**Comparing the performance of different chrysanthemum explants for direct *in vitro* multiple shoot production**

R.S. Telem1\*, R. Sadhukhan1, H. K. Sarkar1, N. Mandal2, S.H.Wani3 and Prabhat Kumar1

R.S. Telem- Department of Genetics, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal-741252, India. e-mail: [telem.ratan@gmail.com](mailto:telem.ratan@gmail.com)

R. Sadhukhan- Department of Genetics, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal-741252, India. e-mail: [drsadhukhan@gmail.com](mailto:drsadhukhan@gmail.com)

H. K. Sarkar- Department of Genetics, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal-741252, India. e-mail: [hemensarkar@rediffmail.com](mailto:hemensarkar@rediffmail.com)

N. Mandal- Department of Agricultural Biotechnology, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal-741252, India.e-mail: [nirman\_bckv05@yahoo.com](mailto:nirman_bckv05@yahoo.com)

S.H.Wani- Division of Plant Breeding and Genetics, SKUAST-K, Shalimar Srinagar, Kashmir- 191 121, India.e-mail: [shabirhussianwani@gmail.com](mailto:shabirhussianwani@gmail.com)

Prabhat Kumar- Department of Genetics, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal-741252, India. e-mail: [prani.ubkv@gmail.com](mailto:prani.ubkv@gmail.com)

**Abstract**

Different explants of local chrysanthemum cultivars available in West Bengal including leaf, shoot tip and ray floret were employed to compare their efficiency for direct *in vitro* regeneration system. The explants were inoculated in Murashige and Skoog (MS) media supplemented with varied combinations of indole acetic acid (IAA), benzylaminopurine (BAP). The auxins indole -3-butyric acid (IBA) was used to induce rooting. Maximum multiple shoots production occurred at 1.0 mgl-1 BAP and 0.1 mg/L NAA for leaf explants, 2.0 mg/L BAP and 0.2 mg/L NAA combination for shoot tip explants and BAP (4.0 mgl-1) + NAA (0.1 mgl-1) for ray floret explants. Among the treatments, IBA 1.0 mg /L for both leaf and shoot tip regenerated plants and 0.5 mg /L for ray floret regenerates proved to be the best for promoting root regeneration as compared to the other treatments tried. Among the various carrier substrates tested for acclimatization, soil + sand + FYM (1:2:1) fortified with ½ strength MS plant salt mixture proved to be ideal substrate as maximum plant survived and a maximum of 82.3 % survivability was obtained from shoot tip derived plantlets.Therefore, shoot tip explants are the most suitable type of explants for plant regeneration of chrysanthemum through direct somatic embryogenesis. However, direct plant regeneration through ray floret explants will also be useful to recover the flower colour mutants.

**Key words**: *Chrysanthemum morifolium*, MS medium, BAP, NAA

**Introduction**

Chrysanthemum (*Chrysanthemum morifolium* Ramat.), which belongs to the family Asteraceae, is highly valued worldwide as a cut flower with its diverse floral types and colors (Teixeira da Silva, 2003). For modern and industrialized horticulture, the cut flower industry, perhaps different from any other industry, is always in demand and in need of new varieties to routinely attend the continuous flower consumer demands. Consumer preferences change and show new and sometimes uncommon features. Therefore, the priority of the flower and ornamental plant biotechnology segments should be the generation of novel plant and flower types (Hutchinson *et al.,* 1982). Due to its high popularity and demand it becomes one of the first commercial targets for micro propagation and thus tissue culture can be utilized for its large scale production (Levin *et al.,* 1988). Micro propagation and other *in vitro* techniques have been used for plans which present particular problems in conventional horticulture (Fay, 1992). The regeneration of plants from tissue culture is an important and essential component of biotechnological research. High frequency regeneration of plants from the *in vitro* cultured tissue is a pre-requisite for successful application of tissue culture techniques for crop improvement (Akter, 2001).Therefore, the present study was undertaken to determine the efficiency of different explants including leaf, shoot tip and ray floret for direct *in vitro* multiple shoot regeneration.

**Materials and Methods**

The experiment was conducted at the Plant Tissue Culture Laboratory, Department of Agricultural Biotechnology, Faculty of Agriculture, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal, India, during 2011 in completely randomized design (CRD) with three replications and each replication has five culture bottles with five explants in each bottle.

**Explant preparation and surface sterilization**

The leaf and shoot tip explants were collected from 4 months old chrysanthemum plant, grown at the experimental field of All India Coordinated Research Project (AICRP) on floriculture, Mandouri centre, BCKV, West Bengal, India. Ray florets from 10-15 days old flower heads were collected for the study. The collected materials were brought to the laboratory and washed thoroughly with running tap water for 20-30 min. Shoot tips of about 0.5-1 cm were then excised with the help of scalpel and forceps.

The explants were washed in running tap water properly to remove any dirt and impurities and treated with 0.01% of antifungal (Bavistin) solution for 15 minutes. Later, the explants were dipped in 70% ethanol for 60 seconds and then rinsed with sterile water for 3-4 times. The explants were transferred to 0.1-1.0% of HgCl2 solution with 2-3 drops of Tween-20 for 2 minutes. Then the explants were rinsed four to five times in sterile distilled water with 5 min duration each.

**Shoot regeneration**

The basal medium consisted of Murashige and Skoog (1962) macro and micro salts, with 3% sucrose, 0.8% agar and 100 mgl-1myoinositol for induction of shoot organogenesis. Its pH was adjusted to 5.8-6 and the medium was transferred to culture bottles (40 ml in each bottle) and autoclaved at 121°C for 30 min. The effect of different combinations of BAP (0, 1, 2, 3, 4, 5 mgl-1) and NAA (0, 0.1, 0.2 mgl-1) on induction of direct organogenesis from leaf, shoot tip and ray floret explants was tested by supplementation into MS medium in varied concentrations. The surfaced sterilized leaf explants were cut into 0.5 m2 slices uniformly and placed on the culture medium horizontally , the basal portion of the shoot tip was cut to eliminate the HgCl2 affected tissue at the base and placed with the basal portion facing the culture medium and surface sterilized ray florets were given a small cut at the petiolar end to remove the HgCl2 affected tissue and mechanical wounding in the explants by brushing the ray floret surfaces was made and placed horizontally on the medium. All these operations were aseptically done inside the laminar airflow. The cultures were incubated in a growth chamber at temperature of 25 ± 2ºC under white fluorescent light (2000 lux) and photoperiodic regime of 16 hrs light and 8 hrs dark cycle.

For shoot regeneration, the data was recorded for different parameters including days for shoot formation, shoot formation percentage and average number of shoots per explants. Shoot formation percentage was calculated after one week, while all the other parameters were taken after eight weeks interval.

**Root Induction**

The rooting media comprised of half strength MS ingredients devoid of vitamins. The sucrose level was also reduced to 20 gml-1. For faster and better induction of rooting, Indole -3- butyric acid (IBA) was used. The well grown, elongated shoots were transferred to the rooting media containing different concentrations of IBA (0, 0.5, 1 mgl-1) and the response was observed. The number of root per explant and root length was recorded at 3 weeks and 4 weeks intervals.

**Acclimatization of Regenerated Plants**

Eight weeks old rooted shoots or plants were first removed from the media bottles and washed thoroughly with sterile water to remove agar, and then dipped in an antifungal solution (Bavistin 0.01%). The plantlets were then transferred to clean plastic cups containing different carrier substrates viz. autoclaved soil, sand and soil + sand + FYM (1:2:1) and kept at hardening chamber at 25 ± 2ºC and 80% RH. After 4 - 5 weeks of hardening, plants were transferred to earthen pots and kept in the field. The survival percentage of the plantlets was recorded.

**Statistical analysis**

The 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). The data were analyzed, using statistical analysis system (SAS) programme, version 6 (1985).

**Results and Discussion**

**Responses of leaf explants on multiple shoot formation and rooting**

From table 1 it is clear that there is highly significant response of leaf explants at different media combination for different parameters. The best results was obtained from MS media supplemented with 1.0 mgl-1  BAP + 0.1 mgl-1 NAA where days for shoot was obtained at a minimum period of 25.3 days, shoot formation percentage was maximum (24.4%) and also higher number of shoots per explants (15.6) followed by the combinations 1.0 mgl-1 BAP + 0.2 mgl-1 NAA. Results of the present study confirmed the works of Sivanesan and Murugesan (2008) who stated that a combination BAP and IAA is responsible for increased in shoot length. On the other hand, adequate rooting (rhizogenesis) was observed in ½ MS without IBA, but the number and length of roots increased with increasing IBA concentrations with maximum number of roots and root length at 1 mgl-1 (Table 2). The present findings are in close proximity with the reports of Karim *et al.* (2003) who observed that the maximum length of roots in half strength MS medium supplemented with 0.2 mgl-1 IBA.

**Responses of shoot tip explants on multiple shoot formation and rooting**

Maximum shoot production 12.6 at 2.0 mgl-1  BAP and 0.2 mgl-1 NAA (Table 1) and maximum shoot initiation of 92.6% was recordec using shoot tips as explants whereas minimum performance was shown by the controls. Similar results were quoted by Karim *et al.* (2002) who described 1.0 mgl-1 BAP as the best BAP concentration as it produced 91% of shoot initiation in chrysanthemum while using shoot tips as explant. One can visualize from the results regarding average number of roots per plantlet (Table 2) that ½ MS + 1.0 mgl-1 IBA is better than all the other treatments used, as it significantly exerts its effect in showing maximum roots per plantlet (25.2) and longest roots (4.26 cm) on the 4 weeks of innoculation. The results showed the supremacy of higher dose of IBA over lower dose. These results are also supported by the findings of Faisal and Amin (2000), Sarkar and Shaheen (2001).

**Responses of ray floret explants on multiple shoot formation and rooting**

Highest number of shoots per microshoot (14.3) was recorded in the cultures on MS medium supplemented with BAP (4.0 mgl-1) + NAA (0.1 mgl-1) and also maximum shoot formation percentage of 97.5% followed by the combinations 4.0 mgl-1  BAP + 0.2 mgl-1 NAA. (Table 1). These results lend support from the report of earlier workers (Liu and Gao, 2007; Park *et al.*, 2007; Waseem *et al*., 2011). Maximum roots per plantlet (11.35) and root length (12.36 cm) was obtained in ½ strength MS supplemented with 0.5 mgl-1 IBA followed by 1.0 mgl-1 IBA treated media (Table 2). Among the treatments, IBA 0.5 mgl-1 proved to be the best for promoting root regeneration when compared to the other auxins tried.

**Hardening of *in vitro* grown plants**

Among the various carrier substrates (Table 3) tested for acclimatization, sterilized soil + sand + FYM (1:2:1) fortified with ½ strength MS plant salt mixture proved to be ideal substrate as maximum plant survival (85.33 %) in this substrate followed by sand (70.5%) and soil (45.56%) alone during the study. Similar result was reported by Padmadevi *et al.* (2009) where highest survival (76.50%) was obtained in sand followed by sand + pot mixture (68%) in *in vitro* regenerated chrysanthemum. During the present investigation, it was also observed that the survival percentage of the plantlets derived from different explants varies. In general, plantlets obtained from shoot tip explants followed by leaf explants recorded maximum survival percentage across the various substrates used and minimum was recorded in ray florets derived plantlets. This may be due to difference in tissue differentiation as leaf and shoot tip are meristematic tissue they undergo only dedifferentiation while ray floret have to undergo both redifferentiation and dedifferentiation.

**Conclusion**

Plant hormones are among the most important physiological factors affecting the growth of plants *in vitro*. The major differences in the response of different plants and different explants to tissue culture conditions lie in the ratio of auxins to cytokinins (Skoog and Miller, 1957). In the present study, although leaf explants produced maximum number of shoots per explants and shoot formation percentage, the survival percentage of the regenerated plants is less than the shoot tips regenerated plants. Therefore, shoot tip may be consider as an ideal explants for multiple shoot production. Standardization of a micropopagation protocol for chrysanthemum is significantly useful for crop improvement also, since a successful *in vitro* regeneration protocol is an essential prerequisite for tissue culture based crop improvement programmes.

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**Table 1. Effects of different combinations of BAP and NAA on shoot regeneration from different explants of Chrysanthemum**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Media combinations** | | **Leaf explant** | | | **Shoot tip explant** | | | **Ray floret explant** | | |
| **BAP mgl-1** | **NAA mgl-1** | **Days for shoot formation** | **Shoot formation %** | **Average No. of shoot/ explant** | **Days for shoot formation** | **Shoot formation %** | **Average No. of shoot/ explant** | **Days for shoot formation** | **Shoot formation %** | **Average No. of shoot/ explant** |
| 0 | 0 | 52.6A | 11.2E | 7.5E | 40.2A | 74.2F | 4.0F | 58.33A | 7.12J | 2.4G |
| 1.0 | 0.1 | 25.3G | 24.4A | 15.6AB | 28.4E | 90.4AB | 5.6E | 57.00AB | 17.23I | 4.2A |
| 2.0 | 0.1 | 28.2F | 20.5BC | 10.5D | 20.3G | 91.6A | 10.5B | 52.24DE | 30.26G | 6.5B |
| 3.0 | 0.1 | 30.5E | 21.9B | 12.7C | 25.5F | 88.4C | 8.2C | 50.42E | 25.75H | 7.2BD |
| 4.0 | 0.1 | 34.0C | 19.5C | 9.7D | 34.6B | 87.2C | 9.4B | 30.26I | 97.5A | 14.3C |
| 5.0 | 0.1 | 32.0D | 18.7C | 11.6CD | 30.2D | 84.5D | 6.9D | 55.46BC | 52.34C | 10.2EF |
| 1.0 | 0.2 | 26.4G | 22.4B | 14.8B | 26.4F | 91.2A | 5.4E | 54.34CD | 25.75H | 6.8B |
| 2.0 | 0.2 | 28.6F | 20.6BC | 12.7C | 18.4H | 92.6A | 12.6A | 40.57G | 34.21F | 8.3D |
| 3.0 | 0.2 | 32.7D | 21.5B | 7.7E | 27.5EF | 85.6CD | 7.7C | 35.47H | 50.23D | 9.5E |
| 4.0 | 0.2 | 35.4C | 18.7C | 8.3DE | 35.4B | 84.7D | 8.8BC | 45.68F | 70.14B | 11.3F |
| 5.0 | 0.2 | 37.6B | 17.8CD | 10.3D | 32.5C | 82.6E | 7.0CD | 55.46BC | 43.12E | 8.4D |

Means within a column followed by different letters are significantly different according to Duncan’s Multiple Range test (P˂0.05)

**Table 2: Effect of IBA concentration on rooting at 2 weeks and 4 weeks after transfer**

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Treatments** | **Leaf explant** | | | | **Shoot tip explant** | | | | **Ray floret explant** | | | |
| **½ MS + IBA (mgl-1)** | **Root Number** | | **Root Length** | | **Root Number** | | **Root Length** | | **Root Number** | | **Root Length** | |
| **2 weeks** | **4 weeks** | **2 weeks** | **4 weeks** | **2 weeks** | **4 weeks** | **2 weeks** | **4 weeks** | **2 weeks** | **4 weeks** | **2 weeks** | **4 weeks** |
| ½ MS only | 6.36B | 8.67C | 1.54A | 2.72B | 9.8C | 15.6C | 2.34B | 3.52B | 3.42C | 4.56C | 3.25C | 4.24C |
| ½ MS + 0.5 mgl-1 | 8.25A | 11.26B | 1.26B | 3.13B | 12.5B | 22.4B | 2.85A | 3.76A | 9.56A | 11.35A | 10.56A | 12.36A |
| ½ MS + 1.0 mgl-1 | 9.51A | 13.24A | 1.78A | 3.57A | 14.4A | 25.2A | 3.25A | 4.26A | 7.22B | 8.44B | 6.24B | 7.23B |

Means within a column followed by different letters are significantly different according to Duncan’s Multiple Range test (P˂0.05)

|  |  |  |  |
| --- | --- | --- | --- |
| **Treatments** |  | **Survival %** |  |
| **Leaf explant** | **Shoot tip explant** | **Ray foret explant** |
| Soil | 40.67C | 45.56C | 38.65C |
| Sand | 67.54B | 70.5B | 65.36B |
| Soil + Sand + FYM (1:2:1) | 80.75A | 85.33A | 76.85A |

**Table 3: Effect of substrate fortified with ½ strength MS solution** **on acclimatization of micropropagated plants from different explants**

Means within a column followed by different letters are significantly different according to Duncan’s Multiple Range test (P˂0.05)