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BIOTECHNOLOGY

Estimating the efficiency of different explants for direct *in Vitro* multiple shoots development in chrysanthemum

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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 mg/L 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 mg/L) + NAA (0.1 mg/l) 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.

Highlights

- The commercial chrysanthemum cultivars are mostly propagated vegetatively through cuttings and suckers.
- Tissue culture, an important aspect of biotechnology can be implemented to enhance the yield of planting material through enhanced availability of distinguished planting stock possessing the useful characters.
- Different explants of local chrysanthemum cultivars including leaf, shoot tip and ray floret were employed to compare their efficiency for direct *in vitro* regeneration system.
- Shoot tip explants were the most suitable type of explants for plant regeneration of chrysanthemum through direct somatic embryogenesis.

Keywords: *Chrysanthemum morifolium*, MS medium, BAP, NAA, multiple shoots

Chrysanthemum, commonly known as Autumn Queen belongs to the family *Asteracea*. It is globally one of the most important cut flower and pot plants with its distinct floral types and colors grown in many parts of the world (Teixeira da Silva, 2003). The most commonly available chrysanthemum

on commercial scale is *Chrysanthemum morifolium* Ramat. It is a highly attractive and charming short day plant, which behaves both as an annual as well as a perennial flowering herb. The commercial cultivars are mostly propagated vegetatively through cuttings and suckers. Tissue culture, an important aspect of biotechnology can be implemented to enhance the yield of planting material through enhanced availability of distinguished planting stock possessing the useful characters. Micro propagation is the constant multiplication of preferred genotypes implying *in vitro* culture techniques. Therefore, this approach brings a sound platform for the generation of huge amount of genetically homogeneous disease free plants (Nalini 2012). *In vitro* culture of chrysanthemum was immensely helpful for large scale production of explants in a brief duration (Dao *et al*. 2006). Tissue culture approach in chrysanthemum are being utilised as a technique for mutation induction and also a way for micropropagation of macromutation and chimeric flowers favourably to generate new mutants in genuine form and promote generation of a large number of new chrysanthemum genotypes (Verma *et al.* 2012a). The present horticulture sector particularly the cut flower industry concievably vary from any other industrial sector as there is always in appeal for the need of novel varieties to regularly meet the extended users demands. Users preferences also advance and desire novel and noteworthy characters (Misra *et al.* 2003). Indirect regeneration via callus was stated from stem, petal and shoot tips, but adventitious shoot production from the initial callus phase may display somaclonal variation and chimeras while direct shoot regeneration from leaf or stem explants may remove this conditions (Verma *et al.* 2012b). The regeneration of plants from tissue culture is an important and essential component of biotechnological research (Ju *et al.* 2011, Manchanda *et al.* 2014). 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, Guleria and Gowda 2015). The efficiency of recovery of multiple shoots in chrysanthemum differs due to many reasons including type of explants used (Zhou *et al.* 2014, Naing *et al.* 2015). 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

Experimental site

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 2011-2012 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 (BASF, India) 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 $HgCl_2$ 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 mg/l myoinositol 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 mg/L) and NAA (0, 0.1, 0.2 mg/L) 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 m^2 slices uniformly and placed on the culture medium horizontally , the basal portion of the shoot tip was

cut to eliminate the $HgCl_2$ 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 $HgCl_2$ 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 regeneration

The rooting media comprised of half strength MS ingredients devoid of vitamins. The sucrose level was also reduced to 20 g/l. 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 mg/L) 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 mg/L BAP + 0.1 mg/L 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 mg/L BAP + 0.2 mg/L 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. Similar results were also reported by Kumar *et al.* (2004) in gerbera. 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 1mg/L (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 mg/l IBA.

Responses of shoot tip explants on multiple shoot formation and rooting

Maximum shoot production 12.6 at 2.0 mg/L BAP and 0.2 mg/L NAA (Table 1) and maximum shoot initiation of 92.6% was recorded using shoot tips as explants whereas minimum performance was shown by the controls. The result were in close proximity with the findings of Alizadeh *et al.* (2004). 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. One can visualize from the results regarding average number of roots per plantlet (Table 2) that ½ MS + 1.0 mg/l IBA is better than all the other treatments used, as it significantly exerts

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 mg/L	NAA mg/L	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
Mean		33.03	19.75	11.04	29.04	86.64	7.83	48.66	41.24	8.10
SEm±		2.27	1.03	0.82	1.96	1.59	0.74	2.85	7.75	0.99
CD		7.15	3.25	2.58	6.18	5.01	2.33	8.98	24.42	3.12

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	Root Number		Root Length		Root Number		Root Length		Root Number		Root Length	
(mg/L)	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 mg/L	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 mg/L	9.51a	13.24a	1.78a	3.57a	14.4a	25.2a	3.25a	4.26a	7.22b	8.44b	6.24b	7.23b
Mean	8.04	11.06	1.53	3.14	12.23	21.07	2.81	3.85	6.73	8.12	6.68	7.94
SEm±	0.92	1.32	0.15	0.25	1.33	2.85	0.26	0.22	1.79	1.97	2.12	2.37
CD	5.60	8.03	0.91	1.52	8.09	17.34	1.58	1.34	10.89	11.99	12.90	14.42

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 substrate fortified with ½ strength MS solution on acclimatization of micropropagated plants from different explants

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
Mean	62.98	67.13	60.28
SEm±	0.53	0.50	0.51
CD	1.59	1.50	1.53

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

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), Nalini (2012).

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 mg/L) + NAA (0.1 mg/L) and also maximum shoot formation percentage of 97.5% followed by the combinations 4.0 mg/L BAP + 0.2 mg/L NAA. (Table 1). These results lend support from the report of earlier workers (Liu and Gao, 2007; Park *et al.* 2007). Maximum roots per plantlet (11.35) and root length (12.36 cm) was obtained in ½ strength MS supplemented with 0.5 mg/L IBA followed by 1.0 mg/L IBA treated media (Table 2). Among the treatments, IBA 0.5 mg /L proved to be the best for promoting root regeneration when compared to the other auxins tried. These findings corroborate the reports of Waseem et al. (2011); Minas (2008) in chrysanthemum.

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. These observations are quite close to the results of Mandal and Datta, 2005; Nahid *et al*. 2007. Similar result was also 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 assumes significance

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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