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Sterilization, germination and shoot proliferation from *Chrysanthemum coronarium* L. seeds

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ABSTRACT

Chrysanthemum coronarium, popularly called as garland chrysanthemum, is an annual highly cross-pollinated species and propagated by seed. The different flower forms viz., single, semi-double and double types are available in the species. However, maintaining such types is difficult due to high self incompatibility and heterozygosity due to outcrossing. Therefore development of homozygous lines is not possible by conventional methods due to segregation of progenies. Micropropagation offers production of large number of disease free, true to type planting material within short period of time irrespective of growing season for cultivation per se or for complementing crop improvement programme. Aseptic condition of culture medium is one of the main aspects for micro propagation. It was achieved by sterilization procedures using chemical treatments. Out of different surface sterilants pre tried, it was evident that the maximum seed germination (73.31 %) was obtained when seeds were surface sterilized with carbendazim (0.2%), ridomil (0.2%) and hydroxy quinoline citrate (8-HQC) (200 ppm) for 2 hours followed by ethanol (70%) for 30 sec and $HgCl_2$ (0.1%) for 3 minutes. Further, the seeds cultured on half strength Murashige and Skoog (MS) medium and gibberelic acid (GA_3) (2.0 mg L^{-1}) resulted in highest seed germination (51.34 %). Highest number of shoots micro-shoot^{-1} was obtained when the microshoots were sub cultured on half strength MS medium and GA_3 (0.5 mg L^{-1}).

***Keywords*:** Chrysanthemum, micropropagation, seed germination, shoot proliferation, surface sterilization and tissue culture

Chrysanthemum coronarium L. is an annual herbaceous ornamental weed, native to the Mediterranean regions (Basta *et al*., 2007), is commonly known as 'garland chrysanthemum' or 'crown daisy' and belongs to Asteraceae family. Its leaves has antioxidant, antibacterial, antifungal and anti-cancerous properties (Zheng *et al*., 2004). It is highly cross-pollinated in nature and conventionally propagated by seed. Classical breeding has limitations such as restricted gene pool, self-incompatibility due to differences in ploidy level and the polygenic control of growth and flowering characters. Plant tissue culture is an effective technique for plant micropropagation by using parts of plants such as cells, tissues and organs, cultured on synthetic medium under sterile conditions. Outcrossing due to high self incompatibility and thereby heterozygosity does not allow to get true to type plants through seed propagation. Therefore, *in vitro* propagation offers large-scale multiplication of true to the type disease-free plants from few explants (Bajaj *et al*., 1992) within a short period of time. *C. coronarium* is highly heterozygous self-incompatible species and shows wide spectrum of variability when grown by seed. To multiply desirable true to type's tissue culture is an alternative. Keeping these considerations, it is necessary to standardize protocol for surface sterilization, culture medium for seed germination and shoot proliferation in *C. coronarium*.

MATERIALS AND METHODS

Collection of seeds

Soon after harvest mature seeds of *Chrysanthemum coronarium* L. were collected from research farm (Latitude 28º38′N, Longitude 77º12′E and Altitude 228.4m above mean sea level) of Division of Floriculture and Landscaping, IARI, New Delhi. The study was carried out at the Central Tissue Culture Laboratory, LBS Centre, IARI, New Delhi during 2017-18.

Surface sterilization of seeds

Cleaned seeds were soaked in water for 30 minutes and non floating seeds were selected for the experiment. They were washed under running tap water for 30 minutes followed by washing with Teepol® and thereafter rinsed thrice with double sterile distilled water (T_0); and this treatment was common and followed prior to all other treatments as well. Thereafter, the seeds were washed with ethanol 70% (v/v) for 30 sec (T_1). Seeds were subjected to different surface sterilization treatments of carbendazim (0.2% w/v), ridomil (0.2% w/v) and 8- Hydroxyquinoline (HQC) (200 ppm) for 2 h followed by ethanol (70%) for 30 sec (T_2) and the same treatment that of T_2 was followed by $HgCl_2$ (0.1%) for 3 min (T_3) and $HgCl_2$ (0.1%) for 4 min (T_4) to establish contamination free cultures. These surface sterilized seeds were cultured on MS medium (Murashige and Skoog, 1962) (½ strength) + sucrose

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(30 g L^{-1}) + agar powder (7.0 g L^{-1}) for germination. Initially these seeds were kept in dark for one week followed by 16 h (57 µmol m^{-2} s^{-1}) photoperiod.

Media preparation and sterilization

The MS basal culture media was prepared using double distilled water. All the stock solutions, and sucrose were added to the double distilled water and final volume of the medium was made using a measuring cylinder. Thereafter, equal volume of media was distributed to each beaker for different treatment combinations followed by simultaneous addition of required quantity of plant growth regulators with the help of micropipette to each beaker according to the respective treatment combinations. The pH of the medium was adjusted to 5.78-5.8 with the addition of 1 N KOH or 1 N HCL using digital pH meter. For the gelling of culture medium, as per the treatment wherever required, agar powder (7.0g L^{-1}) was added to the culture medium at the luke warm stage. Then, medium was heated to melt the agar by continuous stirring to prevent agar from settling and charring. Thereafter, the hot media were dispensed into labeled glass vessels, closed the lids with non-absorbent cotton plugs and packed for autoclaving. Then culture media was sterilized at 121ºC temperature and 15-20 lbs/inch² pressure for 15-20 minutes. After that, the autoclave pressure was released and the media were removed, cooled down and taken to the aseptic conditions.

Culture medium for seed germination

The treatment T_0 contained only ½ MS medium devoid of agar. On the other hand, all other treatments contained Sucrose (30 g L^{-1}) and Agar powder (7 g L^{-1}).The treatments T_1 and T_2 has ½ and full strength MS salts, respectively. Further MS ½ strength was supplemented with GA_3 (1 mg L^{-1}) in treatment T_3, GA_3 (2 mg L^{-1}) in treatment T_5 and GA_3 (3 mg L^{-1}) in treatment T_7. Similarly full strength MS media was supplemented with GA_3 (1 mg L^{-1}) in treatment T_4, GA_3 (2 mg L^{-1}) in treatment T_6, and GA_3 (3 mg L^{-1}) in the treatment T_8.

Culture medium for shoot proliferation

To identify the optimum strength of basal medium (MS) for shoot proliferation, all the treatments T_0 to T_7 consisted of 30g/L mg/L sucrose and 7 g/L mg/L agar powder. Except the treatment T_0 with full MS salts, all others consisted of ½ strength MS salts. The treatments T_2 and T_3 were supplemented with GA_3 0.5 mg/L and 1 mg/L, respectively. On the other hand, the treatments T_4, T_5, T_6, and T_7 were supplemented with Benzylaminopurine (BAP) at 0.5 mg/L, 1 mg/L,1.5 mg/L, and 2 mg/L, respectively.. After shoot proliferation *in vitro* raised plantlets were cultured on MS (1/2 strength of salts) + 60 g/L sucrose + 7 g/L agar + 0.5 mg/L NAA for induction of rooting.

Experimental design and statistical analysis

The experiments were laid out in completely randomized design (CRD) with three replications per treatment. The data was analyzed using online OPSTAT software, HAU, Hisar, UK. All the percentage data was subjected to angular transformation before statistical analysis.

RESULTS AND DISCUSSION

Surface sterilization of seeds

Among different surface sterilization treatments for successful *in vitro* seed germination, the treatment T_3 was found to be the superior one and recorded reasonably less bacterial (11.11 %), fungal (23.33 %) infection and maximum contamination-free cultures (65.56%). Though T_4 recorded least bacterial (10.00%), fungal (22.22%) infection and maximum contamination free cultures (67.78%) but due to toxicity of $HgCl_2$ treatment for prolonged duration, seeds did not germinate (Table1). Some of the surface sterilants like fungicides (systemic/ contact), biocides (8-HQC), chemical sterilants like ethanol, sodium / potassium hypochlorite and mercuric chloride individually or in combinations along with antibiotics are known as pre-treatments to reduce the microbial load from the surface of the explants. Mederos and Lopez (1991) also reported that the use of fungicide benomyl in combination with $HgCl_2$ reduced fungal infection in *Pistacia atlantica*. However, pre- treatments with higher concentrations and for prolonged duration might become toxic and result in poor growth and low establishment of cultures in marigold (Kumar *et al*., 2017). The above treatments were also found to be highly effective for the establishment of axenic cultures in rose (Prasad, 1995), chrysanthemum (Verma *et al*., 2012), and bougainvillea (Kumari *et al*., 2016).

Seed germination

The treatment T_5 resulted in highest seed germination (51.34 %) in least days (3.89) (Fig.1) It was also statistically at par with respect to shoot length (4.72 cm) with treatment T3 recording maximum shoot length (5.12cm). Majumder *et al.* (2014) reported the effective use of GA_3 in culture establishment using shoot tip as explant in marigold cv. Pusa Narangi Gainda. GA_3 in combination with BAP was found significantly better for culture establishment during *in vitro* propagation of White marigold (Misra and Datta, 2001). Gibberellic acid is known to break seed dormancy and promote seed germination and thereby overall development of seedlings. The better root length in GA_3 enriched medium was explained by Brian and Hemming (1955) as the activity of endogenous auxin and auxin-derivatives also increased with the application of GA_3.

Table 1: Effect of seed surface sterilization treatments on contamination

Treatments	Bacterial contamination (%)	Fungal contamination (%)	Total Contamination (%)	Contamination free culture (%)
T_0: Control (double distilled water)	34.44 (35.85)	44.44 (41.77)	78.89 (62.63)	21.11 (27.32)
T_1: Ethanol (70%) for 30 sec	27.77 (31.75)	43.33 (41.14)	71.11 (57.52)	28.89 (32.43)
T_2: Carbendazim (0.2%) + Ridomil (0.2%) + 8-HQC (8Hydroxyquinoline) (200 ppm) for 2h followed by ethanol (70%) for 30 sec	24.44 (29.60)	41.11 (39.81)	65.56 (54.13)	34.44 (35.82)
T_3: Carbendazim (0.2%), Ridomil (0.2%) + 8-HQC (200 ppm) for 2h followed by ethanol (70%) for 30 sec + $HgCl_2$ (0.1%) for 3 min	11.11 (19.42)	23.33 (28.87)	34.44 (35.91)	65.56 (54.04)
T_4: Carbendazim (0.2%), Ridomil (0.2%) + 8-HQC (200 ppm) for 2h followed by ethanol (70%) for 30 sec and $Hgcl_2$ (0.1%) for 4 min	22.22 (28.09)	32.22 (34.56)	67.78 (55.39)	10.00 (18.26)
SEm (±)	**1.63**	**1.809**	**1.648**	**1.64**
LSD (0.05)	**5.19**	**5.77**	**5.260**	**5.26**

Note: Figures in parentheses are angular transformed values

Table 2: Effect of different culture media on *in vitro* seed germination

Treatments	Number of days to germination	Seed germination (%)	Shoot length (cm)	Root length (cm)
T_0: MS (1/2 strength of inorganic salts) liquid	3.78	34.00 (35.64)	4.95	3.56
T_1: MS (1/2 strength) + Sucrose (30 g L^{-1}) + Agar powder (7 g L^{-1})	4.22	23.86 (29.22)	3.50	3.86
T_2: MS (Full strength) + Sucrose (30 g L^{-1}) + Agar powder (7 g L^{-1})	4.33	25.69 (30.04)	3.98	3.75
T_3: MS (1/2 strength) + Sucrose (30 g L^{-1}) + GA_3 (1.0 mg L^{-1}) + Agar powder (7g L^{-1})	4.67	36.67 (37.25)	5.12	6.12
T_4: MS (Full strength) + Sucrose (30 g L^{-1}) + GA_3 (1.0 mg L^{-1}) + Agar powder (7 g L^{-1})	4.56	20.62 (26.83)	4.70	4.81
T_5: MS (1/2 strength) + Sucrose (30 g L^{-1}) + GA_3 (2.0 mg L^{-1}) + Agar powder (7 g L^{-1})	3.89	51.34 (45.75)	4.72	5.00
T_6: MS (Full strength) + Sucrose (30 g L^{-1}) + GA_3 (2.0 mg L^{-1}) + Agar powder (7 g L^{-1})	4.00	15.48 (23.05)	4.90	4.82
T_7: MS (1/2 strength) + Sucrose (30 g/L) + GA_3 (3.0 mg L^{-1}) + Agar powder (7 g L^{-1})	3.89	15.34 (22.66)	4.84	4.83
T_8: MS (Full strength) + Sucrose (30 g/L) + GA_3 (3.0 mg L^{-1}) + Agar powder (7 g L^{-1})	3.56	36.43 (37.10)	4.91	5.26
SEm (±)	**0.30**	**2.34**	**0.33**	**0.38**
LSD (0.05)	**NA**	**7.01**	**0.97**	**1.14**

Note: Figures in parentheses are angular transformed values

Table 3: Effect of different culture media on number of shoot proliferation per micro shoots

Treatments	No. of shoots per micro shoot after 15 days	No. of shoots per micro-shoot after 30 days	No. of shoots per micro-shoot after 45 days	Visual observations
T_0: MS (Full strength of salts) medium supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} agar powder	2.00	9.89	20.56	-
T_1: MS (1/2 strength of salts) medium supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} agar powder	2.33	10.11	23.78	-
T_2: MS (1/2 strength of salts) medium supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} agar powder + GA_3 (0.5 mg L^{-1})	3.11	17.56	44.22	The shoots were normal with dark green colour
T_3: MS (1/2 strength of salts) medium supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} agar powder + GA_3 (1.0 mg L^{-1})	4.33	10.22	21.56	-
T_4: MS (1/2 strength of salts) medium supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} agar powder + BAP (0.5 mg L^{-1})	3.22	15.78	43. 67	The shoots were normal with dark green colour
T_5: MS (1/2 strength of salts) medium supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} agar powder + BAP (1.0 mg L^{-1})	4.11	10	33.55	-
T_6: MS (1/2 strength of salts) medium supplemented with 30 g/L sucrose and 7 g L^{-1} agar powder + BAP (1.5 mg L^{-1})	3.22	10.89	28.11	Shoots were deformed
T_7: MS (1/2 strength of salts) medium supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} agar powder + BAP (2.0 mg L^{-1})	3.22	11.78	29.44	Shoots were deformed
SEm (±)	**0.41**	**1.26**	**2.15**	
LSD (0.05)	**1.25**	**3.81**	**6.50**	

Shoot proliferation

In the present study, ½ MS was found to be the better medium than full MS with regards to number of microshoots at all the stages of crop growth observed (Table 3). At 45 days after sub culturing, maximum number of shoots per micro shoot (44.22) was obtained in treatment T_2 (Fig. 2). The comparison of the cultures produced by the MS medium supplemented with gibberellic acid indicated that the quality of shoots was better than BAP at higher concentration (>0.5 mg L^{-1}) and low concentration of BAP in respect of colour of micro shoots, leaf development and shoot length (visual observation). The higher concentrations of cytokinins (T_6 and T_7) at the later stages of cultures shows vitrification due to hyper action of cytokinins. In *Chrysanthemum morifolium*, Sangwan *et al.* (1987) reported better multiple shoot formation when explants were cultured on MS media supplemented with GA_3. Highest shoot proliferation was also reported when the shoot tips of marigold were transferred to MS + 2.0 mg/L BAP + 0.1 mg L^{-1} Napthalene Acetic Acid (NAA)+ 0.5 mg L^{-1} GA_3 by Majumder *et al.* (2014). Pratibha *et al.* (2000) reported better proliferation of shoots in F_1 sterile hybrids of *Tagetes erecta* without intervening callus and vitrification of shoots in ½ MS medium having 2 mg/L BAP and 30 g/L sucrose for one week and subsequently sub-culturing in a lower concentration of BAP (0.5 mg/L).The shoot proliferation is largely due to the action of BAP. An optimum dose of BAP enhances axillary branching resulting in multiple shoot formation.

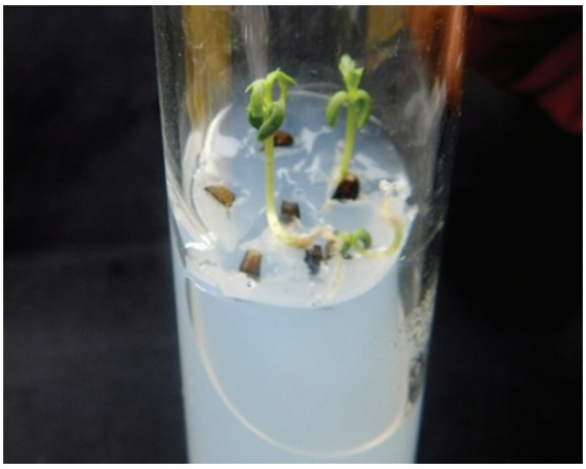

Fig.1: Seed germination [MS (1/2 strength) + Sucrose (30 g L^{-1}) + GA_3 (2.0 mg L^{-1}) + Agar powder (7g L^{-1})]

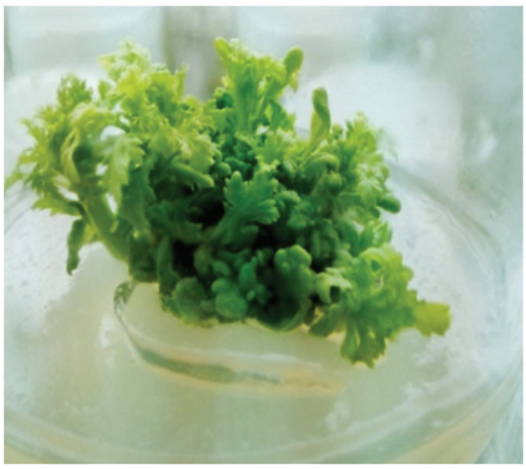

Fig. 2: Shoot proliferation [MS (1/2 strength of salts) +30 g L^{-1} sucrose and 7 g L^{-1} agar powder + GA_3 (0.5 mg L^{-1})]

The increased dose of auxins and cytokinins induces more callus formation and retards the axillary branching. During shoot proliferation process generally higher concentration are avoided as it leads to more callus formation. After shoot proliferation the plantlets of coronarium cultured on MS + 60 g L^{-1} sucrose + 7 g L^{-1} agar +0.5 mg L^{-1} NAA resulted highest per cent rooting (90%). Kumar *et al.* (2004) obtained similar results with maximum rooting in gerbera on half strength MS medium with 0.5 mg L^{-1} NAA + 0.5 mg L^{-1}.

The pre-treatment of *C. coronarium* seeds with carbendazim (0.2%), ridomil® (0.2%) and 8-HQC (200 ppm) for 2 hours followed by ethanol (70%) for 30 sec and $HgCl_2$ (0.1%) for 3 minutes for Surface sterilization was found to be the best treatment with respect to minimal bacterial and fungal contamination. The seeds cultured on MS medium (1/2 strength) + Sucrose (30 g L^{-1}) + GA_3 (2.0 mg L^{-1}) + Agar powder (7 g L^{-1}) resulted in highest seedling formation (51.34 %) and maximum shoot length (4.72 cm). Maximum number of shoots per micro-shoot were obtained when the shoots were sub cultured on MS (1/2 strength of salts) supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} agar powder + GA_3 (0.5 mg L^{-1}). maximum rooting was obtained when plantlets were transferred to MS medium + 60 g L^{-1} sucrose + 7 g L^{-1} agar +0.5 mg L^{-1} NAA. The above standardized protocol would help to isolate true to type plants in annual chrysanthemum. These homozygous lines can be utilized in breeding of pure genetic material.

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