Organogenesis and anatomical study of gamma rays induced mutant of chrysanthemum (*Chrysanthemum morifolium* Ramat.) from ray florets

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Abstract

We developed an efficient in vitro regeneration protocol for the isolation, rapid multiplication and field establishment of Pink Incurving novel mutant from ray florets of chrysanthemum. Earliest (8.33 ± 1.17 d) and highest (86.67 ± 2.03%) callogenesis was observed on Murashige and Skoog (MS) medium supplemented with 10 mg l^{-1} kinetin (KIN) and 2 mg l^{-1} α-Naphthalene acetic acid (NAA). MS medium supplemented with 10 mg l^{-1} KIN + 0.1 mg l^{-1} NAA was found most effective to induce maximum regeneration of micro shoots (96.33 ± 1.95%) while maximum shoot proliferation (98.33 ± 0.88%) was obtained when the medium was supplemented with 5 mg l^{-1} KIN + 0.01 mg l^{-1} NAA + 0.2 mg l^{-1} GA_3. Good shoot elongation was noticed after 30 days of transfer onto a medium comprising of 0.50 mg l^{-1} GA_3. The elongated microshoots were best rooted on half-strength MS medium supplemented with 0.5 mg l^{-1} NAA. The rooted plantlets were successful acclimatized in 3 weeks in glass jar with polypropylene cap filled with peat + Soilrite® (1:1) and transferred to greenhouse for flowering.

The plants obtained through this technique produced the true-to-type flowers in υM_2 generation. The isolated mutant was found stable for the selected characters. Histological investigations revealed that unorganized callus got organized and turned into meristematic zone to produce shoot primordia and further developed into multiple shoots as the concentration of growth regulators changed.

Keywords: Chrysanthemum, Histological analysis, *In vitro*, Organogenesis, Ray florets, Regeneration.

Introduction

Chrysanthemum (*Chrysanthemum morifolium* Ramat.) is economically important flower crop and widely used as cut flower and pot plant in the international floriculture trade. Chrysanthemum is also known for its culinary, medicinal and ethnopharmacological use[27]. In the modern and industrialized floriculture, novelty is always in demand and there is need for new varieties and colour to meet consumer demand and satisfaction[8,19]. Nowadays, this flower crop has earned huge popularity due to wide range of flower shades and colours, size, long vase life and diversity in height and growth[2,3,18]. The classical breeding applied for improvement of chrysanthemum has some limitations including restricted gene pool, longer ray florets that prevent timely pollination, parental ploidy level differences, self-incompatibility and non-synchronous flowering.[23,33]

The alternate breeding method is mutation breeding that can be employed in chrysanthemum for improvement of flower colour, size, form, plant height, growth habit and sensitivity to light quality/quantity[7]. Through mutagenesis, a total of 3218 varieties have been released in the world of which chrysanthemum contributes major share with 279 varieties and out of this India has commercially released 46 mutant cultivars in chrysanthemum. The chemical mutagens have low penetration into buds, shoots and stems leading to low competence and difficulty in producing promising mutant, so these mutagens are not extensively used[29].

Therefore, mutation breeding induced by physical mutagens is one of the effective methods to develop new cultivars for floriculture industry[15]. The irradiation is used for mutagenesis by which a large number of promising mutants emerge in the form of complete mutants or partial chimeras.

The appearance of partial chimeras after treatment with physical and chemical mutagens are the main bottleneck in chrysanthemum due to occurrence of chimeric tissues and mutated cells along with the normal cells invariably. The mutated cells struggle with adjacent non-mutated cells for their service as a result of mutagenic dose. In the diplontic selection if mutated cells survive, they will be expressed in plants[8].The expression of chimeras differed from small portion of a ray florets/flower to single flower and/or a portion of a branch to the entire branch. Even though, it is possible to separate a portion of a branch or an entire branch, if this was completely mutated by normal stem cutting, it is complicated to isolate such mutants which are expressed as new colour in a flower or in a single ray floret.[19]

Similarly, a small sector of a mutated branch or flower cannot be separated and regenerated by available traditional propagation techniques (stem cutting). For that reason, a large number of novel mutants appeared due to induced mutagenesis are lost every year.[4] *In vitro* regeneration of chrysanthemum plant from different explants such as stem, receptacle, florets, floret segment, epidermis, shoot tip, pedicel have been reported[2,27,30].

The efforts are directed to isolate such types of novel chimeras in pure form by *in vitro* regeneration from the ray florets of chrysanthemum[4,7]. The efficiency of recovery of solid mutants after gamma rays irradiation in chrysanthemum depends upon the type of explants used. The regeneration from ray florets results in 100 percent recovery of solid mutants[19] while the shoot buds are less dependable as they originate from multiple cell layers whereas somatic embryo originates from single cell.[28]

In another report, it was suggested that the response of floral parts to a variety of PGRs, applied as alone or in combinations with other PGRs need to be studied in a wider range of cultivars[27]. The PGRs combination use for the regeneration a mutant may not be useful for the regeneration of another mutant. Hence, many novel mutants were lost due to unavailability of array of protocol for the regeneration of novel mutants. Several researchers used different combinations of BAP and NAA for regeneration in chrysanthemum[7,9,27] but little information is available on the use of KIN and NAA which gave excellent response and can be useful for the isolation for many mutants. Our experiment revealed that a combination of KIN and NAA is able to induce regeneration from ray florets to isolate new and solid mutant of chrysanthemum.

The detailed histological investigations were found handy to characterize the type of morphogenic competent cells[26] which can be targeted to induce more regeneration of micro shoots by manipulating growth regulators regimes in culture medium. For better utilization of tissue culture techniques in crop improvement, there is a need to understand the histological events which take place during regeneration.

Histological investigations on *in vitro* regenerated micro shoots showed that the shoot buds originate mainly from the epidermal cells of wounded tissues without callus formation[10]. This study reports the successful *in vitro* regeneration from one such novel mutant that produced pink colour incurving florets. Histological studies were carried out to confirm the origin of the shoot buds during different regeneration stages to get the solid mutant.

Material and Methods

Plant material and selection and preparation of explants: The chimeric ray floret explants were collected from one of the promising mutant which appeared after gamma irradiation (^{60}Co) from an exotic cultivar Thai Chen Queen (Fig. 1a). The fully expanded ray florets (Fig. 1b–c) were taken as explants from field grown plant. The collected explants were soaked in 0.1% Teepol® solution for 8-10 min followed by washing under running tap water for 20 min. Thereafter, explants were pre-treated with 0.1% mancozeb 45 (BASF India Ltd., Mumbai) + 0.1% carbendazim (Rallis India Ltd., Mumbai) + 200 mg l^{-1} 8-hydroxyquinoline citrate (HQC) solution for 3 h. The explants were then surface sterilized with 0.1% mercuric chloride for 4 min followed by rinsing in sterilized double distilled water for 4 times.

Culture conditions: The individual ray floret (fig. 1d) notched the scalpel and inoculated onto MS medium supplemented with 3% sucrose (*w/v*), 0.72% agar-agar (*w/v*) (HiMedia Lab., Mumbai, India) with different concentrations and combinations of Kinetin (KIN), α-Naphthalene acetic acid (NAA) and Gibberellic acid (GA_3) at different stages. The growing medium pH was accustomed to around 5.8±0.1 and autoclaved at 121ºC for 15 min at 15 psi (1.05 kg cm^{-2}). The aseptic work like inoculation, subculture etc. was carried out in the laminar air flow chamber. The cultures were incubated in the culture room after inoculation and provided with 16/8 h light/dark (40.5 µmol m^{-2} s^{-1}) photoperiod at 25±1ºC temperature and 60-70% relative humidity.

Medium for *in vitro* morphogenesis: The Murashige and Skoog (MS) basal medium containing different concentrations of KIN, NAA and GA_3 were used in different combinations for callus initiation shoot regeneration and shoot multiplication. The proliferated shoots were separated and transferred to elongation medium with 4 micro shoots per glass jar. The shoot elongation was composed by basal MS medium supplemented with 0.25, 0.5 and 1.0 mg l^{-1} of GA_3.

Rooting, acclimatization and establishment of plantlets in the greenhouse: The elongated micro shoots obtained from initiated cultures of ray florets were shift to glass jars having half-strength MS medium containing different concentrations of NAA (0.25, 0.5 and 1.0 mg l^{-1}). The rooted plantlets with height of 4.5-5.0 cm were transferred from *in vitro* to *ex vitro* environment in glass jar which was filled with peat + Soilrite® (1:1). After 3 weeks of acclimatization, plants were transferred to polyhouse for further growth and development. The plants were provided with optimal growing conditions for growth and flowering. The flowering occurred 70-80 days after transplanting in the greenhouse.

Histological study of shoot morphogenesis: For histological examination, samples of callus were taken after 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28 and 30 days intervals. All the samples were fixed in FAA [5:5:90; formalin, acetic acid (glacial) and alcohol respectively (v/v/v)] at different regeneration stages for 48 h and transferred to 70% alcohol for storage (-20°C) until the samples were taken for microscopy. The samples were first stained with hematoxylin and then passed through dehydration and clearing series in alcohol followed by embedding in paraffin wax at 58°C.[13] The sections of 18 µm thickness were cut with rotary microtome (Uchida Yoko Co. Ltd., Tokyo, Japan).

The prepared slides were de-waxed by dipping in xylene for 10 min and stained with 2% safranin-fast green for general examination. Thereafter, all the slides were cleaned with clove oil[12]. Finally, permanent slides were made by covering with no. 1 Canada balsam followed by drying at 60°C for 2-3 d in an oven. The slides were examined under a compound

microscope (Nikon Eclipse 50i 100x) and photographs were taken by using a Nikon digital camera (Nikon Coolpix-8400, Nikon Corporation, Japan).

Experimental plan and statistical analysis: Experiments were carried out in completely randomized design. Each treatment had 15 tubes with three replications (each tube had only one ray floret). All the data were analyzed by using the statistical software SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The means were separated by Tukey's HSD test at 5% level of probability. Means are presented with standard error (± SE).

Results

Callus initiation from ray florets: The ray florets were cultured on MS medium supplemented with different combinations of KIN and NAA. The cultures were sub cultured between 10- 12 days of inoculation. The results revealed that among different combinations used, the earliest callus initiation (8.33 ± 1.17 d) and frequency of callus initiation increased and reached maximum 86.67 ± 2.03 percent when explants were cultured on MS medium supplemented with 10.0 mg l^{-1} KIN and 2.0 mg l^{-1} NAA as compared to other treatments (fig. 2).

The organogenic calli were developed better when concentration of KIN increased from 5.0 mg l^{-1} to 10.0 mg l^{-1} in combination with NAA 2.0 mg l^{-1} while further increase (15 mg l^{-1}) in the concentration of KIN reduced the callus initiation significantly and also took more time for callus initiation (43.33 ± 0.88 d). The organogenic callus initiation was first observed on wounded parts of the explants which later covered the entire ray floret and turned green.

Effect of KIN and NAA on morphogenesis: The morphogenesis started with little callus formation on ray florets followed by regeneration occurred on the wounded parts of ray florets (fig. 1e–f). It was evident from the data presented in table 1 that callus cultures on MS medium supplemented with 10 mg l^{-1} KIN and 0.1 mg l^{-1} NAA took minimum number of days (30.00 ± 0.58) for shoot regeneration followed significantly ($p \leq 0.05$) by those cultured on 10 mg l^{-1} KIN and 0.5 mg l^{-1} NAA (34.67 ± 0.88 d). The highest shoot regeneration (96.33 ± 1.95%) with maximum number of micro shoots per callus (8.33 ± 0.67) was observed in the cultures subjected to 10.0 mg l^{-1} KIN and 0.1 mg l^{-1} NAA after 30 d of regeneration (table 1). The shoot regeneration percentage from organogenic calli of ray florets were decreased when optimum concentration of KIN and NAA (10 mg l^{-1} KIN and 0.1 mg l^{-1} NAA) increased to 15.0 KIN + 0.1 NAA (55.33 ± 2.91%) or decreased to 5.0 mg l^{-1} KIN and 1.0 mg l^{-1} NAA (38.67 ± 1.86%).

Nearly every combination of KIN and NAA used in this experiment was able to induce some micro shoots from callus, but simultaneously callus formation was also observed in these combinations. The regeneration on MS medium supplemented with 10 mg l^{-1} KIN and 0.5 mg l^{-1} NAA and 10 mg l^{-1} KIN and 1.0 mg l^{-1} NAA was significantly ($p \leq 0.05$) low as compared to media supplemented with 10 mg l^{-1} KIN and 0.1 mg l^{-1} NAA.

Effect of plant growth regulators on shoot proliferation: The regenerated micro shoots were separated and cultured on MS medium supplemented with different PGRs. Data presented in table 2 showed that KIN and NAA significantly affected the shoot proliferation of regenerated micro shoots (fig. 3a). The maximum shoot proliferation (98.33 ± 0.88%) and average number of micro shoots per explant (9.33 ± 0.67) after 30 d of proliferation (3 sub-culture) was observed when the shoots were transferred onto a medium supplemented with 5.0 mg l^{-1} KIN + 0.01 mg l^{-1} NAA + 0.2 mg l^{-1} GA_3 whereas medium containing 7.5 mg l^{-1} KIN + 0.1 mg l^{-1} NAA + 0.2 mg l^{-1} GA_3 showed the lowest proliferation (table 2). After 30 d shoot multiplication rate increased along with increased concentrations of KIN from 3.0 to 5.0 mg l^{-1} with NAA 0.01, 0.05 and 0.1 mg l^{-1} but further increase in the concentration of KIN (5.0 to 7.5 mg l^{-1}) reduced the shoot proliferation coefficient. Shoot multiplication rate also significantly decreased with increasing the concentrations of NAA (0.01 to 0.1 mg l^{-1}).

When the optimum concentration (5.0 mg l^{-1}) of KIN increased to 7.5 mg l^{-1} and NAA increased from and 0.01 to mg l^{-1}, there was significant reduction in the number of shoots per micro shoot (9.33 ± 0.67 to 1.00±58). The application of optimum concentrations and combinations of KIN and NAA produced maximum number of shoots per micro shoot whereas supplementation of GA_3 improved the general growth of cultures besides controlling the vitrification (data not shown). Three sub-cultures (30 d of proliferation) on culture medium containing 5.0 mg l^{-1} KIN + 0.01 mg l^{-1} NAA + 0.2 mg l^{-1} GA_3 resulted in the highest shoot multiplication and shoots per micro shoot were recorded.

Effect of GA_3 on micro shoots elongation: The micro shoots elongation generally involves separation of micro shoots from the proliferation medium and sub-culture on elongation medium. Proliferated micro shoots with 1-1.5 cm height were transferred to MS medium supplemented with different concentrations of GA_3 (0.25, 0.5 and 1.0 mg l^{-1}) for the elongation. The results presented in fig. 4 regarding to shoot elongation revealed that maximum (3.61 ± 0.05 cm) increased in shoot length was recorded after 25 d of culture on MS medium supplemented with 1.0 mg l^{-1} GA_3 followed by 0.50 mg l^{-1} GA_3 (3.07 ± 0.12 cm).

The shoots qualities were not good on the culture medium supplemented with 1.00 mg l^{-1} GA_3 as compared to 0.25 and 0.50 mg l^{-1} GA_3. The micro shoots elongated on 1.00 mg l^{-1} GA_3 were lanky and translucent, whereas the micro shoots on MS medium supplemented with 0.50 mg l^{-1} GA_3 were sturdy and strong with well-developed dark green expanded leaves. Elongation of micro shoots is therefore important to

attain the optimum plantlets height so that they could be transferred for rhizogenesis with high success rate during hardening.

Rooting and acclimatization of plantlets: The rooting of plantlets is essential for well establishment of plantlets as well as for reduction in the death during acclimatization and in the field. The best results in root initiation, maximum percentage of rooting, maximum number of roots/shoot and maximum root length were recorded on half-strength MS supplemented with 0.50 mg l^{-1} NAA (fig. 3b). Perusal of data presented in table 3 indicated that minimum days required for root initiation (7.33 ± 1.20), maximum rooting (100.00 ± 0.00%), highest number of roots per plant (12.63 ± 0.33) and maximum length of roots (10.67 ± 0.47) were recorded on half-strength MS medium supplemented with 0.5 mg l^{-1} NAA which was significantly ($p \leq 0.05$) followed by NAA 0.25 mg l^{-1} and NAA 1.0 mg l^{-1}. Since the rooted plants cannot be sub-cultured, well rooted plantlets after 20d on rooting medium were transferred on half-strength MS medium supplemented with 0.5 mg l^{-1} NAA in glass jars with polypropylene lids filled with peat + Soilrite® (1:1). This gave the best results in terms of maximum survival percentage, plant height, number of leaves per plant and minimum period for their transfer to greenhouse.

The plantlets moistened time to time with half-strength MS medium without any growth regulators, calcium, organics and sucrose. After 3 weeks of acclimatization, plants were ready to transfer in the greenhouse (fig. 3c). When flowering occurred in the plants which were transplanted under greenhouse, bring about true-to-type flowers (fig. 3d) which appeared in υM_1 generation and morphologically characterized by using DUS (Distinctness, Uniformity and Stability) test descriptors (data not shown).

Histological basis of shoot regeneration: The histological sections from the organogenic callus with intense stain gave detailed information about meristematic tissues formation (Fig. 5a). The organized group of cells in the organogenic callus exhibited formation of meristematic tip (Fig. 5b). Such organized activity of organogenic callus gave rise to initial stage of formation of shoot apical meristem after 25 d of culture (Fig. 5c). The organized cell division from such zones turned meristematic to produce distinct shoot bud primordia possessing apical meristem (AM) and leaf primordia (LP) which emerged to produce micro shoots (Fig. 5d). The micro shoots were visible after 30 to 40 d of inoculation of ray florets on different growth medium.

The histological study confirms that the meristematic tissues were formed from the induced calli. The meristematic tissue cell differentiated into different part for instance apical meristem, leaf primordia, shoot bud etc. Therefore, it can be concluded from the results that plants originate from single mutated cell and it would be a stable mutant.

Fig. 1: *In vitro* regeneration of Pink Incurving novel mutant. (a) Chrysanthemum cv. Thai Chen Queen (Parent); (b) Novel pink colour incurving mutant in υM_1 generation induced through gamma rays; (c) Ray florets of Pink Incurving mutant, taken as explant; (d) Wounded ray floret on the culture medium; (e) Callus induction and organogenesis from wounded parts of ray floret on the culture medium supplemented with 10 mg l–1 KIN and 0.1 mg l–1 NAA; (*f*) Stereomicroscopic view of the organogenic calli.

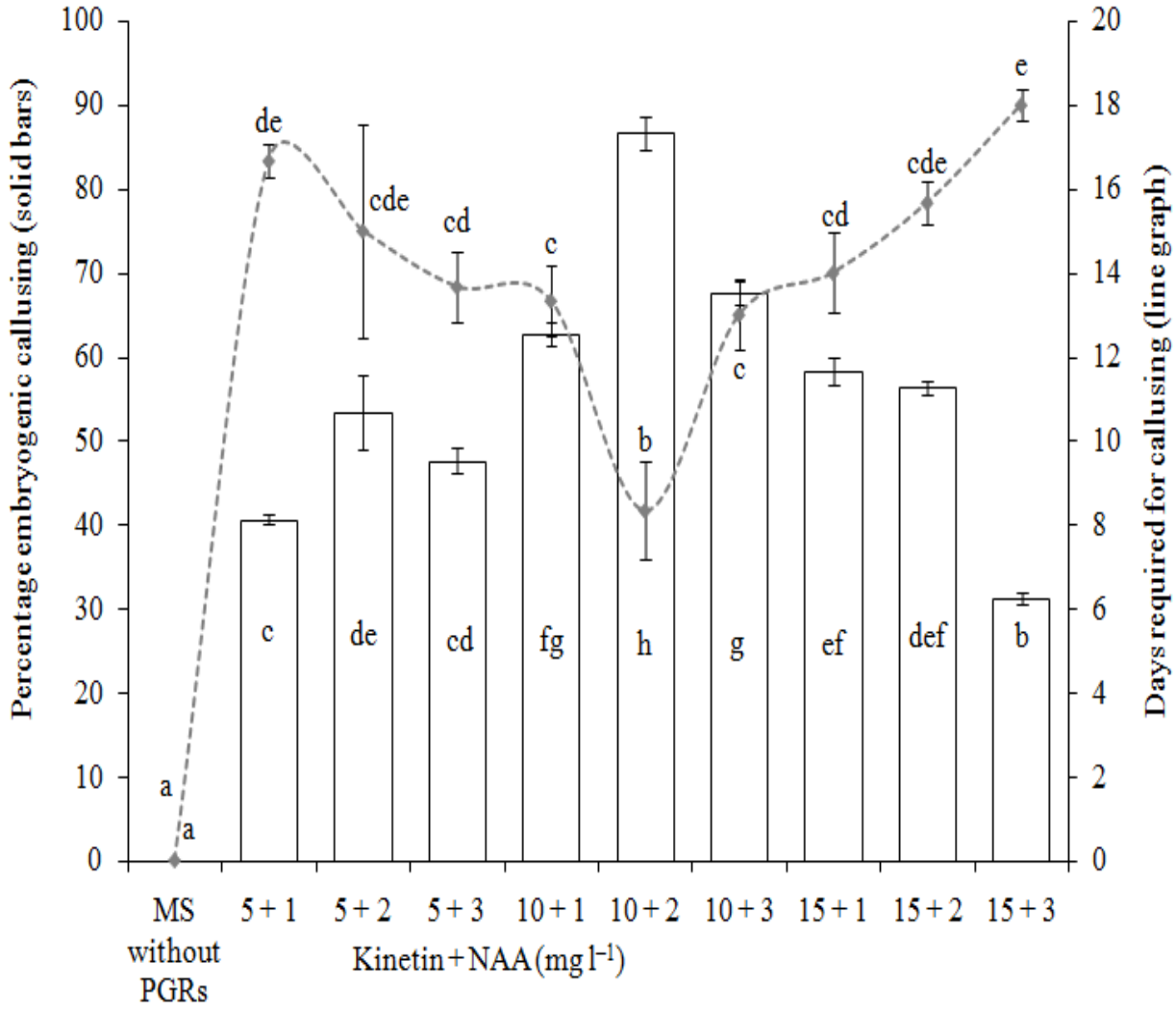

Fig. 2: Effect of different concentrations and combinations of PGRs in MS medium on callusing percentage and days taken for organogenic callus initiation from the ray florets of Pink Incurving novel mutant. Data are mean of three replicates with error bars representing the ± SE. Same letters on the line and bar graphs do not differ significantly when compared by Tukey's HSD test at 5% level of significance.

Table 1
Influence of KIN and NAA on shoot regeneration from chimeric ray florets of novel mutant of chrysanthemum. Each value was recorded between 3 to 7 weeks of culture.

KIN + NAA (mg l^{-1})	Days to shoot regeneration	Explants forming shoots (%)	Number of micro shoots per callus
0.0 + 0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
5.0 + 0.1	43.33 ± 1.86^{ef}	38.67 ± 1.86^{bc}	3.00 ± 0.58^{bcd}
5.0 + 0.5	40.67 ± 1.20^{de}	52.78 ± 1.47^{de}	4.67 ± 0.33^{de}
5.0 + 1.0	37.00 ± 1.15^{cd}	45.56 ± 0.29^{cd}	5.00 ± 0.58^{de}
10.0 + 0.1	30.00 ± 0.58^{b}	96.33 ± 1.95^{g}	8.33 ± 0.67^{f}
10.0 + 0.5	34.67 ± 0.88^{bc}	66.67 ± 1.67^{f}	5.33 ± 0.33^{e}
10.0 + 1.0	36.67 ± 1.33^{cd}	69.67 ± 0.88^{f}	4.33 ± 0.33^{cde}
15.0 + 0.1	43.33 ± 0.88^{ef}	55.33 ± 2.91^{e}	3.33 ± 0.33^{cde}
15.0 + 0.5	47.33 ± 0.88^{f}	45.00 ± 2.89^{cd}	2.33 ± 0.33^{bcd}
15.0 + 1.0	48.33 ± 0.88^{f}	33.33 ± 1.67^{b}	1.00 ± 0.58^{ab}

Data are mean of three replicates with ± SE. Same superscript letters in a column do not differ significantly when compared by Tukey's HSD test at 5% level of significance

Table 2
Effect of different concentrations of KIN and NAA on shoot proliferation and total number of micro shoots.

KIN + NAA + GA_3 (mg l^{-1})	Shoot proliferation (%)	Number of shoots per micro shoot
0.0 + 0.0 + 0.0	6.33 ± 0.88^{a}	0.67 ± 0.33^{a}
3.0 + 0.01 + 0.2	33.67 ± 0.88^{c}	3.67 ± 0.67^{abc}
3.0 + 0.05 + 0.2	59.67 ± 1.45^{d}	4.67 ± 0.33^{bc}
3.0 + 0.1 + 0.2	57.67 ± 1.45^{d}	6.67 ± 1.20^{bcd}
5.0 + 0.01 + 0.2	98.33 ± 0.88^{f}	9.33 ± 0.67^{d}
5.0 + 0.05 + 0.2	74.67 ± 1.45^{e}	6.67 ± 0.67^{bcd}
5.0 + 0.1 + 0.2	71.33 ± 1.33^{e}	7.00 ± 0.58^{cd}
7.5 + 0.01 + 0.2	39.33 ± 0.67^{c}	4.67 ± 0.88^{bc}
7.5 + 0.05 + 0.2	33.33 ± 1.67^{c}	3.33 ± 0.33^{ab}
7.5 + 0.1 + 0.2	13.33 ± 1.67^{b}	1.00 ± 0.58^{a}

Data are mean of three replicates with ± SE. Same superscript letters in a column do not differ significantly when compared by Tukey's HSD test at 5% level of significance

Table 3
Influence of different concentration of NAA on *in vitro* rooting.

NAA (mg l^{-1})	Days to root initiation	Rooting (%)	Average number of roots/shoot	Root length (cm)
0.0	24.67 ± 0.88^{c}	36.33 ± 0.88^{a}	2.37 ± 0.33^{a}	3.33 ± 0.58^{a}
0.25	15.33 ± 0.33^{b}	89.33 ± 2.91^{b}	5.63 ± 0.58^{b}	7.00 ± 0.58^{bc}
0.5	7.33 ± 1.20^{a}	100.00 ± 0.00^{c}	12.63 ± 0.33^{c}	10.67 ± 0.47^{c}
1.0	18.67 ± 1.45^{b}	94.00 ± 2.00^{bc}	7.43 ± 0.67^{b}	8.33 ± 1.11^{b}

Data are mean of three replicates with ± SE. Same superscript letters in a column do not differ significantly when compared by Tukey's HSD test at 5% level of significance

Fig. 3: *In vitro* proliferation, acclimatization and flowering of Pink Incurving novel mutant.
(a) Profuse shoot multiplication from the regenerated shoots; (b) Copious rooted plantlets which were cultured on half-strength MS medium supplemented with 0.5 mg l–1 NAA; (c) Acclimatized plantlets in glass jars containing peat and Soilrite® (1:1, v/v); (d) Solid Pink Incurving mutant type of flowers produced on plants in υM_2 generation under field conditions after 70 d of planting.

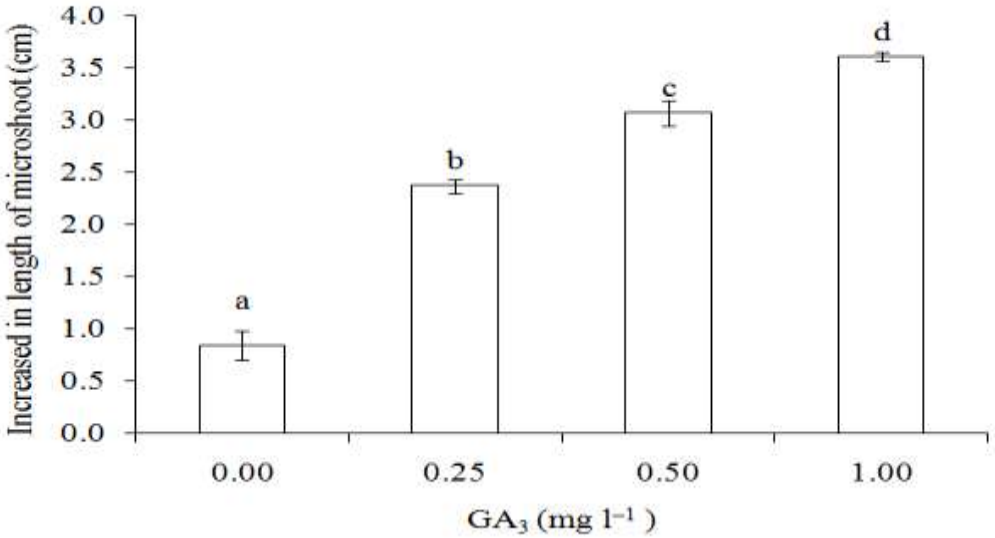

Fig. 4: Influence of different concentration of GA_3 on shoot elongation (expressed in increased length from the original length) of microshoots of Pink Incurving novel mutant. Data are mean of three replicates with error bars representing the ± SE. Different letters on the bar graphs differed significantly when compared by Tukey's HSD test at 5% level of significance.

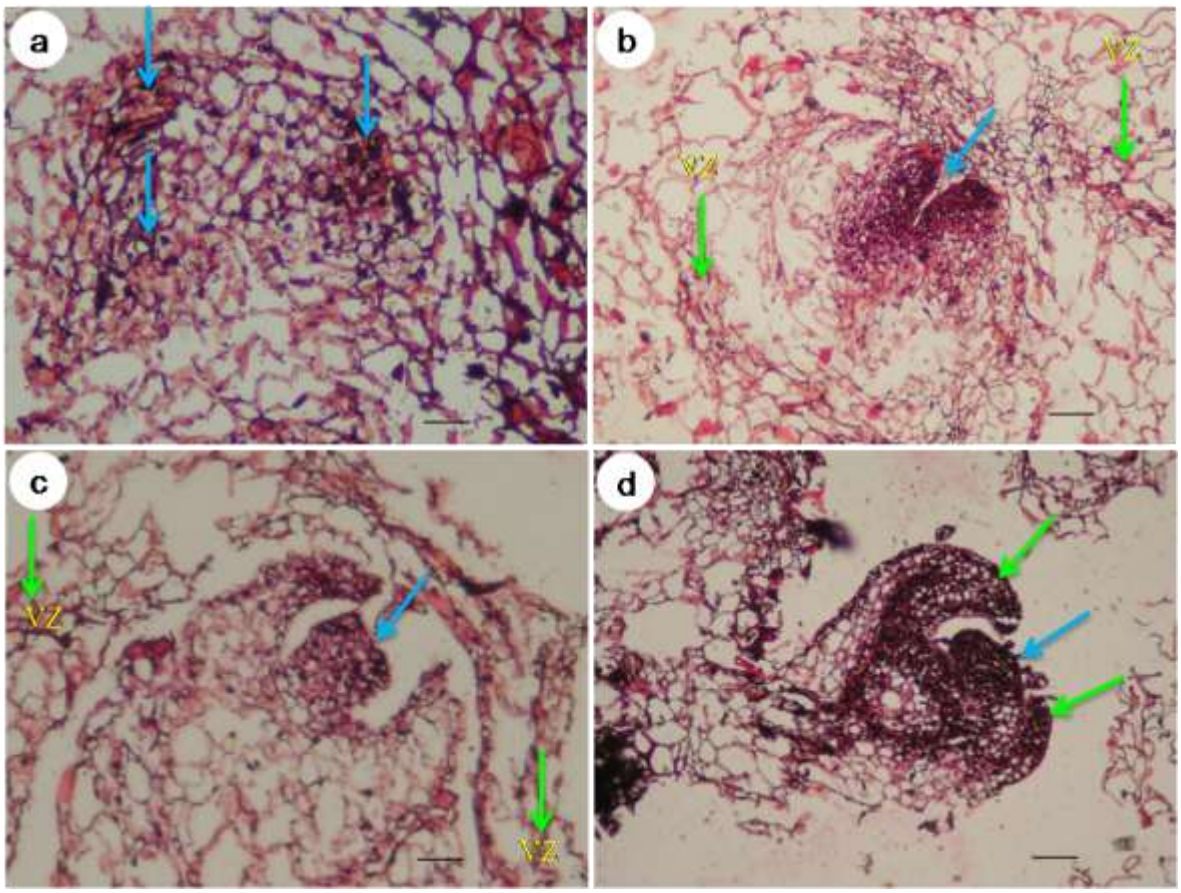

Fig. 5: Histological sections of organogenic callus from ray floret of chrysanthemum novel mutant on MS medium supplemented with 10 mg l^{-1} KIN and 0.1 mg l^{-1} NAA. (a) Blue arrows shows the multiple shoot regeneration from the organogenic callus; (b) Organogenic callus showing the formation of meristematic tip at very early stage pointed out by blue arrow, and vascular zone (VZ) by green arrows after 20 d of culture; (c) Formation of shoot apical meristem surrounded by vascular zone (AM indicated by blue arrow and VZ by green arrows respectively) at initial developmental stage; (d) Blue arrow shows the completely formed apical meristem (AM) and green arrows show completely formed leaf primordia (LP). *Magnification bars* = 500 µm.

Discussion

Undifferentiated tissue production is caused by changes in the level of cytokinins induced as a result of increase in the endogenous auxin level[11]. This may be due to the direct exposure of wounded tissues to the culture medium containing high concentration of KIN in combination with NAA, which resulted in rapid cell division and callogenesis. Callus induced by wounding in ray floret was also described by several workers in chrysanthemum. Organogenic callus formation was observed on chrysanthemum leaves when the medium was supplemented with either KIN and BAP at 1 or 2 mg l^{-1} with 1 or 2 mg l^{-1} 2,4-D[25]. High frequency of callus induction on leaves (100%) and stem (63%) of chrysanthemum was noticed by using low concentration of BA (0.5 mg l^{-1}) and NAA (1.5 mg l^{-1})[34]. The frequency of callus initiation from the leaves explants of chrysanthemum was 100% when medium was supplemented with either BAP 4.44 µM and IAA 57.08 µM or BAP 4.4 µM and NAA 5.37 µM[26]. The favourable response of chrysanthemum ray florets for higher levels of KIN and NAA could be due to the varietal differences.[8]

For the formation of organogenic calli low concentration of BA and high concentration of NAA were found most effective[34]. KIN and NAA in combination in the culture medium resulted in the highest callus initiation with good morphogenic response from the nodal and ray floret explants in chrysanthemum[19]. Therefore, it is evident that appropriate auxin and cytokinin concentrations are essential for callus initiation in each species and variety of chrysanthemum[23]. The auxins alone induce callus production while auxins and cytokinins are responsible for differentiation of callus into organized cells.[31]

Cytokinins, in combination with auxins, have been reported to induce the regeneration from leaf explants in chrysanthemum[16]. These results suggest that a high level of endogenous auxins may be present which prevent shoot regeneration while culturing nodal explants of chrysanthemum[32]. It was concluded that for the successful regeneration, a combination of auxin and cytokinin was essential since the individual application failed to produce shoots[14]. The highest number of regenerated shoots (45.0%) from callus initiated ray florets was obtained by supplementations of 9.3 µM kinetin and 4.9 µM IBA on MS medium[28]. Other research reports indicated that neither auxin nor cytokinins alone are effective in inducing direct shoot organogenesis from ray florets in chrysanthemum during the study of the effects of growth regulators on direct shoot organogenesis from mutated ray florets[8].

The earlier reports indicated that best multiplication was observed in chrysanthemum by using combinations of BA and NAA from epicotyl and hypocotyl explants[17], ray florets[21] and nodal segments[32]. Cytokinins are usually added into the tissue culture media to the stimulate proliferation[6] but the physiological disorder such as short and compact shoots, fasciations and rosettes, shoot tip necrosis, vitrification etc.[1] were prevalent in the shoot proliferating cultures when these were continuously cultured on the KIN rich medium that leads to death of micro shoot in subsequent sub-culture. Therefore, to improve the *in vitro* growth of cultures, GA_3 was added in the proliferation medium.

The micro shoots may be rooted with or without an intermediate shoot elongation phase to reduce the culture cycle[23,27]. However, elongation of micro shoots on elongation medium makes them ready for induction of *in vitro* adventitious rooting. MS medium, supplemented with 0.2 mg l^{-1} GA_3 had the greatest effect on elongation while 0.05 mg l^{-1} GA_3 with 0.2 mg l^{-1} BA showed minimal effect on elongation of begonia plantlets[24]. They concluded that BA in elongation medium was limiting factor in adventitious shoot elongation but the contrasting result was also reported in chrysanthemum[21] who found that MS medium supplemented with BAP (1.0 mg l^{-1}) and GA_3 (0.5 mg l^{-1}) resulted in desirable shoot elongation.

It is well known that BA reduces the height of the shoots while GA_3 induces the internodes elongation.[18] Other report indicated that higher elongation could be achieved by growing adventitious shoots of chrysanthemum on hormone free MS basal medium supplemented by 3% sucrose. The excellent growth of the micro shoots made them elongated with thick, sturdy and strong stem and well developed dark green expended leaves GA_3. However, our results also show that GA_3 was pre-requisite for the elongation as well as for healthy micro shoots of chrysanthemum.

Root initiation and root elongation in chrysanthemum were also reported by several workers using different concentrations of GA_3, cytokinines and auxins[5,7,21,32]. The successful root initiation of elongated shoots depends on number of factors such as strength of basal medium, sucrose level and concentration of auxins etc[23]. It is well known that rooting process in certain plant species may proceed best when the overall salt strength of the medium is reduced. In some cases, reduction in the salt strength may eliminate the need for auxins for rooting. Furthermore, the application of NAA may accelerate the rooting process by increasing cell division at the wounded end of the micro shoots resulting in rapid rooting with excellent root quality in a very short time. The acclimatization of the plantlets of chrysanthemum after 3 weeks of hardening was reported by several workers[19,22,27].

The direct shoot organogenesis was also confirmed through the histological studies from tissues of chimeric petals[4], leaves and stems[34] in chrysanthemum. Histological investigation revealed that morphogenic cells are induced within 30 d of culture of explants on medium containing KIN and NAA. Adventitious shoots were visible after 60–70 days of culture on the induction medium from the leaf explants in *Primulina tabacum*[35]. Direct organogenesis from the leaf and petiole of *Solanum aculeatissimum* was also recoded[10]. Regenerating shoots were dome-shaped and surrounded by leafy primordia, which were well connected

to vascular tissues of the explant. When adventitious shoots or somatic embryos are cultured *in vitro*, regeneration takes place from the L1 or L2 layer of the cell. The plant regenerated from the L1 or L2 layer produces genetically stable plants.[4,5]

Conclusion

From the present study, it can be inferred that the combinations of KIN and NAA had a favourable influence on the formation of meristematic zones in the callus mass which ultimately produced shoot primordia followed by micro shoots, which gave rise to stable solid mutant plant. The regeneration protocol standardized in this experiment would be helpful in isolation of solid mutants which are not possible to isolate employing other methods. Besides this, the methodology is also useful for the *in vitro* regeneration of novel mutants for which protocol is not available. The histological study may provide a base for the histological study of other plants.

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