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Optimization the sterilization and acclimatization protocol for micropropagation of commercial cultivar chrysanthemum 'Maghi White'

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Abstract
The present investigation utilized different explants namely shoot tips, nodal segments and internodal segments of chrysanthemum cv. "Maghi White" for sterilization with different concentrations of mercuric chloride with respect to variations in time durations. It revealed that treatment with 0.1% mercuric chloride for 1 minute duration for shoot tip explants and that for 3 minutes duration for nodal and internodal segments was found best for maximum survival of the explants. For rooting, MS medium supplemented with IBA (1.00 mg/l) exhibited best results. And earliest root emergence (11.28 days), root length (5.53 cm) and percent rooting (85.18) were obtained on this medium. Potting mixture containing 75% Cocopeat + 25% Perlite was found most suitable for hardening of *in vitro* raised plantlets.

Keywords: *Chrysanthemum morifolium*, micro propagation, shoot regeneration, rooting and hardening

Introduction
Chrysanthemum (*Chrysanthemum morifolium* Ramat) a member of family Asteraceae, is an economically important ornamental plant in the global floriculture industry both as cut flower and as pot plant. It stands world second most economically important floricultural crop following rose with 35 percent share in the total cut flower production. The technique of year round blooming based upon scientific research in the field of photoperiodism and genetics has lead to immense increase in the utility and popularity of chrysanthemum worldwide.
Chrysanthemum can be propagated by seeds, suckers and stem cuttings. Its multiplication by seed is very rare because plants raised from seeds are not true to type and in addition to this, growth and development of plantlets is very slow, weak and poor. Multiplication through root suckers is limited as the numbers of suckers produced are very less and also take long time to flower. Moreover, a chance of diseases transfer from the mother plant to the suckers is very high (Waseem *et al*., 2008) [42]. Also, non-uniform performance of the plants is of major concern since the suckers are not of the same age. In commercial propagation of chrysanthemum through stem cuttings, the cuttings are taken repeatedly from the same mother plant which results into a very high chance of viral infection in the propagating material, thereby increasing production costs. Non availability of quality planting material in adequate quantity is one of the challenges faced by the floricultural industry. Thus to have virus free true to type planting material in a relatively shorter period within a small space and making it available throughout the year, micro propagation is the most viable option
Chrysanthemum has the capability of regeneration from leaf, shoot tip, node, inter-node, petal *etc*. through tissue culture techniques (Kumari and Varghese, 2003; Waseem *et al*., 2011) [17, 41]. Therefore, the present study focused for optimization of cultivar chrysanthemum "Maghi White for micro propagation

Materials and Methods
The present investigation on "Micropropagation studies in chrysanthemum (*Chrysanthemum morifolium Ramat*)" was conducted by the Division of Vegetable Science and Floriculture, at Tissue Culture Laboratory, School of Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Faculty of Agriculture, Chatha Jammu during the year 2014-15 where as the mother stock of selected "Maghi White" grown at Experimental Area-I, Division of Vegetable Science and Floriculture, Faculty of Agriculture, Chatha.

Preparation of Explants material

The explants (shoot tips, nodal segments and internodal segments) of chrysanthemum cv "Maghi White" were thoroughly washed under running tap water for 30 minutes and then after washing the explants were treated with fungicides (Bavistin @ 0.1% + Dithane M - 45 @ 0.2%) for 10 minutes and then rinsed with distilled water and treated with 70% ethanol for 20 seconds.

Standardization of Surface sterilization of explants

The explants were treated with most effective surface sterilization method of mercuric chloride under aseptic conditions with different concentrations (0.05 and 0.1 percent) as well as different durations (1-5 minutes) and rinsed thrice with sterilized distilled water. The explants were then inoculated on test tubes and flasks containing culture medium (MS medium supplemented with different growth regulators and incubated at 25±2 ºC temperature in 16 hours continuous fluorescent light (3500 lux) followed by dark periods of 8 hours.

Percent survival of explants

The percent survival of explants was calculated after 1 week of inoculation to find the cultures response to different mercuric chloride concentrations and durations. The percent survival was calculated by the following formula:

$$\frac{\text{Number of cultures survived}}{\text{Total number of cultures inoculated}} \times 100$$

Standardization of shoot regeneration medium

These study already carried by the Dogra S *et al*. in which the sterilized explants of size 1.5 cm cultured on basal MS medium performed well upto the establishment of aseptic cultures.

Standardization of rooting medium

For rooting, we standardized the multiplied shoots were cultured on ½ MS medium supplemented with different concentrations of NAA and IBA and various parameter of observations recorded such as days taken to root emergence, Percent rooting, Number of roots and Length of root as discussed in the result section.

Standardization of hardening medium

In order to acclimatize the *in vitro* raised chrysanthemum plantlets, a study on different hardening treatment was carried out. Uniformly rooted plantlets were taken from the culture vessels with the help of forceps. The agar medium from roots was washed gently under running tap water and after removal of adhering media; the plantlets were transplanted, 4 weeks after root initiation, into pots containing media of cocopeat and perlite. The data on percent survival of explants was calculated after 6 weeks of hardening into different growing media.

$$\frac{\text{Number of plants hardened}}{\text{Total number of plants taken for hardening}} \times 100$$

Results

The results of the present investigation with regard to sterilization and acclimatization protocol have been carried out in chrysanthemum "Maghi Whitecrop" ornamental crop.

Surface sterilization of Explants

a. Nodal explants

The data depicted in Table 1 statistically significant variations for percent survival of the nodal explants. The highest survival (63.64%) in the nodal explants was recorded, when they were exposed to mercuric chloride for 3 minutes (T_3) where as the minimum survival (30.17%) was obtained when exposed to mercuric chloride for 1 min. (T_1). Interaction among concentrations and durations showed that 3 minute exposure (T_3) to 0.1% concentration of $Hgcl_2$ resulted in highest survival (82.66%) with nodal explants in chrysanthemum where as minimum survival (20.33%) was obtained at 1 minute exposure (T_1) to 0.05% mercuric chloride.

Table 1: Effect of surface sterilization treatments on percent survival of nodal explant in chrysanthemum (*Chrysanthemum morifolium* Ramat) cv. Maghi White

Notation	Duration of exposure (Minutes)	Concentration of mercuric chloride (%)		Mean
		0.05	0.1	
T_1	1.0	20.33 (26.79)	40.00 (39.21)	30.17 (33.00)
T_2	2.0	24.63 (29.68)	51.03 (45.57)	37.83 (37.62)
T_3	3.0	44.62 (41.89)	82.66 (65.37)	63.64 (53.63)
T_4	4.0	50.27 (45.13)	46.39 (42.91)	48.33 (44.02)
T_5	5.0	56.05 (48.45)	35.55 (36.58)	45.80 (42.51)
	Mean	39.18 (38.39)	51.12(45.93)	

b. Shoot tip explants

The data presented in showed statistically significant variations for percent survival of shoot tip explants. The data revealed the highest survival (61.59%) in the shoot tip explants on exposure to mercuric chloride for 1 minute (T_1) where as the minimum survival (31.63%) was obtained at 5 minute exposure (T_5) in the Table 2. When explants were treated with different concentrations of $HgCl_2$ percent survival was found to be higher with 0.1% $HgCl_2$ than 0.05%. Among interactions 1 minute exposure (T_1) to 0.1% concentration resulted in highest survival (72.62%) of explants in chrysanthemum where as minimum survival (30.18%) was obtained at 5 minute exposure (T_5) to 0.05% mercuric chloride.

Table 2: Effect of surface sterilization treatments on percent survival of shoot tip explant in chrysanthemum (*Chrysanthemum morifolium* Ramat) cv. Maghi White.

Notation	Duration of exposure (Minutes)	Concentration of mercuric chloride (%)		Mean
		0.05	0.1	
T_1	1.0	50.56 (45.30)	72.62 (58.44)	61.59 (51.87)
T_2	2.0	51.17 (45.65)	60.79 (51.21)	55.98 (48.43)
T_3	3.0	55.55 (48.16)	53.61 (47.05)	54.58 (47.61)
T_4	4.0	44.09 (41.59)	45.61 (42.46)	44.85 (42.02)
T_5	5.0	30.18 (33.31)	33.08 (35.10)	31.63 (34.20)
	Mean	46.31 (42.80)	53.14 (46.86)	

c. Internodal explants

The data presented in the revealed statistically significant

variations for percent survival of internodal explants. The highest survival (52.41%) was recorded; when they were exposed to mercuric chloride for 3 minutes (T_3) where as the minimum survival (30.25%) was obtained at 1 minute exposure (T_1) to mercuric chloride in the Table 3. Among different concentrations, 0.1% mercuric chloride treatment resulted in maximum survival (45.64%). Interaction between various concentrations and durations of mercuric chloride exhibited highest survival (65.08%) of internodal explants at 3 minute exposure (T_3) to 0.1% $Hgcl_2$ in chrysanthemum where as minimum survival (25.47%) was obtained at 1 minute exposure (T_1) to 0.05% mercuric chloride.

Table 3: Effect of surface sterilization treatments on percent survival of internodal explant in chrysanthemum (*Chrysanthemum morifolium* Ramat) cv. Maghi White

Notation	Duration of exposure (Minutes)	Concentration of mercuric chloride (%)		Mean
		0.05	0.1	
T_1	1.0	25.47 (30.23)	35.04 (36.29)	30.25 (33.25)
T_2	2.0	31.22 (33.94)	40.59 (39.55)	35.90 (36.75)
T_3	3.0	39.73 (39.05)	65.08 (53.75)	52.41 (46.40)
T_4	4.0	42.48 (40.65)	46.99 (43.27)	44.74 (41.49)
T_5	5.0	40.85 (39.71)	40.48 (39.49)	40.67 (39.60)
	Mean	35.95 (36.72)	45.64 (42.47)	

Rooting

Effect of different rooting hormones on *in vitro* rooting of multiplied shoots has been given in Table 4.

a. Days taken to root emergence: Table 4 depicted that the earliest root emergence (11.28 days) was recorded on ½ MS medium +1.00 mg/ l IBA (T_9) followed by ½ MS medium + 0.50 mg/ l NAA (T_3) 13.55 days while maximum number of days to root emergence were observed on ½ MS medium (T_1) when no rooting hormone was used.

b. Percent rooting: The data in the Table 4. revealed that highest rooting (85.18%) was observed in ½ strength medium + 0.75 mg/l NAA (T_4) which was at par with ½ strength Ms. + 1.00 mg/l IBA (T_9) 80.00%, ½ strength Ms. + 0.75 mg/l IBA (T_8) 77.98% and ½ strength Ms. + 1.00 mg/l NAA (T_5) 77.92%. The minimum rooting (37.03%) was obtained with ½ MS medium (T_1) when no rooting hormone was used.

c. Number of roots: The highest number of roots (9.18) was obtained with ½ MS + 0.50 mg/ l NAA (T_3) where as minimum numbers of roots (3.00) were obtained on ½ MS (T_1) when no rooting hormone was used Table 4.

d. Root length (cm): The longest root length (5.53 cm) was obtained when ½ strength MS medium was fortified with 1.0 mg/l IBA (T_9) while the shortest root was obtained when no rooting hormone was used (Table 4).

Table 4: Effect of different rooting hormones on *in vitro* rooting of multiplied shoots in chrysanthemum (*Chrysanthemum morifolium* Ramat) cv. Maghi white

Notation	Treatments	Days taken to root emergence	Percent rooting	Number of roots	Root length (cm)
T_1	½ MS Medium	20.44	37.03 (37.45)	3.00	1.61
T_2	½ MS medium + 0.25 mg/L NAA	14.19	46.57 (43.00)	4.25	3.27
T_3	½ MS medium + 0.50 mg/L NAA	13.55	71.84 (57.98)	7.98	4.30
T_4	½ MS medium + 0.75 mg/L NAA	14.43	85.18 (67.33)	7.53	4.02
T_5	½ MS medium +1.00 mg/L NAA	15.04	77.92 (61.95)	5.51	3.52
T_6	½ MS medium + 0.25 mg/L IBA	16.05	56.07 (48.48)	5.12	3.58
T_7	½ MS medium + 0.50 mg/L IBA	16.91	60.00 (50.75)	6.11	4.04
T_8	½ MS medium + 0.75 mg/L IBA	14.06	77.98 (61.98)	7.10	4.07
T_9	½ MS medium +1.00 mg/L IBA	11.28	80.00 (63.43)	9.18	5.53
	SE (m) ±	0.36	1.84	0.30	0.30
	C.D (P=0.05)	1.08	5.51	0.90	0.91

Hardening

Effect of different media composition on percent survival of the tissue culture raised plantlets of chrysanthemum has been given in Table 5 Among the growing media used for hardening of plantlets, percent survival was significantly highest (83.86%) in the media containing 75% cocopeat + 25% Perlite (T_5) followed by 70.66 percent survival in the media having 50% cocopeat + 50% perlite (T_4) where as the lowest survival (34.41%) was recorded with 100% perlite (T_1) alone.

Table 5: Effect of different media composition on percent survival of the tissue culture raised plantlets of chrysanthemum (*Chrysanthemum morifolium* Ramat) cv. Maghi White

Notation	Treatments	Percent survival
T_1	Cocopeat	39.99 (39.20)
T_2	Perlite	34.41 (35.86)
T_3	Cocopeat 25% + Perlite 75%	52.76 (46.51)
T_4	Cocopeat 50% + Perlite 50%	70.66 (57.40)
T_5	Cocopeat 75% + Perlite 25%	83.86 (66.33)
	SE (m) ±	3.44
	CD (P = 0.05)	11.00

Discussion

The discussions of the present investigation with regard to sterilization and acclimatization protocol have been carried out in chrysanthemum "Maghi Whitecrop" ornamental crop are given in the following components.

a) Sterilization of Explants

In vitro propagation comprises of various stages *viz*., selection of explants, aseptic culture establishment, multiplication and acclimatization. But the most important and challenging is sterilization of explants for aseptic culture establishment. The problem is more exacerbated when the explants are sourced directly from field grown plants since surfaces of plant carry wide range of microbial contamination. To avoid these sources of infection, the explants are treated with different sterilants. In chrysanthemum cv. Maghi White, explants namely nodal segment, shoot tip and internodal segments were sterilized by exposing to different concentrations and durations of mercuric chloride.

The data presented in Tables 1 and 2 showed significant variations for percent survival of nodal and internodal explants, respectively. Maximum survival in nodal segments (82.66%) as well as in internodal segments (52.41%) was obtained when treated with 0.1% $HgCl_2$ for 3 minute durations (T_3), while minimum survival (20.33% & 25.47%, respectively) was obtained when explants were exposed to 0.05% concentration for 1 minute (T_1). The lower concentration resulted in contamination due to presence of microbes. These results are in conformity with previous studies on chrysanthemum (Mandal *et al*., 2002; Datta *et al*., 2005; Chitra *et al*., 2006 and Padmadevi *et al*., 2009) [7, 8, 6, 27].

A significant variation for percent survival of shoot tip explants was recorded when treated with $HgCl_2$ at different concentrations and durations (Table 2). In shoot tip explants, 0.1% concentration of mercuric chloride with the shortest duration of 1 minute (T_1) was found to be the most efficient sterilizing protocol as it gave (72.62%) contamination free explants. However, 0.5% $HgCl_2$ for 5 minute duration (T_5) showed the lowest survival (30.18%). There are many reports of surface sterilization in plant tissue culture using $HgCl_2$ (Anburaj *et al*., 2011; Preeti *et al*., 2011 and Sen *et al*., 2013) [3, 30, 45]. However there are reports that exposure to mercuric chloride may have negative effects on survival rate of explants (Danso *et al*., 2011). A large period of exposure with mercuric chloride can also lead to burning and death of explants. The present results showing deleterious effects of $HgCl_2$ at higher concentration /durations and these are in agreement with other reports (Jhonson *et al*., 2005; Wesely *et al*., 2011 and Sen *et al*., 2013) [15, 43, 45].

b) Rooting

The process of rooting on micro shoots in chrysanthemum is strongly affected by the type and concentration of growth regulators utilized in MS medium. In most species, efficient rooting was observed on medium containing auxins. Naphthalene acetic acid and Indole Butyric acid are most commonly used hormones for root induction. In the present studies, effect of different concentrations of NAA and IBA when supplemented in half strength MS medium for rooting of multiplied shoots showed a statistically significant behaviour for all the parameters (Table 2).

b.1) Days taken to root emergence

1.00 mg/l IBA (T_9) concentration proved to be best for earliest root formation while maximum number of days taken to root formation (20.44) was observed in ½ strength MS medium with no rooting hormone. In earliest root formation, the highest concentration of IBA had radically did better and proved its superiority over all the other treatments. Similar results have been obtained by Minas (2008) [22] in chrysanthemum.

b.21) Percent rooting

The data in the Table 2 revealed very high and encouraging results with respect to percent rooting. Highest rooting (85.18%) was observed in ½ strength medium + 0.75 mg/l NAA (T_4) which was at par with ½ strength Ms + 1.00 mg/l IBA (80.00%), ½ strength MS medium + 0.75 mg/l IBA (77.98%) and ½ strength MS medium + 1.00 mg/l NAA (77.92%). Maximum percent rooting was also achieved by Rashid *et al*., 2009 [32] and Waseem *et al*., (2011) [41] in chrysanthemum at higher concentrations of IBA and NAA.

b.3) Number of roots

Maximum numbers of roots (9.18) were produced using 1.00 mg/l IBA on ½ strength MS medium (Table 2). Waseem *et al*. (2011) [41] also found that IBA is a better rooting hormone than NAA with respect to number of roots.

b.4) Root length (cm)

The longest root length (5.53 cm) (Fig 4.4d) was obtained when ½ strength MS medium was fortified with 1.0 mg/l IBA (T_9). IBA is considered as the most effective auxin in root induction. Our results are conformity with findings of Rashid *et al*., 2009 [32] and Waseem *et al*., 2011 [41], who suggested IBA as the best auxin for root induction and development.

c) Hardening

Use of suitable growing media is essential for hardening of tissue culture raised plants. It directly affects the development. A good growing medium provides sufficient anchorage or support to the plant, serves as reservoir for nutrient, water, allows oxygen diffusion to the roots and permit gaseous exchange between the roots and atmosphere outside the root substrate.

Cocopeat a soilless substrate, is considered as a good growing media component with acceptable pH, electrical conductivity and other chemical attributes. However, cocopeat has been recognized to have high water holding capacity which causes poor air-water relationship, leading to low aeration within the medium, thus affecting the oxygen diffusion to the roots. Incorporation of coarser materials into cocopeat could improve the aeration status of the media. Perlite is among the possible coarser materials could be used to improve the air-water relationship of cocopeat. It is very useful for increasing aeration and drainage within the container because of its uniformity and lightness. The possibility of combining perlite with cocopeat to be used as growing media was examined in this study.

The data presented in the table 3, showed significant variations for percent survival. The highest survival of the tissue culture raised plants (83.86%) was recorded in the hardening media composed of cocopeat 75% + perlite 25% (T_5) followed by 50% cocopeat +50% perlite media (70.66%). These findings are in accordance with the findings of Trifunovic *et al.* (2006) [14] and Tymosuzuk *et al.* (2014) [39].

Conclusion

It can be concluded from the present studies that 0.1% concentration of mercuric chloride found optimum for maximum survival of nodal explants when exposed for 3 min duration. For shoot regeneration, nodal explants performed better than shoot tip when cultured on MS medium fortified with 2.0 mg/ l BAP+ 0.5 mg/l NAA. Mutliplication rate of regenerated shoots was found higher on the standardized regeneration media. Better results for rooting were obtained on ½ strength MS medium containing 1.0 mg/l BAP. The *in vitro* raised plantlest grew well in the hardening media composed of 75% cocopeat and 25% perlite. Thus, the protocol standardized for micropropagation of chrysanthemum cv. Maghi White can be utilized for mass multiplication and for further studies.

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