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In vitro isolation, regeneration and purification of yellow mutant in chrysanthemum (*Chrysanthemum morifolium*) cv. Lalit through ray floret regeneration

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ABSTRACT

Mutations, induced or spontaneous, play an important role in inducing genetic variations in chrysanthemum (*Chrysanthemum morifolium* Ramat.). Isolation and purification of mutated tissue is impossible through conventional methods that results complete loss of the precious mutants due to lack of suitable techniques to isolate them through conventional methods. In the present investigation, an effort was made to develop efficient ray floret regeneration protocols to isolate, purify and establish a novel mutant which spontaneously appeared as chimera in the form of yellow flowers in chrysanthemum (*Chrysanthemum morifolium*) cv. Lalit (white). Maximum survival (82.0%) and callus formation (90.28%) in minimum duration (6.6 days) were recorded when the ray florets were pre-treated with mancozeb-45 (0.2%) + carbendazim (0.2%) + 8-HQC (200 mg/l) for 3 h followed by surface sterilized with $HgCl_2$ (0.1%) for a duration of four minutes and cultured on Murashige and Skoog (MS) medium supplemented with 6-Benzylaminopurine (BAP) (4.0 mg/l) and NAA (1.0 mg/l). The maximum regeneration of micro-shoots (80%) from the ray floret induced callus was recorded on MS medium fortified with BAP (4.0 mg/l), NAA (0.5 mg/l) and gibberellic acid (GA_3) (0.1 mg/l). MS medium supplemented with BAP (4.0 mg/l) + NAA (0.05 mg/l) + GA_3 (0.1 mg/l) was found to be best for highest micro-shoot proliferation (92.0%). Highest rooting (86.0%) was induced after culturing the micro-shoots individually on half-strength MS medium fortified with 0.5 mg/l NAA and 50 g/l sucrose. Successful acclimatization of *in vitro* raised plantlets was done in glass jar with polypropylene cap filled with a mixture of sterilized coco-peat, soilrite and perlite (1:1:1) supplemented with half-strength MS inorganic salts. After 3-4 weeks of acclimatization, the plantlets were successfully transferred to ambient conditions and compared with the parent variety Lalit. The *in vitro* raised plants produced all bright yellow flowers as compared to the original variety Lalit with white flowers.

Key words: Chrysanthemum, Growth regulators, *In vitro* regeneration, Mutation breeding, Novel mutant

Chrysanthemum (*Chrysanthemum morifolium* Ramat.) is one of the most beautiful ornamental plants with varied uses such as cut flower, pot plant, garland making, decoration and garden display purposes. It is one of the important cut flowers in the international market and ranks 2ndin the global cut flower trade after rose (Datta and Gupta 2012). A wide range of variability in respect of flower form, shape, size and colour, flowering duration and photo-sensivity exists in this crop. The florist chrysanthemum is propagated commercially through vegetative means, i.e. rooting of shoot tip cuttings, rooted suckers and micro-propagation. In vegetatively propagated plants such as chrysanthemum, mutation breeding method is very suitable for its improvement through induction of variability, early detection and ease of maintaining mutants in subsequent generations by vegetative means. Cytologically, *Chrysanthemum morifolium* is a polyploid with chromosome number ranging from 2X to 22X, besides a number of aneuploids, and belongs to family Asteraceae. Mutation breeding has played a vital role in the improvement of chrysanthemum and exploiting the variation in the form of new desirable mutants. Chimera formation or reversion of a variant to its parental type is the major problem in the development of a new variety through mutation. Isolation and purification of desirable mutated tissue is impossible through conventional techniques which result in complete loss of the precious mutants due to lack of suitable techniques. Broertjes *et al*. (1976) proposed that *in vitro* regeneration techniques could solve the problem of chimera formation and purification of mutants. Mutation breeding method using *in vitro* technique has been applied in chrysanthemum by several researchers (Bhattacharya *et al.* 1990, Chakrabarty *et al.* 1999, Mandal *et al.* 2000, Mandal and Datta 2005, Prasad *et al.* 2008, Kumar *et al.* 2012). Different explants, such as stem, flower receptacle,

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whole floret, floret segment, epidermis, shoot tip etc. have been used to regenerate plants *in vitro* (Bhattacharya *et al.* 1990). Efficiency of the recovery of solid colour mutants in chrysanthemum was variable in different type of explants used. The ray florets gave the maximum (100%) recovery of solid colour mutants (Mandal *et al.* 2000). However, reports on successful establishment of novel mutants using irradiation and *in vitro* regeneration protocols are limited in chrysanthemum (Prasad *et al.* 2008). Several experiments were conducted at Central Tissue Culture Laboratory, Division of Floriculture and Landscaping, IARI, New Delhi by combining the mutation breeding and tissue culture techniques and released seven new varieties of chrysanthemum namely Pusa Anmol, Pusa Centenary, Pusa Aditya, Pusa Chitraksha, Pusa Kesari, Pusa Sona and Pusa Anurodaya through ray floret regeneration techniques. In similar line of work, a chimeral plant producing beautiful bright yellow and white flowers at the same plant was observed in chrysanthemum cv. Lalit which otherwise has white flowers. This mutation was considered as spontaneous/ natural sectorial type of mutation. The yellow spray varieties of chrysanthemum are in a great demand in the flower market. Hence, the solid yellow flowers, appeared spontaneously in chrysanthemum cv. Lalit (White), were selected for the present investigation to isolate, purify and regenerate the clonal plants using ray florets as explants to develop a new stable yellow coloured genotype.

MATERIALS AND METHODS

The experiment was conducted at Central Tissue Culture Laboratory, Division of Floriculture and Landscaping, IARI, New Delhi during 2014-15. The ray florets of solid yellow flowers appeared spontaneously in chrysanthemum cv. Lalit having white flowers were selected as explants for the present investigation. First, the ray florets were washed thoroughly with running tap water for 30 min followed by 2 min washing with a solution containing few drops of liquid detergent (Teepol). Subsequently explants were washed thrice in tap water followed by one washing with double distilled water. Thereafter, to minimize the microbial load, the explants were pre-treated with a solution containing Bavistin (carbendazim 0.2%), Ridomil (0.2%) and 8-hydroxyquinoline citrate (200 ppm) for 2 h on an incubator shaker with 80 rpm at 24°C temperature. Then, the pre-treated explants were shifted to laminar air-flow chamber and treated with 0.1% $HgCl_2$ for 4 min followed by three washings with sterilized doubled distilled water. The individual ray floret was pinched with a sterile needle and then inoculated onto MS medium (Murashige and Skoog 1962) supplemented with 7.0% gelling agent (agar-agar type-1), 3% sucrose and different concentrations of BAP, NAA and GA_3 for different micro-propagation stages like callus induction, shoot regeneration, shoot proliferation, elongation and rooting. The pH of the medium was adjusted to 5.7 to 5.8 before autoclaving at 121°C and 20 psi for 20 min. The cultures were incubated in controlled atmosphere culture room provided with a constant photoperiod of 16/8 h light/ dark regimes at 24 ± 1°C temperature having a light intensity of 3000 lux at plant level provided by cool white fluorescent tubes. The temperature of the culture room and photo-period were maintained by automatic temperature and photo-period control devices. For callus induction, the surface sterilized ray florets were inoculated on MS medium supplemented with different concentrations of cytokinins, i.e. kinetin (5.0 and 10.0 mg/l) or BAP (3.0, 4.0 and 5.0 mg/l) along with NAA (1.0 and 2.0 mg/l). MS medium fortified with different concentrations of cytokinins, i.e. kinetin (5.0 and 10.0 mg/l) or BAP (3.0, 4.0 and 5.0 mg/l) along with NAA (0. 5 and 1.0 mg/l) was used for the adventitious regeneration of micro-shoots from ray florets and MS medium supplemented with different concentrations of cytokinins, i.e. kinetin (3.0, 5.0 and 10.0 mg/l) or BAP (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) along with a constant concentration of NAA (0.05 mg/l) and GA_3 (0.1 mg/l) was used for shoot proliferation. To prepare the micro-shoots suitable for rhizogenesis, these were transferred to MS medium supplemented with GA_3 (0.5 and 1.0 mg/l) and a constant dose of sucrose (40 g/l). Half-strength MS medium supplemented with different concentrations of auxins namely NAA (0.5 and 1.0 mg/l) or indolebutyric acid (IBA) (0.5 and 1.0 mg/l) along with an increased dose of sucrose (50 g/l) was used for *in vitro* rooting on to the well developed and elongated shoots. The rooted plants were transferred to glass jar fitted with polypropylene cap and filled with a mixture of sterilized cocopeat, soilrite and perlite (1:1:1) for their acclimatization. After 2-4 weeks of hardening, the *in vitro* raised plants were transferred to ambient field conditions for comparison with its parent variety Lalit. The experiments were laid out in completely randomized design (CRD). Each treatment had 10 units with five replications. All the percentage values were angularly transformed to data transformation before calculating ANOVA.

RESULTS AND DISCUSSION

Culture establishment and callus induction

The yellow ray florets treated with a solution containing Bavistin (carbendazim) (0.2%), Ridomil (0.2%), and 8-hydroxyquinoline citrate (200 ppm) for 2 h on an incubator shaker with 80 rpm and at $24C^0$ temperature followed by surface sterilized with $HgCl_2$ (0.1%) for 4 min. and inoculated on MS medium supplemented with different concentrations of cytokinins and auxin showed good response in respect of contamination free culture establishment/survival (%) and callus induction (%) (Table 1). Data presented in Table 1 indicate that maximum survival (82.0%) and callusing (90.28 %) were observed after 6.6 days when the surface sterilized and pinched (wounded) ray florets were cultured on MS medium supplemented with BAP (4.0 mg/l) and NAA (1.0 mg/l) (T-7) as compared to those cultured on the other media. Present findings are in the line with the earlier work done by Kumar *et al.* (2012) who reported that the wounded parts of ray florets showed faster callusing as compared to those inoculated

Table 1 Effect of BAP, kinetin and NAA on callus induction in ray floret explants of chrysanthemum cv. Lalit

Treatment	Medium	Survival (%)		Callusing (%)		Duration required for callusing (days)
		Mean	Angular value	Mean	Angular value	
T-0	MS Blank	60.00	51.672	0.00	0	0.00
T-1	MS + Kinetin (10.0 mg/l) + NAA (1.0 mg/l)	70.00	59.852	81.43	67.121	11.00
T-2	MS + Kinetin (10.0 mg/l) + NAA (2.0 mg/l)	72.00	55.688	86.43	73.081	9.00
T-3	MS + Kinetin (5.0 mg/l) + NAA (1.0 mg/l)	66.00	59.676	75.12	60.448	10.20
T-4	MS + Kinetin (5.0 mg/l) + NAA (2.0 mg/l)	70.00	51.460	74.48	59.967	9.80
T-5	MS + BAP (5.0 mg/l) + NAA (1.0 mg/l)	74.00	61.006	85.00	72.292	9.40
T-6	MS +BAP (5.0 mg/l) + NAA (2.0 mg/l)	76.00	59.549	86.78	70.953	9.20
T-7	MS + BAP (4.0 mg/l) + NAA (1.0 mg/l)	82.00	61.263	90.28	75.787	6.60
T-8	MS + BAP (4.0 mg/l) + NAA (2.0 mg/l)	70.00	64.188	88.33	72.057	7.40
T-9	MS + BAP (3.0 mg/l) + NAA (1.0 mg/l)	70.00	62.626	75.95	60.986	7.20
T-10	MS + BAP (3.0 mg/l) + NAA (2.0 mg/l)	62.00	58.220	81.00	66.795	8.60
	CD	N A		14.681		1.829
	SE(m)	5.032		5.134		0.64
	CV	19.185		18.584		17.797

without wounding. The callus initiation was observed first on wounded parts of the ray florets (Fig 1b) that turned green, which later on got spread over the whole ray floret. It may be due to the direct exposure of tissue to the culture medium containing higher levels of cytokinin in combination with auxin (NAA), which resulted in rapid cell division and callus formation (Dash *et al.* 2000). It was suggested by Nahid *et al.* (2007) that BAP is more crucial for callus induction in chrysanthemum. The earliest callus induction and vigorous callus growth was noticed in the explants cultured on BAP supplemented MS medium than those on kinetin based medium. The present results on survival and callusing percentage are in conformity with earlier findings of Nahid *et al.* (2007).

Adventitious micro-shoot regeneration and proliferation

New shoot apices develop directly on the ray florets having very small creamish-green callus when cultured on MS medium supplemented with different combinations of cytokinins and auxins. Data on the response of ray floret explants to various culture media in respect of regeneration (%), days required for regeneration and number of micro-shoots/explant are presented in Table 2. Highest regeneration (80 %), maximum number of shoots/explant (6.2) in minimum duration (19.6 days) was recorded in the ray florets cultured on MS medium supplemented with BAP (4.0 mg/l), NAA (0.5 mg/l) and GA_3 (0.1 mg/l) as compared to those cultured on the other media. These observations are quite similar to the results of other

Table 2 Effect of BAP, kinetin and NAA on regeneration in ray floret explants of chrysanthemum cv. Lalit

Treatment	Medium	Regeneration (%)		Duration required (days)	No. of shoots/ Expl.
		Mean	Angular Value		
T-0	MS Blank	0.00	0	0	0
T-1	MS + Kinetin (10.0 mg/l) + NAA (0.5 mg/l) + GA3 (0.1 mg/l)	42.00	40.268	37	3.6
T-2	MS + Kinetin (10.0 mg/l) + NAA (1.0 mg/l) + GA3 (0.1 mg/l)	42.00	40.268	45.6	4.8
T-3	MS + Kinetin (5.0 mg/l) + NAA (0.5 mg/l) + GA3 (0.1 mg/l)	76.00	60.752	27.2	5.8
T-4	MS + Kinetin (5.0 mg/l) + NAA (1.0 mg/l) + GA3 (0.1 mg/l)	28.00	31.744	51.2	4
T-5	MS + BAP (5.0 mg/l) + NAA (0.5 mg/l) + GA3 (0.1 mg/l)	72.00	58.345	42.8	5
T-6	MS + BAP (5.0 mg/l) + NAA (1.0 mg/l) + GA3 (0.1 mg/l)	54.00	47.288	45.6	4.6
T-7	MS + BAP (4.0 mg/l) + NAA (0.5 mg/l) + GA3 (0.1 mg/l)	80.00	64.003	19.6	6.2
T-8	MS + BAP (4.0 mg/l) + NAA (1.0 mg/l) + GA3 (0.1 mg/l)	60.00	50.798	32	3.6
T-9	MS +BAP (3.0 mg/l) + NAA (0.5 mg/l) + GA3 (0.1 mg/l)	32.00	34.401	32.8	3.6
T-10	MS + BAP (3.0 mg/l) + NAA (1.0 mg/l) + GA3 (0.1 mg/l)	22.00	27.883	48	3.6
	CD	6.728		9.404	0.937
	SE(m)	2.353		3.288	0.328
	CV	12.698		21.184	18.063

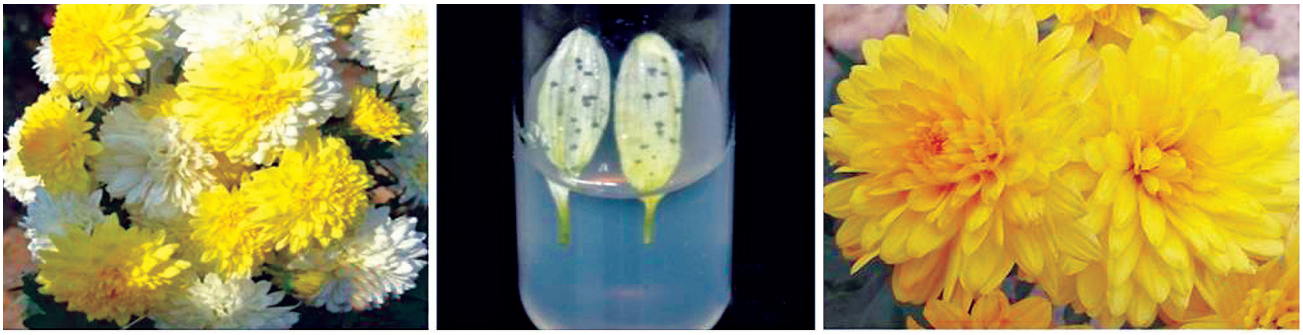

Fig 1 *In vitro* isolation and purification of Yellow mutants through ray floret regeneration in chysanthemum cv. Lalit (white)

Table 3 Effect of BAP, kinetin and NAA on shoot proliferation in chrysanthemum cv. Lalit

Treatment	Medium	Proliferation (%)		No. of shoots after		
		Mean	Angular value	60 days	120 days	180 days
T-0	MS Blank	4.00	7.371	0	0	0
T-1	MS + Kinetin (10.0 mg/l) + NAA (0.05 mg/l) + GA3 (0.1 mg/l)	62.00	52.002	26	32.8	46
T-2	MS + Kinetin (5.0mg/l) + NAA (0.05 mg/l) + GA3 (0.1 mg/l)	72.00	58.22	50.8	74.8	98.8
T-3	MS + Kinetin (3.0 mg/l) + NAA (0.05 mg/l) + GA3 (0.1 mg/l)	44.00	41.472	29.8	42.4	58.8
T-4	MS + BAP (5.0 mg/l) + NAA (0.05 mg/l) + GA_3 (0.1 mg/l)	54.00	47.288	36	48.8	79.2
T-5	MS + BAP (4.0 mg/l) + NAA (0.05 mg/l) + GA_3 (0.1 mg/l)	92.00	77.296	56.4	90.4	122
T-6	MS + BAP (3.0 mg/l) + NAA (0.05 mg/l) + GA_3 (0.1 mg/l)	48.00	43.829	27.2	38.6	88.4
T-7	MS + BAP (2.0 mg/l) + NAA (0.05 mg/l) + GA_3 (0.1 mg/l)	70.00	57.067	41.6	62.8	96.4
T-8	MS + BAP (1.0 mg/l) + NAA (0.05 mg/l) + GA_3 (0.1 mg/l)	66.00	54.359	18.8	23.8	37
	CD	9.550		8.036	5.645	5.534
	SE(m)	3.300		2.79	1.96	1.922
	CV	15.133		19.594	9.519	6.172

workers working on chrysanthemum (Chakrabarty *et al.* 1999, Jaime and Silva 2005, Kumar *et al.* 2012). Direct adventitious shoot regeneration begins with cells that are located either in the epidermis or just below the surface of the explant apparently originating from single cells. However, the response of explants depends on different growth regulators, their concentrations and combinations. Cytokinin, i.e. BAP was necessary to induce adventitious buds (Cheah *et al.* 1978) and usually added to tissue culture media to stimulate proliferation. Highest (92%) micro-shoot proliferation and number of shoots per micro-shoot (122 after 180 days) was recorded in the cultures on MS medium supplemented with BAP (4.0 mg/l) + NAA (0.05 mg/l) + GA_3 (0.1 mg/l) (Table 3). These results lend support from the report of earlier workers (Liu and Gao 2007, Park *et al.* 2007, Waseem *et al.* 2011, Kumar *et al.* 2012). Shoot proliferation in tissue culture might be due to the role of optimum dose of BAP, which enhances axillary branching and multiple shoot formation.

Shoot elongation, rhizogenesis, acclimatization and field establishment

The proliferated micro-shoots were very small, tender, thin, undeveloped and not suitable for the induction of a

Table 4 Effect of gibberellic acid on shoot length (cm) in chrysanthemum variant Lalit

Treatment	Medium	Shoot length (cm) after			Total increase after 45 days
		15 days	30 days	45 days	
T-0	MS Blank	2.40	3.80	5.00	3.10
T-1	MS +GA_3 (0.5 mg/l)	2.80	4.10	6.70	5.20
T-2	MS + GA_3 (1.0 mg/l)	3.20	5.20	9.00	7.50
	CD	0.459	0.673	1.159	0.943
	SE(m)	0.147	0.216	0.372	0.303
	CV	11.755	11.062	12.053	12.854

Table 5 Effect of auxins on rooting of micro-shoots in chrysanthemum variant Lalit

Treatment	Medium	Rooting (%)		Days to root initiation	No. of roots/ shoot	Length of longest root (cm)
		Mean	Angular value			
T-0	MS (1/2 strength)	58.00	49.65	8.00	4.00	8.50
T-1	MS (1/2 strength) + NAA 0.5 mg/l	86.00	70.65	6.40	7.20	4.80
T-2	MS (1/2 strength) + NAA (1.0 mg/l)	66.00	54.36	8.00	4.00	3.80
T-3	MS (1/2 strength) + IBA (0.5 mg/l)	70.00	56.89	6.60	4.40	3.60
T-4	MS (1/2 strength) + IBA (1.0 mg/l)	78.00	62.38	7.60	5.00	4.40
	CD	9.350		N A	1.188	1.050
	SE(m)	3.147		0.506	0.400	0.354
	CV	11.972		15.456	18.179	15.748

very strong root system. Gibberellic acid stimulates the growth of microshoots and makes them elongated, strong and suitable for *in vitro* induction of rooting. Hence, these were cultured individually on MS medium supplemented with GA_3 (0.5 and 1.0 mg/l) along with slightly increased concentration of sucrose (40 g/l) (Table 4). Micro-shoots sub cultured individually on MS medium supplemented with GA_3 (0.5 mg/l) and sucrose (40 g/l) showed very good response with regards to optimum shoot length and stem thickness resulting in an optimum increase in shoot length (5.20 cm) over those grown on other media (Table 4). The micro-shoots cultured on MS medium supplemented with GA_3 (1.0 mg/l) became very long, thin and difficult to manage and exhibited more increase in shoot length (7.50 cm) than those on other media. Gibberellins are known for inducing stem elongation in a number of crops. The elongation of the stems is not due to increased formation of nodes and internodes but results from rapid elongation of internodes, which is due to both cell division and cell elongation (Krishnamoorthy 1981). Successful root induction onto elongated shoots depends on number of factors including strength of basal medium salts, level of sucrose and supplementation of auxins (Rout and Das 1997). Supplementation of various levels (0, 0.5 and 1.0 mg/l) of auxins, i.e. NAA and IBA to 1/2 strength of MS medium containing a constant dose of sucrose (60 g/l) responded well in respect of per cent rooting, duration required for rooting, number of roots/shoot and length of the longest root (Table 5). The micro-shoots cultured on half-strength MS medium supplemented with 0.5 mg/l NAA took minimum days to root initiation (6.40 days) and produced maximum rooting (86 %), higher average number of roots/shoot (7.20) and optimum and manageable length of longest root (4.80 cm) (Table 5). These findings are in the line of reports of Prasad *et al*. (2007), Waseem *et al*. (2011) and Kumar *et al.* (2012). Plantlets were successfully acclimatized by transferring them in glass jars with polypropylene lids each filled with peat + soil rite + soilrite (1:1:1) and moistened with half-strength MS salts (devoid of growth regulators, calcium, organics and sucrose). After 3-4 weeks of acclimatization, the plants were transferred to the field and grown along with parent variety Lalit. The mutant plants produced uniform bright yellow flowers as compared to the parental variety Lalit producing white flowers.

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