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Full Length Research Paper

Rapid plant regeneration of chrysanthemum (*Chrysanthemum morifolium* l.) through shoot tip culture

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Shoot multiplication of chrysanthemum was achieved from shoot tip explant, using MS 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 during the course of study. Low concentration of indole-acetic acid (IAA, 0.1 mg/l) excelled all the other concentrations in almost all the parameters studied when used alone, as maximum shoot initiation (86.6%), shoot per explants (3.9), length of shoots (4.3 cm), number of leaves (10.0) and nodes (4.8) were recorded in it. Intermediate concentration of benzyladenine purine (BAP, 1.0 mg/l) is superior to all the other BAP concentrations used when used alone. MS media fortified with 1.0 mg/l BAP had produced the 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 IAA and BAP were used, significant results regarding the regeneration of chrysanthemum plantlets were achieved. MS media supplemented with intermediate levels of BAP (1.0 and 2.0 mg/l) along with lower concentrations of IAA (0.1 and 0.2 mg/l) showed better results as compared to other concentrations and combinations. Satisfactory rooting response was obtained in half strength MS media supplemented with 0.2 mg/l indole butyric acid (IBA).

Key words: Chrysanthemum, *Chrysanthemum morifolium*, growth regulators, *in vitro* culture, shoot tip culture, indole acetic acid, benzyladenine purine, regeneration, rooting.

INTRODUCTION

Chrysanthemum commonly known as Gul-e-Daudi or Autumn Queen belongs to the family Compositeae (Asteraceae) (Arora, 1990). It is a highly attractive and charming short day plant, which behaves both as an annual as well as a perennial flowering herb. The plant height ranges from $^1/_3$ to 1 m and flowers bloom in early winter with a wide range of color, shape and sizes. They are appreciated for their high keeping quality. Also their ability to produce desired grades and types at anytime during the year adds to their popularity.

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Regeneration through *in vitro* culture has now become a viable alternative to conventional propagation methods. The formation of healthy shoots and higher rates of multiplication is one of the prerequisite of an economically viable micro-propagation protocol. It is possible now to obtain large number of plants from one explant through *in vitro* (Bajaj, 1992). Due to high popularity and demand for chrysanthemum, it has become one of the first commercial targets for micropropagation and thus tissue culture can be utilized for its large-scale production (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). Tissue culture studies on chrysanthemum were first initiated by Morel and Martin in 1952;

they used meristem tip culture to obtain virus free plants. Murashige (1990) stated that clonal plant propagation is the most extensive and visible application of culture. Rapid clonal plant propagation *in vitro* can be obtained through bud or shoot proliferation (Pierik, 1990). Hoque and Fatema (1995) reported that shoot tips showed comparatively better response to multiple shoot regeneration than the nodal segments. Gao et al. (2001) confirmed that stem explants were superior to leaf explants.

In tissue culture, the use of plant growth regulators play a pivotal role in influencing different plant processes comprising mostly of growth, differentiation and development such as culture establishment, shoot initiation, callogenesis, embryogenesis and rooting (Hobbie, 1998). Synthetic growth regulating chemicals that have been found most reliable in stimulating adventitious root production are the auxins including, indole-acetic acid (IAA), naphthalene acetic acid (NAA) and indole butyric acid (IBA) (Arteca, 1996).

Roest and Bokelmann (1973) confirmed that a combination of BAP and IAA was most favourable for shoot bud regeneration. Liu et al. (1994) achieved the highest production frequency of adventitious buds from shoot tip explants of chrysanthemum. Lazar and Cachita (1983) reported that good bud and shoot differentiations were obtained with IAA at 0.1 mg/l + BA at 10 mg/l. Kim and Kim (1998) regenerated shoots on basal media supplemented with 3.0 mg/l BAP and 0.2 mg/l IAA in chrysanthemum. Wankhede et al. (2000) observed that the greatest shoot formation was observed in shoot tip explants grown in an MS medium supplemented with 1.0 mg/l BAP + 2.0 mg/l IAA. Waseem et al. (2007) also reported that the lowest concentration of IAA (0.1 mg/l) showed the best response in all the parameters studied as compared to the other concentrations of IAA. Khan et al. (1994) observed shoot proliferations on MS medium supplemented with 0.5 and 1.0 mg/l BAP. At 2.0 mg/l BAP, shoot regeneration was higher but they were compact and stunted in growth. Gul (2001) also reported that in chrysanthemum, maximum shoot regeneration was observed from stem nodal segments at 0.5 mg/l BAP. Increased levels of BAP in the medium increased the number of shoots but suppressed their growth (Singh and Arora, 1995). Karim et al. (2002) reported that the frequency of multiple shoot regeneration response was 95 and 91%, for nodal segments and shoot tips, respectively, when cultured on the medium containing MS + 1.0 mg/l BAP. Various concentrations of IBA used for root induction showed maximum response (100%) on MS medium containing 0.2 mg/l IBA (Hoque and Fatema, 1995; Hoque et al., 1998; Sarker and Shaheen, 2001). Faisal and Amin (2000) reported that in chrysanthemum, *in vitro* regenerated shoots and roots developed on all media combinations; the maximum number being found in both super yellow and light violet in half strength of MS supplemented with 0.2 mg/l IBA and 0.2 mg/l IAA, respectively. Good rooting of "deep pink' was achieved on half strength basal MS salts supplemented with 0.25 mg/l of IBA or IAA (Rout et al., 1997). 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). Micro-propagation using axillary shoot proliferation from shoot tip culture is the most desirable and safe technique, as it not only minimizes genetic variation but also helps in the formation of healthy shoots and its high rate of multiplication. Therefore, attempts were made to determine the effect of different growth regulators on shoot proliferation and multiplication of shoot tip explant of chrysanthemum.

MATERIALS AND METHODS

The experiments regarding the effect of different concentrations of growth regulators and their combinations on the regeneration and rooting of chrysanthemum plantlets using shoot tips as explants were conducted at the Plant Tissue Culture Laboratory, Institute of Horticultural Sciences, University of Agriculture, Faisalabad, during 2004 in completely randomized design (CRD) with three replications. For the regeneration of chrysanthemum plantlets, MS media was supplemented with varied concentrations of IAA (control, 0.1, 0.3 and 0.5 mg/l), BAP (control, 0.5, 1.0 and 2.0 mg/l) and their combinations. For rooting of chrysanthemum micro-shoots, half strength MS media supplemented with various concentrations of IBA (0.1, 0.2 and 0.5 mg/l) and NAA (0.1, 0.2 and 0.5 mg/l) were used. The following procedure was adopted.

Preparation of explants

The explant material was collected from 6 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 min. Apical shoot tips of about 0.5 cm were then excised with the help of scalpel and forceps.

Sterilization of plant material

The excised explants were dipped in 70% ethanol for 60 s. After pretreatment with ethanol, the explants were rinsed with double distilled water twice, so as to lower the toxic effect of ethanol. Apical shoot tips were then surface sterilized with 1.0% mercuric chloride ($HgCl_2$) for 3 min (Ilahi 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 effects of $HgCl_2$.

Culture of Explants for the regeneration purpose

Explants were cultured on solidified MS media with agar (8 gm/l) and its pH was adjusted to 5.7 before autoclaving at 121°C for 30 min. On cooling of the media, apical shoot tips were cultured in Murashige and Skoog (1962) media containing different concentrations of auxins. One explant in each tube (15 × 2.5 cm) containing 10 ml media was placed. The tubes were covered with autoclaved poly praline sheets after culturing, which were held in place with

Table 1. Effect of different concentrations of IAA on the regeneration of chrysanthemum using shoot tips as explants

Treatments IAA (mg/l)	Shoot initiation %	Av. shoots explant^{-1}	Length of shoots (cm)	Av. leaves shoot^{-1}	Av. nodes shoot^{-1}
T1 = (Control)	30.0 D	1.5 D	2.1 D	3.1 D	2.7 C
T2 = 0.1	86.6 A	3.9 A	4.3 A	10.0 A	4.8 A
T3 = 0.3	73.3 B	3.3 B	3.7 B	8.0 B	4.5 A
T4 = 0.5	60.0 C	2.9 C	3.0 C	7.4 C	3.5 B
LSD (P<0.05)	12.15	0.2455	0.3151	0.25256	0.4375

Means within a column followed by different letters are significantly different according to Duncan's Multiple Range Test (P<.05)

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

Treatments BAP (mg/l)	Shoot initiation %	Av. shoots explant^{-1}	Length of shoots (cm)	Av. leaves shoot^{-1}	Av. nodes shoot^{-1}
T1= Control	30.0 C	1.5 C	2.0 D	3.1 D	2.6 C
T2 = 0.5	76.7 B	3.1 B	4.1 B	9.9 B	4.6 B
T3 = 1.0	93.3 A	4.1 A	5.0 A	11.0 A	5.5 A
T4 = 2.0	66.7 B	2.8 B	3.7 C	8.2 C	4.2 B
LSD (P<.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<.05)

rubber band. The cultured tubes were incubated for 16 h daily light of fluorescent, Philip white tubes with intensified 1000 LUX, at 25 ± 1℃ 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 and 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 and 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

Effect of IAA on the regeneration of chrysanthemum plantlets

Data for shoot initiation percentage from shoot tip explants were calculated on the basis of number of explant producing shoots out of total cultures. The highly significant differences regarding shoot initiation percentage were recovered for different concentrations of IAA showing that T2 (0.1 mg/l IAA) gave the maximum (86.6%) shooting, while T3 (0.3 mg/l IAA) also produced good (73.3%) shoot initiation (Table 1); whereas, the least response was observed in T1 (control) with 30% shoot initiation.

It is evident from the data that T2 (0.1 mg/l IAA) showed its superiority over all the other treatments by producing significantly higher number of shoots per explant (3.9), longer shoots (4.3 cm) leaves per shoot (10.0) and nodes per shoot (4.8) followed by T3 (0.3 mg/l IAA) with 3.3 shoot per explant, 3.7 cm long shoots, 8.0 leaves per shoot and 4.5 nodes per shoot. The least response was observed in T1 (control) for all the parameters

Effect of BAP on the regeneration of chrysanthemum 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 viz; maximum shoot initiation (93.33%), maximum shoots per explant (4.1), longest shoots (5.0 cm) leaves (11.0) and nodes (5.5). Observed in T2 (1.0 mg/l BAP) were, 76.7% shoot initiation, 3.1 shoots per explant, 4.1 cm long shoots, 9.9 leaves and 4.6 nodes per shoot. The least response was exhibited by T1 (control) for all the parameters.

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

Treatments BAP+IAA (mg/l)	Shoot initiation %	Av. shoots explant^{-1}	Av. Shoot Length(cm)	Av. leaves shoot^{-1}	Av. nodes shoot^{-1}
T1= 0.5+0.1	76.7 DEF	6.1 EF	3.9 D	16.0 D	4.4 DE
T2 = 0.5+0.2	66.7 GH	5.3 HI	3.3 FG	14.9 F	3.8 FG
T3 = 0.5+0.5	60.0 HI	5.0 IJ	3.1 G	13.2 H	3.6 GH
T4 = 0.5+1.0	53.3 IJ	4.8 J	2.7 H	12.1 J	3.3 H
T5 =1.0+0.1	93.3 A	7.2 A	5.0 A	17.3 A	5.4 A
T6 =1.0+0.2	90.0 AB	6.9 AB	4.5 BC	17.0 A	5.0 ABC
T7 =1.0+0.5	73.3 EFG	5.8 FG	3.8 DE	14.1 G	4.2 EF
T8 =1.0+1.0	50.0 J	4.4 K	2.4 HI	11.7 K	2.9 I
T9 =2.0+0.1	93.3 A	7.0 AB	4.8 AB	16.7 B	5.2 AB
T10=2.0+0.2	90.0 AB	6.9 AB	4.7 BC	16.3 C	5.1 AB
T11=2.0+0.5	80.0 CDE	6.3 DE	4.0 D	13.5 H	4.5 DE
T12=2.0+1.0	46.7 JK	4.2 K	2.3 I	11.3 L	2.8 I
T13=5.0+0.1	86.7 ABC	6.8 BC	4.4 C	15.8 D	4.9 BC
T14=5.0+0.2	83.3 BCD	6.5 CD	4.1 D	15.3 E	4.7 CD
T15=5.0+0.5	70.0 FG	5.6 GH	3.5 EF	12.7 I	3.9 FG
T16=5.0+1.0	40.0 K	4.0 K	2.1 I	10.7 M	2.6 I
LSD (P<.05)	7.202	0.3408	0.3567	0.3199	0.3936

Means within a column followed by different letters are significantly different according to Duncan's Multiple Range Test (P<.05)

Effect of different concentrations of BAP and IAA on the regeneration of chrysanthemum plantlets

Highly significant differences regarding the effect of different combinations of BAP and IAA on the regeneration of chrysanthemum plantlets are presented in Table 3. The result showed that out of 16 different hormonal combinations, T5, T9, T10 and T6 were highly responsive combinations as they excelled in all the parameters as compared to other combinations, as shown in Table 3.

Maximum shoot initiation (93.0, 93.0, 90.0 and 90.0%), shoots explant (7.2, 7.0, 6.9 and 6.9), shoot length (5.0, 4.8, 4.7 and 4.5 cm), leaves per shoot (17.3, 16.7, 16.3 and 17.0) and nodes per explant (5.4, 5.2, 5.1, 5.0) were recorded in T5 (1.0 mg/l BAP+ 0.1 mg/l IAA), T9 (2.0 mg/l BAP + 0.1 mg/l IAA), T10 (2.0 mg/l BAP + 0.2 mg/l IAA) and T6 (1.0 mg/l BAP + 0.2 mg/l IAA), respectively. The least response was observed in T16 (5.0 mg/l BAP + 1.0 mg/l IAA) and by T12 (2.0 mg/l BAP + 1.0 mg/l IAA) for almost all the parameters studied. All the other treatments showed an intermediate behavior for all the parameters.

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

One can visualize from the results regarding average number of roots per plantlet (Table 4) that T3 (½ MS + 0.2 mg/l IBA) is better than all the other treatments used, as it significantly took the least number of days (5.01), maximum rooting (100%), maximum roots per plantlet (14.3) and longest roots (9.0 cm). It was followed by T6 (½ MS + 0.2 mg/l NAA) and T4 (½ MS + 0.5 mg/l IBA) with 5.4 and 6.0 days, 93.3 and 100% rooting, 13.4 and 11.6 roots per plantlet and 8.5 and 7.7 cm long roots, respectively; whereas, the least response was recorded in T1 (control) for all the rooting parameters as shown in Table 4.

DISCUSSION

The most successful and most widely used discipline of plant tissue culture technique is micro-propagation which refers to the propagation of plants by using meristem tip culture, which is the transfer of apical buds and surrounding leaf primordial to sterile culture conditions (Ali et al., 2004; Mangal et al., 2002). Clonal propagation through *in vitro* culture can enhance multiplication many folds (Sauvaire and Galzy, 1978). Micro-propagation and other *in vitro* techniques have been used for plants which present particular problems in conventional horticulture (Fay, 1992).

During the present investigation, chrysanthemum plant regeneration was studied, using shoot tip as explant. Shoot tips were sterilized with 1.0% mercuric chloride for

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<.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<.05)

3 min and then they were rinsed with double distilled water thrice so as to lower the toxic affects of mercuric chloride. Then these shoot tips were inoculated on MS media supplemented with different concentrations of IAA and BAP alone and their 16 different combinations.

A close observation of the results regarding the effect of different concentrations of IAA on the regeneration of chrysanthemum plantlets revealed that its lowest concentration (0.1 mg/l) favored the regeneration of chrysanthemum plantlets, whereas the response was lowered with an increase in IAA concentration. This might be due to the fact that IAA is not usually considered to be a shoot proliferation growth regulator rather than root promoting regulator, as reported by Lazar and Cachita (1983). Our results gets support from a previous work done by Rout et al. (1997) who stated that the lower the IAA concentration, the higher would be the shoot bud regeneration in chrysanthemum. Bhattacharya et al. (1990) also reported maximum (2.15) shoots per explants in chrysanthemum shoot tips, when MS medium was supplemented with 0.1 mg/l IAA. These results are in agreement with our previous findings (Waseem et al., 2007) that a low concentration of IAA (0.1 mg/l) excelled all the other higher concentrations by producing the best results for shoot proliferation.

Cytokinins are often used to stimulate growth and development, Kn and BAP being in common use (Pierik 1987). The results regarding the performance of different BAP concentration showed that intermediate (1.0 mg/l) concentration of BAP excelled all other treatments, when BAP was used alone. Similar results were quoted by Karim et al. (2002) who described 1.0 mg/l BAP as the best BAP concentration as it produced 91% of shoot initiation in chrysanthemum while using shoot tips as explant.

It could be inferred from the results that 1.0 mg/l BAP is the optimum concentration for the better performance of this particular hormone. The fact that higher dozes fail to manifest their effect could be attributed to an obnoxious effect at higher concentrations, 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 increased number of buds primordial in chrysanthemum (Khan et al., 1994; Haq et al., 1998; Karim et al., 2002; Karim et al., 2003).

Cytokinins along with auxins also play a vital role in shoot regeneration in chrysanthemum (Karim et al. 2003). A very close examination of the result, revealed that T5 (1.0 mg/l BAP + 0.1 mg/l IAA) was the paramount combination of BAP and IAA, which showed the best response towards chrysanthemum regeneration closely followed by T9 (2.0 mg/l BAP + 0.1 mg/l IAA), T6 (1.0 mg/l BAP + 0.2 mg/l IAA) and T10 (2.0 mg/l BAP + 0.2 mg/l IAA), which also produced excellent results.

The result showed that the intermediate levels of BAP (1.0 and 2.0 mg/l) along with the lower concentrations of IAA (0.1 and 0.2 mg/l) had a favorable effect on the regeneration of chrysanthemum plantlets using shoot tips as explants. The favorable effect of intermediate concentration of BAP (1.0 mg/l) and the lowest concentration of IAA (0.1 mg/l) was observed when both the growth regulators were used alone, as stated earlier. The reason may be as BAP belongs to cytokinin group, so it would have a tendency towards shoot development (Vijaya et al., 1991) and thus its influence on the shoot development of chrysanthemum was not affected by the lower concentrations of IAA; whereas the higher concentrations of IAA had affected its influence on shoot development. Similar results were also reported by Singh and Arora (1995) who stated that increased levels of BAP in the medium increased the number of shoots but suppressed their growth. An addition of IAA does not supplement the effect of BAP on shoot morphogenesis.

These results are in agreement with previous findings

of Karim et al. (2003) who stated that a combination of 1.0 mg/l BAP + 0.1 mg/l IAA had produced the longest shoots of 4.5 cm in the shoot tips of chrysanthemum. The results also concluded that the highest (5.0 mg/l) and lowest (0.5 mg/l) concentrations of BAP with the combination of higher concentrations of IAA that is 0.5 and 1.0 mg/l showed least response towards regeneration of chrysanthemum using shoot tip explants. Almost similar results are quoted by a number of research workers stating that different combinations of BAP + IAA had affected the regeneration of chrysanthemum plantlets (Prasad et al., 1993; Dikshit et al., 1997; Wankhede et al., 2000; Bhattacharya et al., 1990 and Karim et al., 2003).

For root induction and its development in chrysanthemum micro-shoots, half strength MS media supplemented with different concentrations of IBA and NAA were compared. The results showed the supremacy of IBA over NAA. Many research workers have obtained similar results (Himstedt et al., 1991; Khan et al., 1994; Hoque and Fatima, 1995; Choi et al., 2002) as they stated that maximum rooting in chrysanthemum was observed when ½ MS medium was used supplemented with 0.2 mg/l IBA; whereas, an intermediate level of 0.2 mg/l for IBA and NAA was very useful for root induction and development in chrysanthemum. These results are also supported by the findings of Hoque et al. (1998), Singh and Arora (1995), Faisal and Amin (2000), Sarkar and Shaheen (2001) and Karim et al. (2002) who reported that 0.2 mg/l IBA had produced maximum results regarding the rooting of chrysanthemum micro-shoots.

Conclusion

Both growth regulators (IAA and BAP) showed their significant effect on the regeneration of chrysanthemum plantlets using shoot tip culture. Low concentration of IAA (0.1 mg/l) and intermediate level of BAP (1.0 mg/l) showed better results amongst their respective concentrations, when used alone. However, when their combinations were tested, MS media supplemented with lower concentrations of IAA (0.1 and 0.2 mg/l) and intermediate concentrations of BAP (1.0 and 2.0 mg/l) showed far more better results regarding regeneration of chrysanthemum plantlets as compared to other combinations. For rooting of chrysanthemum micro-shoots, half strength MS media supplemented with 0.2 mg/l IBA gave the most promising results.

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