anthos’ meaning flower. Chrysanthemum is ranked second next only to rose in importance among the floriculture crops in the world. It is grown for its aesthetic as well as commercial value. A large number of cultivars of chrysanthemum are grown worldwide, and they exhibit wide variation in respect of growth habit, size, colour and shape of blooms that make the chrysanthemum an excellent flower crop. Chrysanthemum belongs to family Asteraceae and originated in Asia. It is introduced as “garden mums” to Europe between 17th and 18th century and in present days is produced as cut flowers, potted and garden plants. The number of chrysanthemum cultivars is incredibly large with more than 15,000 listed in Japan alone while the National Chrysanthemum Society of Britain lists over 6000 cultivars [1]. To achieve the desired outcome of any tissue culture procedure, choosing the correct explant is essential with minimal delays, besides proper sterilisation of the explants is the pre-requisite step leading to the development of a successful protocol for *in vitro* propagation. Inadequate sterilisation may result in a total loss of explants incubated on the sucrose containing medium and the incubation conditions which are most favourable for microbial growth. Any minute contamination on the explant (i.e., a single live spore or a bacterial cell) after sterilisation can create havoc. Mercuric chloride being a potent sterilant is also known to compromise membrane integrity of live plant tissue. Therefore, longer duration may result in decreased explant survival as against shorter one. Moreover, use of a final dip in ethyl alcohol further improves survival of explants significantly. Concentrated ethyl alcohol is a powerful sterilant that kills bacterial and fungal spores by instantly drawing out water out of them. Along with the water, ethyl alcohol removes any traces of Hg^{2+} ions from the explant tissue.Washing explants under running tap water for 30 minutes can assist in obtaining sterile cultures by physically removing some of the contaminants, especially with field-grown material. In this study after washing explants with tap water, different sterilants like mercuric chloride, sodium hypochlorite or ethyl alcohol for the disinfection singly or in combination with a fungicide as carbendazim have been used. For successful micro-propagation, the first objective is to achieve maximum culture asepsis as possible by using the sterilants. In the process of sterilisation of explants, the type, concentration and duration of the sterilant treatment are the important factors not only to achieve a desirable level of asepsis, but also to ensure that explants survive the undesirable toxicity effects. Keeping these factors under consideration, the present investigation attempted to evaluate the safe and effective use of sterilant treatments in order to achieve maximum explant survival.

2. MATERIALS AND METHODS

Different types of explants shoot tips, petals, nodal segments and leaf segments of chrysanthemum morifolium cvs. “Candid” and “Flirt” were cut from freshly dugout plants and thoroughly washed with tween-20 followed by 100 or 200 ppm carbendazim treatment for 30 minutes. Further treatment with mercuric chloride 0.1% dip for 2 and 4 minutes followed by 70% ethyl alcohol dip for 30 seconds was done under laminar air flow. Explants were given final 3

washings with sterile water before placing on the medium. In this 12 cultures per treatment were maintained each replicated four times. Data recorded was subject to statistical analysis using ANOVA.Materials used and details of methodologies followed in the foregoing study are presented in the following sections.

2.1 Materials

Stock Plants: Chrysanthemum cultivars "Candid" (Red) and "Flirt" (Blood red) were used in the current study (Plate 1). The stock plants of the two cultivars were grown in pots under polyhouse and experimental farm of the Division of Floriculture and Landscape Architecture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar.

Chemicals: The major and minor elements, sucrose, agar, and organic compounds like amino acids, vitamins and plant growth regulators required for preparation of media during the present investigation were procured from Hi-media, India.

Glassware: The borosilicate glassware used in the experiments was procured from Borosil. Glassware was washed with detergent followed by thorough washing with tap water. The glassware was then dried in hot air oven at 100 ºC for 24 hours before use.

Culture media: Murashige and Skoog [2] medium was employed during the course of present investigation (Table 1). In addition to above, some modifications in Murashige and Skoog [2] medium were also employed details of which have been provided in the appropriate sections.

2.2 Methods

Preparation of stock solutions: Different types of stock solutions of macro elements, micro elements and organics were prepared for each medium and stored in reagent bottles for use. Usually four types of stock solutions (A-D) are made for Murashige and Skoog [2] basal medium. However, for longer storage seven types of stock solutions (A, B, C, D, E, F, and G) of Murashige and Skoog [2] are generally prepared in the Tissue culture Laboratory of the Division of Floriculture and Landscape Architecture and same was followed in the current investigation (Table 2). During the entire investigation period, 200 ml of each stock solution was prepared each time which was sufficient for making 20 litres of medium. For making one litre of medium, 10 ml of each stock solution was added to 600 ml of double distilled water in a beaker and final volume made to one litre by adding double distilled water.

Seven stock solutions were used in the preparation of MS medium. Stock solution (A/B/D) containing only one chemical was prepared by dissolving required quantity of each chemical (quantity of chemical required for making 20 litres of medium) in 150 ml of double distilled water and final volume made to 200 ml with double distilled water. Thus stock solution with a strength of 100 X was obtained. Stock solution containing more than one chemical (C/E/F/G) was prepared by weighing the required quantity of each chemical (quantity of chemical required for making 20 litres of medium) separately and dissolved to the last particle in double distilled water followed by mixing them together slowly with continuous stirring. Final volume of 200 ml of the mixture was made by addition of double distilled water. Stock solutions were stored in corning reagent bottles at 4ºC.

A) *Dendranthemum morifolium* L. cv. "Candid" B) *Dendranthemum morifolium* L. cv. "Flirt"

Plate 1. Chrysanthemum cultivars

Table 1. Chemical composition of Murashige and Skoog (1962) medium

Components	Chemical formula	Quantity (mg l^{-1})
Macronutrients		
Ammonium nitrate	NH_4NO_3	1650
Potassium nitrate	KNO_3	1900
Calcium chloride, dihydrate	$CaCl_2.2H_2O$	440
Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$	370
Potassium phosphate, monobasic	KH_2PO_4	170
Micronutrients		
Potassium iodide	KI	0.83
Boric Acid	H_3BO_3	6.2
Manganese sulfate, tetrahydrate	$MnSO_4.4H_2O$	22.3
Zinc sulphate, heptahydrate	$ZnSO_4.7H_2O$	8.6
Sodium molybdate, dihydrate	$Na_2MoO_4.2H_2O$	0.25
Cupric Sulfate, pentahydrate	$CuSO_4.5H_2O$	0.025
Cobaltous chloride, hexahydrate	$CoCl_2.6H_2O$	0.025
Ethylenediaminetetraacetic acid disodium salt	$Na_2EDTA.2H_2O$	37.3
Ferrous sulphate, heptahydrate	$FeSO_4.7H_2O$	27.8
Organic supplements		
Myo inositol	$C_6H_{12}O_6$	100.00
Glycine	$C_2H_5NO_2$	2.00
Nicotinic acid	$C_6H_5NO_2$	0.5
Pyridoxine hydrochloride	$C_8H_{11}NO_3.HCl$	0.5
Thiamine hydrochloride	$C_{12}H_{17}ClN_4SO.HCl$	0.1

Table 2. Composition and preparation of stock solutions for Murashige and Skoog (1962) medium

Stock	Components	Chemical formula	Qty. mgl^{-1}	Qty. for 20 litres	Qty. of stock solution	Conc. of stock solution
A	Ammonium nitrate	NH_4NO_3	1650	33.00 g	200 ml	100 X
B	Potassium nitrate	KNO_3	1900	38.00 g	200 ml	100 X
C	Potassium iodide	KI	0.83	16.6 mg	200 ml	100 X
	Boric acid	H_3BO_3	6.2	124 mg		
	Potassium phosphate, monobasic	KH_2PO_4	170	3.4 g		
	Molybdic Acid (Sodium Salt)•$2H_2O$	$Na_2MoO_4.2H_2O$	0.25	5 mg		
	Cobaltous chloride hexahydrate	$CoCl_2.6H_2O$	0.025	0.5 mg		
D	Calcium chloride, dihydrate	$CaCl_2.2H_2O$	440	8.8 g	200 ml	100 X
E	Magnesium sulphate, heptahydrate	$MgSO_4.7H_2O$	370	7.4 g	200 ml	100 X
	Zinc sulphate, heptahydrate	$ZnSO_4.7H_2O$	8.6	172 mg		
	Manganese Sulfate, tetrahydrate	$MnSO_4.4H_2O$	22.3	446 mg		
	Cupric Sulfate pentahydrate	$CuSO_4.5H_2O$	0.025	0.5 mg		
F	Ethylenediaminetetra acetic acid disodium salt	$Na_2EDTA•2H_2O$	37.3	746	200 ml	100 X
	Ferrous Sulfate heptahydrate	$FeSO4.7H_2O$	27.8	556 mg		
G	Glycine	-----	2.00	40 mg	200 ml	100 X
	Nicotinic acid	-----	0.5	10		
	Pyridoxine HCl	-----	0.5	10		
	Thiamine HCl	-----	0.1	2		

The required quantity of cobalt chloride was too small (0.5 mg) to be weighed properly on the available digital balance. This quantity was multiplied by 100 and the quantity obtained (50 mg) was dissolved in 100 millilitres of water. Then one millilitre of this solution was added to stock solution C and final volume of 200 ml was made by adding double distilled water**.** Similar method was used for adding required quantity of cupric sulphate and thiamine to their respective stock solutions.

For the preparation of the iron stock solution (stock solution F), required quantities of $FeSO_4.7H_2O$ and Na_2EDTA were dissolved separately in 75 ml double distilled water. Na_2EDTA solution was heated over hot plate and stirred to dissolve it completely. It was then added to $FeSO_4.7H_2O$ solution gently and stirred well to get a homogeneous light yellow solution. Final volume (200 ml) of the mixture was made by adding double distilled water and stored in an amber-coloured reagent bottle.

Preparation of stock solutions of growth regulators: Basal medium was supplemented with different types of growth regulators. Small quantity of Stock solution (50 ml) of each growth regulator was prepared on weekly/monthly basis and stored in refrigerator at 4^oC. Stock solutions of benzyl-aminopurine (BAP), kinetin, naphthalene-acetic-acid (NAA), indole-3-aceticacid (IAA) and indole-3-butyric-acid (IBA) were prepared by dissolving 50 mg of each growth regulator in 25 ml of 0.50 N NaOH and then volume was made to 50 ml with double distilled water. However, 2, 4-D was dissolved directly in sterile double distilled water (50 $mg^{-50\ ml}$).

Preparation of culture media: The required quantity of sucrose (usually 30 g l^{-1}) and myo-inositol was dissolved in double distilled water in a beaker. Required quantity of stock solutions containing macro elements, microelements, vitamins and growth regulators were added as per the treatment requirement. Final volume of the medium was made with the addition of double distilled water. The pH of medium solution was adjusted at 5.7 by adding 1N NaOH or 1 N HCl drop wise with a micropipette. Care was taken that electrode does not come in direct contact with the walls of beaker and bulb of electrode remains completely immersed in the solution. The medium was slightly heated in microwave oven and then required quantity of agar was added, stirred well with scalpel or stirrer and again heated in microwave oven to boil so as to dissolve the agar completely. The medium was allowed to cool for few minutes and subsequently dispensed into culture vessels. Non absorbent cotton plugs covered with news paper were used to plug the culture vessels.

Sterilisation of culture media: Culture media in test tubes/flasks was sterilised by autoclaving in a horizontal autoclave at 121°C and 1.05 kg cm^{-2} (15 psi) for 20 minutes. This high temperature not only kills bacteria and fungi, but also their heat-resistant spores. Flasks and test tubes were placed in autoclave when it had developed the required pressure. Lid of the autoclave was closed and vacuum valve opened to remove air from the autoclave chamber. Vacuum value was closed and steam inlet valve opened. Pressure indicator lamp (green lamp) switches off when required pressure (15 psi) is developed inside the autoclave chamber. Time for autoclaving was counted from the switching off of the pressure indicator lamp of the autoclave. After autoclaving the test tubes and flasks for the required time, electric supply of the autoclave was switched off and exhaust valve opened to release the pressure slowly. Autoclaved media was allowed to cool overnight at room temperature and used when required within 10 days.

Transfer area and aseptic manipulations: All the aseptic manipulations like surface sterilisation, preparation and inoculation of explants and subsequent sub-culturing were carried out in the laboratory using laminar air flow cabinet where pure air is forced out continuously so that no contaminant is allowed to enter the cabinet during working time. Pure air is generated by forcing the air to pass through a series of fine filters which remove dust and other potential contaminants. The working table of laminar air flow chamber was first surface sterilised with absolute alcohol and then by ultraviolet light for 30 minutes. The forceps, scalpel and Petri-dishes were first steam sterilised in an autoclave at 121^oC for 20 minutes. These instruments were further sterilised at the start of inoculation by pouring some ethyl alcohol over them in the laminar hood followed by flaming. During inoculation forceps and blade were flame sterilised several times. Hands were cleaned and wiped with Hi-Care disinfectant gel before working.

Culture incubation: The cultures were generally incubated at 24 ± 1^oC in an air conditioned culture room with a 16/8 hour light/dark regime and a light intensity of 3500 lux.

Collection of explants: Actively growing shoots under polyhouse were collected from the healthy plants of chrysanthemum cvs. "Candid" and "Flirt". The shoots were excised from the mother plants with help of a sharp blade and placed in a beaker containing distilled water and brought to the laboratory for isolation of explants.

Different types of explants used during the course of present investigation were shoot tips, petals, nodal segments and leaf segments. Shoot tips were mainly collected from the secondary branches of the mother plant. The explants were placed in cleanly washed flasks containing distilled water for further processing in the laboratory. Explants were extracted from the plants growing in the field. The explants were excised from the first upper node than originating from second or third node and were cut approximately to the size of 5 cm before final processing.

The young leaves just below the apical meristem were separated from the shoots with the help of scissors. They were cut into small pieces and placed in flasks containing distilled water for further processing in the laboratory. After removing few outer leaves, the explants were placed in clean flasks containing distilled water before further processing.

Standardisation of experiments: Different types of experiments were conducted during the present investigation aimed at the development of a protocol for *in vitro* sterilisation of the two chrysanthemum cultivars. Details regarding the methodologies adopted are given in the sections that follow.

Explant surface sterilisation and inoculation: Explant surface sterilisation is the first vital step for the initiation of cultures *in vitro.* Final steps in surface sterilisation were performed under the aseptic conditions of laminar flow hood. Knives and forceps were flame sterilised before use. While making incisions, knives and forceps were flame sterilised to avoid spread of contamination between different parts of the explants. Details of surface sterilisation procedures for different explants are given in the following sections.

Shoot tips: Shoot tips brought to the laboratory were washed with running tap water several times to remove any adhering dirt or dust particles. The outer leaves except few young were removed to reduce shoot tips to manageable size and there after given a vigorous shake for 30 minutes in Tween-20 surfactant solution (few drops) fortified with required concentration of fungicide concentration. The surfactant along with the fungicide was washed off under running tap water followed by a final washing with single distilled water. After initial cleaning the explants were latter brought to laminar flow hood for treatment with mercuric chloride. The shoot tips were given final 3 rinses with sterile water. Then all the leaves were removed from shoot tips and were placed on the medium for aseptic inoculation conforming to their original polarity. The details regarding the composition of various sterilant formulations and duration of application are given in the Table 3.

Nodal segments, leaf segments and petals: Explants on arrival to the laboratory were run through the same surface sterilisation procedure as followed in the shoot tips. In case of nodal segments two keeled leaves were removed and finally cut to the size of 1.0, 2.0 and 3.0 cm by giving both top and basal recut to the segments in order to remove the sterilant damaged tissues, while as leaf explants were reduced to the uniform size of 1 cm segments. Petals from both

Table 3. Composition of sterilant formulations and duration of application for surface sterilisation of various explants

S_1:	Mercuric chloride 0.1% for 2 minutes
S_2:	Mercuric chloride 0.1% for 4 minutes
S_3:	Sodium hypochlorite 0.1% for 2 minutes
S_4:	Sodium hypochlorite 0.1% for 4 minutes
S_5:	Carbendazim 100 ppm for 30 minutes + S_1+ Ethyl alcohol 70% for 10 seconds
S_6:	Carbendazim 100 ppm for 30 minutes + S_2+ Ethyl alcohol 70% for 10 seconds
S_7:	Carbendazim 200 ppm for 30 minutes + S_1+ Ethyl alcohol 70% for 10 seconds
S_8:	Carbendazim 200 ppm for 30 minutes + S_2+ Ethyl alcohol 70% for 10 seconds
S_9:	Carbendazim 100 ppm for 30 minutes + S_3+ Ethyl alcohol 70% for 10 seconds
S_{10}:	Carbendazim 100 ppm for 30 minutes + S_4+ Ethyl alcohol 70% for 10 seconds
S_{11}:	Carbendazim 200 ppm for 30 minutes + S_3+ Ethyl alcohol 70% for 10 seconds
S_{12}:	Carbendazim 200 ppm for 30 minutes + S_4+ Ethyl alcohol 70% for 10 seconds

the cultivars were cut and placed in distilled water. Finally a horizontal cut was given at the terminal end before placing them on the inoculation medium.

Observations with regard to aseptic cultures (without infection) and explant survival (surviving explants) were made upto 4 weeks of inoculation. Data for following parameters were recorded during the course of investigation.

i) **Asepsis (%) =** Aseptic cultures per treatment per replication / total number of cultures per treatment per replication X 100.

ii) **Survival (%) =** Surviving cultures per treatment per replication / total number of aseptic cultures per treatment per replication X 100.

2.3 Statistical Analysis

Statistical analysis of the data collected for different parameters during the present investigation was subjected to analysis of variance for completely randomized design with four replications [3]. To satisfy model assumptions for analysis of variance, percentage data was subjected to square root transformation as suggested by Steel and Torrie [4].

3. RESULTS AND DISCUSSION

To achieve desired outcome of any tissue culture procedure, choosing the correct explant is essential with minimal delays, besides proper sterilisation of the explants is the pre-requisite step leading to the development of a successful protocol for *in vitro* propagation. Inadequate sterilisation may result in a total loss of explants incubated on the sucrose containing medium and the incubation conditions which are most favourable for microbial growth. Any minute contamination on the explant (i.e., a single live spore or a bacterial cell) after sterilisation can create havoc.

Washing explants under running tap water for 30 minutes can assist in obtaining sterile cultures by physically removing some of the contaminants, especially with field-grown material [5]. After washing explants with tap water, several workers have used different sterilants like mercuric chloride, sodium hypochlorite or ethyl alcohol for the disinfection singly or in combination with a fungicide as carbendazim.

The results of the present investigation regarding the explant sterilisation using mercuric chloride singly or in combination with carbendazim dip have been discussed in the following sections.

3.1 Culture Asepsis of Shoot Tip, Petals, Nodal Segment and Leaf Segment

Four types of explants viz., shoot tip, petals, nodal segment and leaf were surface sterilised with mercuric chloride 0.1% singly for 2 and 4 minutes or in combination with carbendazim surfactant treatment at 100 and 200 ppm for 30 minutes followed by ethyl alcohol 70% for 10 seconds. Significant differences among means were noticed for both explant and sterilant treatments in both the cultivars (Tables 4 and 5). Highest culture asepsis was achieved in shoot tips, followed by petals, nodal segment and leaf segment explants. This may be attributed to the covered position of shoot tips provided by the several leaf whorls, thereby protecting them against any external contamination. While preparing the shoot tips for incubation, the outer leaves are removed under laminar flow hood, thus leaving limited chances for growth of contaminants in the cultures. Petals were incubated under laminar flow hood , thus leaving limited chances for growth of contaminants in the cultures. On the other hand nodal segments are exposed, bearing opposite pair of keeled leaves. This transition area between leaf and stem provides an ideal place for harbouring contaminants causing decrease in culture asepsis percentage. Leaves in chrysanthemum provide more surface area for accumulation of dirt, dust and disease spores. Hence the inoculum load is enhanced in leaves in comparison to other plant parts. This is reflected in lower asepsis percentage in leaf explants of both the cultivars.

Among sterilant treatments, a 2 minute mercuric chloride 0.1 % dip marginally improved culture asepsis of chrysanthemum explants as compared to a 4 minute dip (Tables 4 and 5). However, mercuric chloride 0.1 % irrespective of time duration in combination with higher concentration of carbendazim (200 ppm) for 30 minutes dip followed by ethyl alcohol 70% dip for 10 seconds significantly improved the culture asepsis in all the four explants recorded under S_7 and S_8 treatments in both the cultivars. Mercuric chloride being a reliable disinfectant, most of the researchers have successfully used it singly for the surface sterilisation of chrysanthemum explants. Waseem et al. [6] used nodal segments

of chrysanthemum (*Chrysanthemum morifolium* L) and were sterilised with 1.0% mercuric chloride for three minutes. Mishra et al. [7] standardised an efficient protocol for large scale multiplication of chrysanthemum cv. Yellow Bungalow and obtained good success for both shoot tip and nodal segment explants when pre-treated with 0.2% carbendazim + 200 ppm 8-hydroxy quinnoline citrate (HQC) for 2 h, followed by 3 minutes agitation in 0.1% $HgCl_2$. Hiremath et al. [8] reported use of mercuric chloride 0.1 % as surface disinfectant of shoot tip, nodal segment, leaf and flower bud explants of carnation for a 3 minute dip. Devi and Gupta [9], also employed mercuric chloride 0.1% dip for 2-3 minutes in carnation explants of shoot tip, nodal stem, inter-nodal and leaf segments. Sharma and Srivastava [10] employed a 10 minute 0.1% mercuric chloride dip. In the present studies a five minutes dip appears to be just adequate for a reasonable level of asepsis and to avoid mortality of explants which are only a few cells thick. This is in sharp contrast to observations made earlier [11,10]. Parthasarathy et al. [12] sterilised nodal, internodal and shoot tip explants of Chrysanthemum by washing thoroughly with running tap water for 15 minutes followed by agitation in 5% aqueous solution of Teepol. The explants were repeatedly washed with single distilled water, disinfected with 90% ethanol for 10 seconds and subsequently surface sterilised with $HgCl_2$ solution (0.1% w/v) for 2 minutes followed by repeated rinsing with sterile double distilled water. Cossio and Bussi [13] cultured, actively growing chrysanthemum shoots each with at least one node and were washed and further trimmed after sterilising in 70% ethanol followed by 4% sodium hypochlorite, then placed on nutrient agar substrate in test tubes and kept at 23-25 °C in 16-h days under 4000 lux.

Perusal of data regarding sterilants revealed significantly higher culture asepsis in treatments involving mercuric chloride (0.1%) for 2 minutes duration in combination with carbendazim 200 ppm followed by ethyl alcohol 70% dip for 10 seconds in both the cvs. “Candid” and “Flirt”.

3.2 Survival of Shoot Tip, Petals, Nodal Segment and Leaf

For successful micro-propagation, the first objective is to achieve maximum culture asepsis as possible by using the sterilants. In the process of sterilisation of explants, the type, concentration and duration of the sterilant treatment are the important factors not only to achieve a desirable level of asepsis, but also to ensure that explants survive the undesirable toxicity effects. Keeping these factors under consideration, the present investigation attempted to evaluate the safe and effective use of sterilant treatments in order to achieve maximum explant survival.

In the current study four different explants (shoot tips, petals, nodal and leaf segments) in both the cultivars were employed to generate survival percentage data. However, shoot tips and nodal segments of cv. “Flirt” failed to generate any survival per cent data during the exploratory trials and hence were not included as explant in the final experiment.

Perusal of data (Table 6 and 7) reveal that there were significant differences among the means of culture survival observed for both explant type and sterilant treatments. Shoot tip explant in cv. “Candid” recorded significantly higher survival rate than other explants, while as in cv “Flirt” shoot tip exlant could not survive the sterilant treatment. This might be due the fact that shoot tips in case of cv. “Candid” are highly meristematic tissues which can withstand exposure to mercuric chloride this was followed by petal, nodal segments where survival was significantly better than leaf explants. This can be attributed to the safe position of shoot tips provided by the several outer leaf whorls, which are removed after sterilisation under laminar air flow hood, while preparing them for incubation. While in case of cv. “Flirt” shoot tips and nodal segments showed burning signs even with a minute quantity of mercuric chloride treatment resulting in very less survival,even in case of other explants too. Maximum lethal effect of sterilant treatments was observed in leaf explants, which recorded more mortality rate than other explants in case of cv. “Candid”, while in cv. “Flirt” maximum lethal effect of sterilant treatments was observed in shoot tip and nodal segment explants, which recorded more mortality rate than other explants. Huafang [14], also reported that leaves of tree peony were more sensitive to sterilants compared to other organs.

Among sterilant treatments, perusal of data reveal that longer duration of mercuric chloride (0.1%) exposure had significantly detrimental effect on survival of explants. The combinations involving 2 minute dip in 0.1% mercuric chloride resulted in significantly higher survival than those comprising 4 minute duration in case of cv.

Table 4. Influence of sterilants and explant type on culture asepsis (%) in chrysanthemum (*Dendranthemum morifolium)* cv. "Candid"

Sterilants	Explant type				
	Shoot tip	**Petals**	**Nodal segment**	**Leaf**	**Mean**
S_1: Mercuric chloride 0.1% for 2 minutes	81.25	79.17	72.92	70.83	**76.04**
	(9.01)	(8.89)	(8.53)	(8.41)	**(8.71)**
S_2 :Mercuric chloride 0.1% for 4 minutes	79.17	75.00	70.83	66.66	**72.91**
	(8.89)	(8.66)	(8.41)	(8.16)	**(8.53)**
S_3:Sodium hypochlorite 0.1% for 2 minutes	62.50	60.41	54.17	50	**56.77**
	(7.90)	(7.75)	(7.35)	(7.07)	**(7.52)**
S_4:Sodium hypochlorite 0.1% for 4 minutes	64.58	62.50	56.25	54.16	**59.37**
	(8.03)	(7.90)	(7.49)	(7.35)	**(7.69)**
S_5:Carbendazim 100 ppm for 30 minutes + S_1+ Ethyl alcohol 70% for 10 seconds	87.50	83.33	79.17	75	**81.25**
	(9.35)	(9.13)	(8.89)	(8.66)	**(9.00)**
S_6:Carbendazim 100 ppm for 30 minutes + S_2+ Ethyl alcohol 70% for 10 seconds	83.33	81.25	75.00	72.91	**78.12**
	(9.13)	(9.01)	(8.66)	(8.53)	**(8.83)**
S_7:Carbendazim 200 ppm for 30 minutes + S_1+ Ethyl alcohol 70% for 10 seconds	91.66	89.58	87.50	83.33	**88.02**
	(9.57)	(9.46)	(9.35)	(9.13)	**(9.37)**
S_8:Carbendazim 200 ppm for 30 minutes + S_2+ Ethyl alcohol 70% for 10 seconds	89.58	87.50	81.25	79.16	**84.37**
	(9.46)	(9.35)	(9.01)	(8.89)	**(9.17)**
S_9:Carbendazim 100 ppm for 30 minutes + S_3+ Ethyl alcohol 70% for 10 seconds	66.66	64.58	58.33	56.24	**61.45**
	(8.16)	(8.03)	(7.64)	(7.49)	**(7.83)**
S_{10}:Carbendazim 100 ppm for 30 minutes + S_4+ Ethyl alcohol 70% for 10 seconds	70.83	66.66	62.50	58.33	**64.58**
	(8.41)	(8.16)	(7.90)	(7.64)	**(8.02)**
S_{11}:Carbendazim 200 ppm for 30 minutes + S_3+ Ethyl alcohol 70% for 10 seconds	72.92	70.83	64.58	62.49	**67.70**
	(8.53)	(8.41)	(8.03)	(7.90)	**(8.21)**
S_{12}:Carbendazim 200 ppm for 30 minutes + S_4+ Ethyl alcohol 70% for 10 seconds	75.00	72.92	66.66	64.57	**69.79**
	(8.66)	(8.53)	(8.16)	(8.03)	**(8.34)**
Mean	**77.08**	**74.48**	**69.09**	**66.14**	
	(8.75)	**(8.60)**	**(8.28)**	**(8.10)**	

LSD *(P=0.05)*
Explant 0.094
Sterilant 0.163
*Explant *Sterilant NS*
Figures in the parenthesis are square root transformed values of percentage data

Table 5. Influence of sterilants and explant type on culture asepsis (%) in chrysanthemum (*Dendranthemum morifolium)* cv. "Flirt"

Sterilants	Explant type				
	Shoot tip	**Petals**	**Nodal segment**	**Leaf**	**Mean**
S_1: Mercuric chloride 0.1% for 2 minutes	79.17	72.92	70.83	66.66	**72.39**
	(8.89)	(8.53)	(8.41)	(8.16)	**(8.50)**
S_2 :Mercuric chloride 0.1% for 4 minutes	75.00	70.83	66.66	64.58	**69.27**
	(8.66)	(8.41)	(8.16)	(8.03)	**(8.31)**
S_3:Sodium hypochlorite 0.1% for 2 minutes	56.25	47.92	47.92	45.83	**49.48**
	(7.49)	(6.91)	(6.91)	(6.76)	**(7.02)**
S_4:Sodium hypochlorite 0.1% for 4 minutes	58.33	50.00	54.17	50.00	**53.12**
	(7.64)	(7.07)	(7.35)	(7.07)	**(7.28)**
S_5:Carbendazim 100 ppm for 30 minutes + S_1+ Ethyl alcohol 70% for 10 seconds	83.33	79.17	75.00	72.92	**77.60**
	(9.13)	(8.89)	(8.66)	(8.53)	**(8.80)**
S_6:Carbendazim 100 ppm for 30 minutes + S_2+ Ethyl alcohol 70% for 10 seconds	81.25	75.00	72.92	70.83	**75.00**
	(9.01)	(8.66)	(8.53)	(8.41)	**(8.65)**
S_7:Carbendazim 200 ppm for 30 minutes + S_1+ Ethyl alcohol 70% for 10 seconds	**89.58**	**87.50**	**83.33**	**81.25**	**85.41**
	(9.46)	**(9.35)**	**(9.13)**	**(9.01)**	**(9.23)**
S_8:Carbendazim 200 ppm for 30 minutes + S_2+ Ethyl alcohol 70% for 10 seconds	**87.50**	**83.33**	**81.25**	**79.17**	**82.81**
	(9.35)	**(9.13)**	**(9.01)**	**(8.89)**	**(9.09)**
S_9:Carbendazim 100 ppm for 30 minutes + S_3+ Ethyl alcohol 70% for 10 seconds	64.58	58.33	56.25	54.17	**58.33**
	(8.03)	(7.64)	(7.49)	(7.35)	**(7.63)**
S_{10}:Carbendazim 100 ppm for 30 minutes + S_4+ Ethyl alcohol 70% for 10 seconds	66.66	62.50	58.33	56.25	**60.93**
	(8.16)	(7.90)	(7.64)	(7.49)	**(7.79)**
S_{11}:Carbendazim 200 ppm for 30 minutes + S_3+ Ethyl alcohol 70% for 10 seconds	70.83	64.58	62.50	58.33	**64.06**
	(8.41)	(8.03)	(7.90)	(7.64)	**(7.99)**
S_{12}:Carbendazim 200 ppm for 30 minutes + S_4+ Ethyl alcohol 70% for 10 seconds	72.92	66.66	64.58	62.50	**66.66**
	(8.53)	(8.16)	(8.03)	(7.90)	**(8.15)**
Mean	**73.78**	**68.23**	**66.14**	**63.54**	
	(8.56)	**(8.22)**	**(8.10)**	**(7.93)**	

LSD *(P=0.05)*
Explant 0.091
Sterilant 0.159
*Explant *Sterilant NS*
Figures in the parenthesis are square root transformed values of percentage data

Table 6. Influence of sterilants and explant type on culture survival (%) in chrysanthemum (*Dendranthemum morifolium)* cv. "Candid"

Sterilants	Explant type				
	Shoot tip	**Petals**	**Nodal segment**	**Leaf**	**Mean**
S_1: Mercuric chloride 0.1% for 2 minutes	75.00	72.91	66.66	64.58	**67.79**
	(8.66)	(8.53)	(8.16)	(8.03)	**(8.34)**
S_2 :Mercuric chloride 0.1% for 4 minutes	72.92	70.83	64.58	62.50	**67.70**
	(8.53)	(8.41)	(8.03)	(7.90)	**(8.21)**
S_3:Sodium hypochlorite 0.1% for 2 minutes	56.25	54.16	47.92	45.83	**51.04**
	(7.49)	(7.35)	(6.91)	(6.76)	**(7.13)**
S_4:Sodium hypochlorite 0.1% for 4 minutes	58.33	56.24	50.00	47.92	**53.12**
	(7.64)	(7.49)	(7.07)	(6.91)	**(7.28)**
S_5:Carbendazim 100 ppm for 30 minutes + S_1+ Ethyl alcohol 70% for 10 seconds	81.25	79.16	72.92	70.83	**76.04**
	(9.01)	(8.89)	(8.53)	(8.41)	**(8.71)**
S_6:Carbendazim 100 ppm for 30 minutes + S_2+ Ethyl alcohol 70% for 10 seconds	79.17	75.00	70.83	66.66	**72.91**
	(8.89)	(8.66)	(8.41)	(8.16)	**(8.53)**
S_7:Carbendazim 200 ppm for 30 minutes + S_1+ Ethyl alcohol 70% for 10 seconds	**89.58**	**83.33**	**79.17**	**75.00**	**81.77**
	(9.46)	**(9.13)**	**(8.89)**	**(8.66)**	**(9.03)**
S_8:Carbendazim 200 ppm for 30 minutes + S_2+ Ethyl alcohol 70% for 10 seconds	**83.33**	**81.24**	**75.00**	**72.92**	**78.12**
	(9.13)	**(9.01)**	**(8.66)**	**(8.53)**	**(8.83)**
S_9:Carbendazim 100 ppm for 30 minutes + S_3+ Ethyl alcohol 70% for 10 seconds	62.50	58.33	54.17	50.00	**56.25**
	(7.90)	(7.64)	(7.35)	(7.07)	**(7.49)**
S_{10}:Carbendazim 100 ppm for 30 minutes + S_4+ Ethyl alcohol 70% for 10 seconds	64.58	62.49	56.25	54.17	**59.37**
	(8.03)	(7.90)	(7.49)	(7.35)	**(7.69)**
S_{11}:Carbendazim 200 ppm for 30 minutes + S_3+ Ethyl alcohol 70% for 10 seconds	66.66	64.57	58.33	56.25	**61.45**
	(8.16)	(8.03)	(7.64)	(7.49)	**(7.83)**
S_{12}:Carbendazim 200 ppm for 30 minutes + S_4+ Ethyl alcohol 70% for 10 seconds	70.83	66.66	62.50	58.33	**64.58**
	(8.41)	(8.16)	(7.90)	(7.64)	**(8.02)**
Mean	**71.70**	**68.75**	**63.19**	**60.41**	
	(8.44)	**(8.26)**	**(7.92)**	**(7.74)**	

LSD *(P=0.05)*
Explant 0.093
Sterilant 0.162
Explant *Sterilant NS
Figures in the parenthesis are square root transformed values of percentage data

Table 7. Influence of sterilants and explant type on culture survival (%) in chrysanthemum (*Dendranthemum morifolium*) cv. "Flirt"

Sterilants	Explant type		
	Petals	**Leaf**	**Mean**
S_1: Mercuric chloride 0.1% for 2 minutes	70.83	64.57	**67.70**
	(8.41)	(8.03)	**(8.21)**
S_2 :Mercuric chloride 0.1% for 4 minutes	66.66	62.49	**64.58**
	(8.16)	(7.90)	**(8.03)**
S_3:Sodium hypochlorite 0.1% for 2 minutes	47.92	45.83	**46.87**
	(6.91)	(6.76)	**(6.83)**
S_4:Sodium hypochlorite 0.1% for 4 minutes	54.17	47.91	**51.04**
	(7.35)	(6.91)	**(7.13)**
S_5:Carbendazim 100 ppm for 30 minutes + S_1+ Ethyl alcohol 70% for 10 seconds	75.00	70.83	**72.92**
	(8.66)	(8.41)	**(8.53)**
S_6:Carbendazim 100 ppm for 30 minutes + S_2+ Ethyl alcohol 70% for 10 seconds	72.92	66.66	**69.79**
	(8.53)	(8.16)	**(8.34)**
S_7:Carbendazim 200 ppm for 30 minutes + S_1+ Ethyl alcohol 70% for 10 seconds	**83.33**	**79.16**	**81.25**
	(9.13)	**(8.89)**	**(9.01)**
S_8:Carbendazim 200 ppm for 30 minutes + S_2+ Ethyl alcohol 70% for 10 seconds	**81.25**	**72.91**	**77.08**
	(9.01)	**(8.53)**	**(8.77)**
S_9:Carbendazim 100 ppm for 30 minutes + S_3+ Ethyl alcohol 70% for 10 seconds	56.25	50	**53.12**
	(7.49)	(7.07)	**(7.28)**
S_{10}:Carbendazim 100 ppm for 30 minutes + S_4+ Ethyl alcohol 70% for 10 seconds	58.33	54.16	**56.25**
	(7.64)	(7.35)	**(7.49)**
S_{11}:Carbendazim 200 ppm for 30 minutes + S_3+ Ethyl alcohol 70% for 10 seconds	62.50	56.24	**59.37**
	(7.90)	(7.49)	**(7.69)**
S_{12}:Carbendazim 200 ppm for 30 minutes + S_4+ Ethyl alcohol 70% for 10 seconds	64.58	58.33	**61.45**
	(8.03)	(7.64)	**(7.83)**
Mean	**66.14**	**60.76**	
	(8.103)	**(7.763)**	

LSD *(P=0.05)*
Explant 0.099
Sterilant 0.243
*Explant *Sterilant NS*
Figures in the parenthesis are square root transformed values of percentage data

"Candid", while in cv. "Flirt" the combinations involving less than 2 minute dip even in 0.25% mercuric chloride resulted detrimental effect on survival of explants. Mercuric chloride being a potent sterilant is also known to compromise membrane integrity of live plant tissue. Therefore, longer duration resulted in decreased explant survival as against shorter one. Moreover, use of a final 10 second dip in ethyl alcohol further improved survival of explants significantly. Concentrated ethyl alcohol is a powerful sterilant that kills bacterial and fungal spores by instantly drawing out water out of them. Along with the water, ethyl alcohol removes any traces of Hg^{2+} ions from the explant tissue. Hence, most of the workers have advocated caution in use of mercuric chloride as a sterilant. Mohanty et al. [15] reported use of a final wash in 1% hypertonic KCL solution to draw out any traces of Hg2+ from the explant tissue. Verma et al. [16] reported that surface sterilisation with 0.1% $HgCl_2$ gave the highest survival percentage In another report nodal segment explants of chrysanthemum washed with household detergent to remove the impurities and then washed thoroughly under tap water and then sterilisation was done with 70% alcohol for 2 minutes followed by 1% $HgCl_2$ for 2~3 minutes [17]. Similar inferences were drawn by Singh et al. [18], Sooch et al. [19], Singh and Choudhary [20] and Bora et al. [21] who exposed explants of carnation to mercuric chloride (0.1%) for 2 minute duration in sterilisation process, followed by thorough washing in sterile distilled water several times in order to remove any traces of Hg^{2+} from the explant tissue.

4. CONCLUSION

The study concludes that Carbendazim 200 ppm for 30 minutes + mercuric chloride (0.1%) dip for 2 minutes and final treatment with ethyl alcohol (70%) for 10 seconds proved better for culture asepsis and survival of shoot tips followed by petals, nodal segments and leaf segments in chrysanthemum morifolium cv. "Candid" and thus leads to the standardisation of the shoot tip explant for culture asepsis in both the cultivars and shoot tip and petal explant for culture survival in *chrysanthemum* cv. "Candid" and "Flirt" respectively.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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