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SIGNIFICANCE OF DIFFERENT PLANT GROWTH REGULATORS ON THE REGENERATION OF CHRYSANTHEMUM PLANTLETS (*DENDRANTHEMA MORIFOLIUM* L.) THROUGH SHOOT TIP CULTURE

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Abstract

Shoot multiplication of Chrysanthemum was achieved from shoot tip explant, using MS (Murashige & Skoog) media supplemented with different concentrations and combinations of plant growth regulators. Different parameters including shoot initiation percentage, average number of shoots per explant, length of shoots (cm), number of leaves per shoot and number of nodes per shoot were studied. Lowest concentration (0.5 mg/L) of naphthalene acetic acid (NAA) excelled all the other concentrations in almost all the parameter studied when used alone. Maximum shoot initiation (80.00%), shoot per explants (3.2), length of shoots (3.4 cm), number of leaves (9.5) and nodes (4.5) were recorded in MS media showed supplemented with 0.5 mg/L NAA. Intermediate concentration (1.0 mg/L) of benzyle adenine purine (BAP) its superiority over all the other BAP concentrations used, when used alone. MS medium fortified with 1.0 mg/L BAP had produced maximum shoot initiation (93.3%), shoot per explant (4.1), length of shoots (5.0 cm) number of leaves (11.0) and nodes (5.5).

Similarly, when the combination of different concentrations of NAA and BAP were used, significant results regarding intermediate concentration of regeneration of chrysanthemum plantlets were achieved. MS media supplemented with lower concentrations of NAA (0.5 and 1.0 mg/L) and BAP (1.0 and 1.5 mg/L) showed better results as compared to other concentrations and combinations. Satisfactory rooting response including days to root emergence (5.0), root initiation percentage (100%), roots per plantlet (14.3) and root length (9.0 cm) was obtained in half strength MS media supplemented with 0.2 mg/L Indole butyric acid (IBA).

Introduction

Chrysanthemum (*Dendranthema morifolium* L.) commonly called as Gul-e-Daudi or autumn queen, belongs to the family Compositae (Arora, 1990). It is a vegetatively propagated perennial ornamental plant (Verma *et al.,* 2009) either through root suckers or terminal cuttings. The transfer of disease infection and limited number of suckers are the main problem of chrysanthemum propagation through root suckers whereas the conventional process of shoot cutting is very slow. Murashige (1990) stated clonal plant propagation as the most extensive and visible application of tissue culture. Rapid clonal plant propagation *In vitro* can be obtained through bud or shoot proliferation (Pierik, 1990; Ali *et al.*, 2009). Chebet *et al.,* (2003) reported the use of biotechnological approaches to improve horticultural crop production. Plant growth regulators play a vital role in the regeneration of new plantlets through different *In vitro* culture techniques, as they influence different plant processes comprising mostly of growth, differentiation and development e.g., culture establishment, shoot initiation, callogenesis, embryogenesis, rooting etc., (Hobbie, 1998).

Due to high popularity and demand for chrysanthemum it becomes one of the first commercial targets for micropropagation and thus tissue culture can be utilized for the large-scale production of Chrysanthemum (Levin *et al.,* 1988). A decade ago, the protocols for rapid true to type, disease-free propagation has been developed in chrysanthemum through bud/shoot proliferation (Grewal *et al.,* 1996; Zulfiqar *et al.*, 2009). Many workers in the past have reported micro-propagation of *Chrysanthemum morifolium* Ram. (now *Dendranthema grandiflora* Tzvelv) from shoot tips and axillary buds. Hoque & Fatema (1995) regenerated maximum number of multiple shoot from shoot tip explants when 1.0 mg/L BAP + 1.0 mg/L NAA were added to MS medium. Tripepi (1997) used various combinations of BAP and NAA for the induction of adventitious shoots as they are among the growth regulators used most often for the shoot organogenesis. Hoque *et al.,* (1998) obtained best response towards multiple shoot regeneration on MS medium containing 1.0 mg/L BAP and 0.5 mg/L NAA. Chakarbarty *et al,.*(2000) regenerated ray florets of *Chrysanthemum morifolium* cv. Colchi Bahar shoots on MS medium supplemented with 0.2 mg/L NAA and 1.0 mg/L BAP. Radojevic *et al.,* (2000) also reported that MS medium supplemented with 0.5 mg/L NAA + 1.0 mg/L BAP was the most suitable medium for 13 cultivars of chrysanthemum. Gul (2001) also reported that in chrysanthemum, maximum shoot regeneration was observed at 0.5mg/L BAP. Karim *et al.,* (2002) reported that the frequency of multiple shoot regeneration response was 91%, for shoot tips, when cultured on the medium containing MS + 1.0 mg/L BAP. Karim *et al.,* (2003) reported that maximum frequency of explants produced axillary shoot and the highest number of shoots per explant were obtained when MS medium was fortified with 1.0 mg/L BAP. Kumari & Varghese (2003) reported that regeneration of shoots took place on MS media fortified with various combinations of BAP and NAA. Shanti *et al.,* (2005) reported that shoot tip explants of chrysanthemum inoculated in MS media supplemented with 2.0 mg/L BAP and 0.5 mg/L NAA gave better performance for plantlet regeneration. Trifunovic *et al.,* (2006) achieved induction of morphogenesis in stem segment culture on MS medium supplemented with NAA and BAP (0.5 and 1.0 mg/L, respectively). Misra & Datta (2007) also reported that shoot bud differentiation could be achieved in ray florets in the presence of 0.5 mg/L NAA + 2.0 mg/L BAP. Nahid *et al*., (2007) found 58% shoot initiation in chrysanthemum petals inoculated on MS media supplemented with 1.0 mg/L BAP and 0.1 mg/L NAA. Waseem *et al.,* (2007) reported that the lowest concentration of 0.5 mg/L NAA showed its superiority over the other concentrations, as it produced as high as 80.0% shoot initiation, maximum (3.2) shoots per explant, shoot length (4.0 cm), 9.5 and 4.5 leaves: nodes per shoots.

The efficient rooting of micro-shoots raised is usually obtained by using different plant growth regulators. Synthetic growth regulating chemicals that have been found most reliable in stimulating adventitious root production are the auxins i.e., indole acetic acid (IAA), naphthalene acetic acid (NAA) and indole butyric acid (IBA) (Arteca, 1996). Various concentrations of IBA used for root induction showed maximum response (100%) on MS medium containing 0.2 mg/L IBA (Hoque & Fatema, 1995; Hoque *et al.,* 1998; Sarker & Shaheen, 2001). Faisal & Amin (2000) reported that in chrysanthemum, *in vitro* regenerated shoots roots developed on all media combinations; the maximum number being found in half strength of MS supplemented with 0.2 mg/L IBA and 0.2 mg/L IAA, respectively. Chrysanthemum shoots raised from tissue culture, developed roots within 4-5 days ½ MS + 0.25 mg/L IBA (Khan *et al.,* 1994; Karim *et al.,* 2002). *In vitro* rooting in chrysanthemum was successfully achieved on MS media supplemented with different concentrations of IAA, NAA and IBA (Shatnawi *et al.,* 2009).

Micro-propagation using axillary shoot proliferation from shoot tip culture is the most desirable and safe technique, as it not only minimizes the genetic variation but also helps in the formation of healthy shoots and its high rate of multiplication. Therefore, the attempts were made to determine the effect of different growth regulators on the shoot proliferation, multiplication and rooting of shoot tip explant of chrysanthemum.

Materials and Methods

The experiments regarding the effect of different concentrations of growth regulators and their combinations (NAA, BAP and BAP + NAA) on the regeneration and rooting of chrysanthemum plantlets using shoot tips as explant were conducted at the "Plant Tissue Culture Laboratory; Institute of Horticultural Sciences, University of Agriculture, Faisalabad, during 2007 in completely randomized design (CRD) with three replications. To check the effect of NAA, BAP and their different combinations, standard MS medium (Murashige & Skoog, 1962) with varied concentrations of NAA (Control, 0.5, 1.0 and 1.5 mg/L NAA), BAP (Control, 0.5, 1.0 and 2.0 mg/L BAP) and their various combinations were used. For rooting half strength MS medium supplemented with various concentrations (0.1, 0.2 and 0.5 mg/l) of IBA and NAA were used. The following procedure was adopted.

a. Preparation of explants: The explant material was collected from six months old Chrysanthemum plants, grown at the floriculture garden of the "Institute of Horticultural Sciences". The collected material was brought to the laboratory, and washed thoroughly with running tap water for 30 minutes. Apical shoot tips of about 0.5 cm were then excised with the help of scalpel and forceps.

b. Sterilization of plant material: The excised explants were dipped in 70 % ethanol for 60 seconds. After pretreatment with ethanol, the explants were rinsed with double distilled water twice, so as to lower the toxic affect of ethanol. Apical shoot tips were then surface sterilized with 1.0 % Mercuric chloride ($HgCl_2$) for 3 minutes (Illahi *et al.,* 2007). After the surface sterilization of explants, Mercuric chloride was removed and the explants were rinsed with double distilled water thrice, so as to lower the toxic affects of $HgCl_2$.

c. Culture of Explants for the regeneration purpose: Explants were cultured on solidified MS medium with agar (8 g/L) and its pH was adjusted to 5.7 before autoclaving at 121°C for 30 minutes. On cooling of the media, apical shoot tips were cultured in Murashige & Skoog (1962) media containing different concentrations of growth regulators. One explant in each tube (15 x 2.5 cm) containing 10 ml medium was placed. The tubes were covered with autoclaved poly praline sheets after culturing, which were held in place with rubber band. The cultured tubes were incubated for 16 hours daily light of fluorescent Philip white tubes with intensified 1000 LUX at 25 ± 1°C temperature.

Data was recorded for different parameters including shoot initiation percentage, average number of shoots per explant, average shoot length (cm), average number of leaves per shoot and average number of nodes per shoot.

For rooting, micro-shoots raised were harvested after 6 weeks and each shoot was transferred to a test tube containing 10 ml of half strength MS (Murashige & Skoog, 1962) medium supplemented with different levels of IBA and NAA. Data was recorded for different parameters including days to root initiation, root initiation percentage, average number of roots per explant, average root length (cm). Recorded data were analyzed statistically using analysis of variance technique (ANOVA) and means were compared by Duncan's multiple range test (Steel *et al.,* 1997).

Results and Discussion

Effect of NAA on the regeneration of chrysanthemum plantlets: The data shown in the Table 1 clearly depicted the un-challengeable supremacy of T2 (0.5 mg/L NAA) over all the other treatments as it excelled in all the parameters. Maximum shoot initiation (80%) and number of shoots per explants (3.2) were recorded in T2 (0.5 mg/L NAA) closely followed by T3 (1.0 mg/L NAA) with 70% shoot initiation and 2.9 shoots per explant. Both the treatments showed a non-significant behavior for each other and were significantly at par. Minimum shoot initiation (30%) and minimum number of shoots (1.5) was recorded in T1 (Control). These results are in line with the previous findings of Khalid *et al.,* (1989) who reported that number of shoots were greater with the minimum level of NAA. However, the minimum (1.5) shoots per explant were recorded in T1 (Control).

The longest shoots (4.0 cm), maximum number of leaves (9.5) and nodes (4.5) per shoot were also recorded in T2 (0.5 mg/L NAA) followed by T3 (1.0 mg/L NAA) with 3.4 cm long shoots, 8.1 leaves and 3.9 nodes per shoot, respectively (Fig. 1). However, the least response for all these parameters was recorded in T1 (Control).

Table 1. Effect of different concentration of NAA on the regeneration of chrysanthemum from shoot tip explants.

Treatments	Shoot initiation %	Shoots explant^{-1}	Length of shoots (cm)	Leaves shoot^{-1}	Nodes shoot^{-1}
T1 = (Control)	30.0B	1.5C	2.1 D	3.2 D	2.7 D
T2 = (0.5 mg/L)	80.0A	3.2A	4.0 A	9.5 A	4.5 A
T3 = (1.0 mg/L)	70.0A	2.9A	3.4 B	8.1 B	3.9 B
T4 = (1.5 mg/L)	60.0A	2.5B	2.8 C	6.2 C	3.3 C
LSD (p<0.05)	**24.9**	**0.3**	**0.4**	**0.4**	**0.5**

Means within a column followed by different letters are significantly different according to Duncan's Multiple Range Test (p<0.05)

The results presented in the Table 1 revealed that the lowest concentration of NAA (0.5 mg/L), responded well for the regeneration of chrysanthemum. Similar results were quoted by Waseem *et al.,* (2007) who reported best results for the regeneration of chrysanthemum plantlets at 0.5 mg/L NAA, when used alone as compared to the other NAA concentrations. As NAA concentration increased, a decrease in regeneration capability was observed. This might be due to the reason, that NAA usually don't respond well against shoot proliferation (Vijaya *et al.,* 1991) and is known for its root formation ability (Liu *et al.,* 1994). Concentration increased, a decrease in regeneration capability was observed. This might be due to the reason, that NAA usually don't respond well against shoot proliferation (Vijaya *et al*., 1995) and is known for its root formation ability, (Liu *et al*., 1994).

Effect of BAP on the regeneration of chrysanthemum plantlets: BAP is the most effective plant growth regulator in relation to shoot proliferation, as it belongs to the cytokinin group (Vijaya *et al.,* 1991). Different concentrations of BAP were tested for Chrysanthemum shoot tip explant for the regeneration of plantlets. The highly significant data as shown in Table 2, revealed the superiority of T3 (1.0 mg/L BAP) over all the other treatments in all the parameters. Maximum shoot initiation (93.33%), number of shoots per explant (4.1), shoot length (5.0 cm), leaves per shoot (11.0) and nodes per shoot (5.5) were observed in T3 (1.0 mg/L BAP), followed by T2 (0.5 mg/L BAP) and T4 (2.0 mg/L BAP) producing 76.7 and 66.7% shoot initiation, 3.1 and 2.8 shoots per explant, 4.1 and 3.7 cm long shoots, 9.9 and 8.2 leaves per shoot and 4.6 and 4.2 nodes per shoot, respectively (Fig. 2). The least effect was exhibited by T1 (Control) in all the parameters studied.

These results are also similar to those as obtained by Karim *et al.,* (2002) who described 1.0 mg/L BAP as the best BAP concentration as it had produced maximum shoot initiation in chrysanthemum while using shoot tips as explant. Studies on BAP indicate that this chemical accelerates the development of shoot buds. Similar results were also reported by Ali *et al.,* (2008) who also reported that when BAP was used alone at 1.0 mg/L concentration, highest number of shoots was obtained in all the culture for carnation. By increase in BAP concentration the rate of shoot multiplication was decreased.

It could be inferred from above results that 1.0 mg/L BAP is the optimum concentration for the better performance of this particular hormone. Any plus or minus deviation from this normal concentration of the growth regulator showed poor results. The fact that higher dozes failed to manifest their effect could be attributed to an obnoxious effect at higher concentration, whereas, the ineffectiveness of the lower dose indicated inadequate doze of hormone as a consequence indicating poor performance. Many previous research workers have also confirmed that BAP accelerates the development of the bud initials causing the increased number of buds primordial in chrysanthemum (Karim *et al.,* 2003; Chagas *et al.,* 2004 and Aftab *et al.,* 2008).

Effect of different concentrations of BAP and NAA on the regeneration of chrysanthemum plantlets: Cytokinin with auxin also plays an important role for shoot proliferation. BAP + NAA combination was used to find out proper balance between cytokinin and auxin for shoot proliferation from shoot tip explants of chrysanthemum.

In this experiment, chrysanthemum shoot tips were cultured on MS media supplemented with different concentrations of BAP (1.0, 1.5, 2.0 and 2.5 mg/L) in combination with NAA (0.5, 1.0, 1.5 and 2.0 m/l). Among the different treatments shoot regeneration frequency varied significantly (Table 3). The regeneration frequency could be improved by manipulating the compositions of the hormones in the culture media. The reason is that juvenility played an important role in regeneration is not clear and the number of regenerated shoot buds depends on the composition of culture medium, especially on the levels of PGRS (Rout & Das, 1997; Sajid *et al.*, 2009).

On shoot proliferation medium containing 1.0 mg/L BAP along with lower concentrations of NAA showed better results for all the growth parameters. The data showed that maximum results were reported in T1 (1.0 mg/L BAP+0.5 mg/L NAA), T2 (1.0 mg/L BAP+1.0 mg/L NAA) and T3 (1.0 mg/L BAP+1.5 mg/L NAA) with highest degree of shoot proliferation/initiation (100, 100 and 96.7%), shoots per explant (11.8, 11.1 and 10.2), shoot length (6.0, 6.0 and 5.9 cm) leaves per shoot (19.9, 19.6 and 20.3) and nodes per shoot (6.5, 6.4 and 6.3), respectively. All these three treatments behaved statistically similar for shoot initiation percentage, shoot length and nodes per shoot. However, the least response for all the parameters was recorded in T16 (2.5 mg/L BAP+2.0 mg/L NAA) and T15 (2.5 mg/L BAP+1.5 mg/L NAA) with 50.0 and 53.3% shoot initiation, 4.8 and 4.9 cm long shoots and 4.9 and 5.0 nodes per shoots, respectively. While, all the other treatments showed intermediate response for all the parameters. These results are in accordance with the findings of Hoque *et al.,* (1998) who reported that the best response of 8-10 shoot buds were obtained from the MS medium fortified with 1.0

mg/L BAP + 0.5 mg/L NAA, using shoot tip explant of chrysanthemum. Similarly, Hoque & Fatima (1995) also regenerated the maximum number of multiple shoots from shoot tip explants, when they used MS media fortified with 1.0 mg/L BAP + 1.0 mg/L NAA. Trifunovic *et al.,* (2006) also achieved induction of morphogenesis in stem segment culture on MS medium supplemented with 1.0 mg/L BAP + 0.5 mg/L NAA.

Table 2. Effect of different concentration of BAP on the regeneration of chrysanthemum from shoot tip explants.

Treatments BAP	Shoot initiation %	Shoots explant^{-1}	Length of shoots (cm)	Leaves shoot^{-1}	Nodes shoot^{-1}
T1 = (Control)	30.0 C	1.5 C	2.0 D	3.1 D	2.6 C
T2 = (0.5 mg/L)	76.7 B	3.1 B	4.1 B	9.9 B	4.6 B
T3 = (1.0 mg/L)	93.3 A	4.1 A	5.0 A	11.0 A	5.5 A
T4 = (2.0 mg/L)	66.7 B	2.8 B	3.7 C	8.2 C	4.2 B
LSD (p<0.05)	**13.3**	**0.4**	**0.3**	**0.4**	**0.5**

Means within a column followed by different letters are significantly different according to Duncan's Multiple Range Test (p<0.05)

Table 3. Effect of different concentration of BAP + NAA on the regeneration of chrysanthemum from shoot tip explants.

Treatments (BAP + NAA mg/l)	Shoot initiation	shoots explant^{-1}	Av. Length of shoots	Av. leaves shoot^{-1}	Av. nodes shoot^{-1}
T1 = (1.0+0.5)	100.0 A	11.8 A	6.0 A	19.9 B	6.5 A
T2 = (1.0 +1.0)	100.0 A	11.1 B	6.0 A	19.6 BC	6.4 AB
T3 = (1.0+1.5)	96.7 AB	10.2 C	5.9 AB	20.3 A	6.3 ABC
T4 = (1.0 +2.0)	93.3 BC	8.6 F	5.6 DE	18.0 F	6.0 CD
T5 = (1.5 +0.5)	90.0 CD	9.4 D	5.8 ABC	19.0 D	6.3 ABC
T6 = (1.5 +1.0)	90.0 CD	9.1 DE	5.7 CD	19.3 C	6.2 BC
T7 = (1.5 +1.5)	86.7 D	7.9 G	5.4 EF	17.6 G	5.7 DE
T8 = (1.5 +2.0)	80.0 E	7.3 H	5.2 FG	17.1 H	5.5 EF
T9 = (2.0 +0.5)	90.0 CD	8.7 EF	5.7 CD	18.5 E	6.0 CD
T10 = (2.0+1.0)	90.0 CD	8.2 FG	5.4 E	18.8 DE	5.8 D
T11 = (2.0+1.5)	80.0 E	6.9 HI	5.2 FG	16.0 J	5.4 EF
T12 = (2.0+2.0)	73.3 F	5.9 KL	5.0 GHI	15.5 K	5.2 FGH
T13 = (2.5+0.5)	66.7 G	6.6 IJ	5.1 GH	16.7 I	5.3 FG
T14 = (2.5+1.0)	56.7 H	6.2 JK	5.0 GHI	15.1 L	5.3 FG
T15 = (2.5+1.5)	53.3 HI	5.6 L	4.9 HI	14.4 M	5.1 GH
T16 = (2.5+2.0)	50.0 I	5.1 M	4.8 I	14.2 M	4.9 H
LSD (p<0.05)	**6.351**	**0.4934**	**0.2231**	**0.3489**	**0.3242**

Means within a column followed by different letters are significantly different according to Duncan's Multiple Range Test (p<0.05)

Table 4. Effect of different concentrations of IBA and NAA on the rooting of micro shoots raised from shoot tip explants of chrysanthemum.

Treatments	Days to roots emergence	Root initiation %	Av. Roots plantlet^{-1}	Length of roots (cm)
T1 (½ MS (Control))	8.0 C	66.7 DE	7.7 H	5.8 G
T2 (½ MS + 0.1mg/L IBA)	6.5 F	83.3 ABCD	10.7 E	7.5 D
T3 (½ MS + 0.2mg/L IBA)	5.0 I	100.0 A	14.3 A	9.0 A
T4 (½ MS + 0.5mg/L IBA)	6.2 F	100.0 A	11.6 D	7.7 D
T5 (½ MS + 0.1mg/L NAA)	7.0E	83.3 ABCD	9.3 F	7.1 E
T6 (½ MS + 0.2mg/L NAA)	5.4 H	93.3 AB	13.4 B	8.5 B
T7 (½ MS + 0.5mg/L NAA)	7.5 D	90.0 ABC	8.5 G	6.2 F
LSD (p<0.05)	**0.4**	**17.0**	**0.4**	**0.3**

Means within a column followed by different letters are significantly different according to Duncan's Multiple Range Test (p<0.05)

Over all result tabulated in the Table 3, regarding the effect of different combinations of BAP and NAA on the shoot tip of chrysanthemum, revealed that a lower doze of BAP i.e., 1.0 mg/L excelled all the other concentrations (1.5, 2.0 and 2.5 mg/L) of BAP used. This result clearly demonstrate the fact that 1.0 mg/L BAP is the optimum concentration of BAP, that can provide better response towards shoot proliferation and multiplication. As the BAP concentration increased, a linearly decrease in its performance was observed and thus any addition to this concentration (1.0 mg/L) had shown poorer results for regeneration. On the other hand, the lower dozes of NAA used @ 0.5, 1.0 mg/L exhibited better results as compared with its higher concentration i.e. 1.5 and 2.0 mg/L NAA.

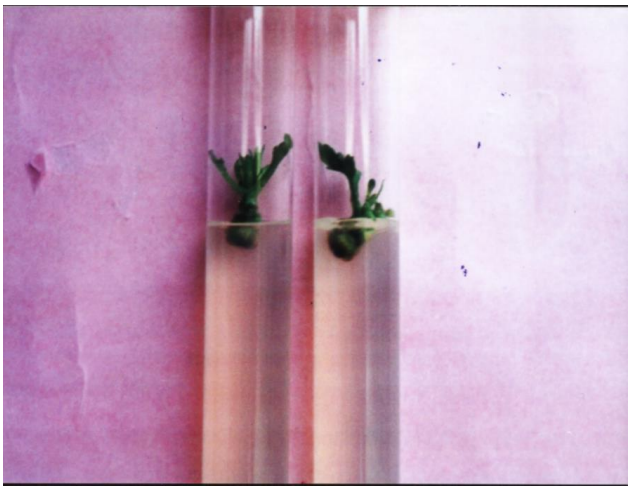

Fig. 1. Initial Shoot Proliferation in MS media supplemented with 0.5 mg/l NAA.

Fig. 2. Shoot multiplication in MS media supplemented with 1.0 mg/l BAP.

Fig. 3. Shoot multiplication in MS media supplemented with 1.0. mg/l BAP + 0.5 mg/l NAA.

Fig. 4. Rooting of micro-shoots in ½ strength MS media supplemented with 0.5 mg/l IBA.

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