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Regeneration of Chrysanthemum (Chrysanthemum morifolium) via somatic embryogenesis and screening of clones for agronomic traits

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Abstract

Chrysanthemum morifolium propagation using conventional method of stem cutting produces weak plants and showed delayed anthesis above 20°C with reduced flower diameter. Therefore, in the present study chrysanthemum plantlets were produced through somatic embryogenic calli to exploit the somaclonal variation for its improvement. Various explants of variety Dante yellow were cultured on LS (Linsmaier and Skoog 1965) medium augmented with various concentrations of KT (Kinetin) and 2,4-D (2,4-dichlorophenoxyacetic acid) and their combinations for callus induction. Embryogenic calli were proliferated and regenerated by using different plant growth regulators. Regenerated plantlets were acclimatized and evaluated for agronomic characteristics and compared with mother plant in a replicated field trial. Results revealed that young leaf explant cultured on LS medium containing 9.02 µM 2,4-D and 11.61 µM KT gave an ample amount of callus. Combination of 0.44 µM BAP (6-benzylaminopurine) and 5.37 µM NAA (1-naphthaleneacetic acid) yielded highest amount of callus proliferation (0.27g ± 0.03). Significant amount of shootlets (25 ± 0.8) from embryogenic callus was observed on the medium augmented with 0.45 µM 2, 4-D. During the field experiment, Clone *S84* showed considerable improvement in flower size as compare to mother plant and found to be a promising clone for commercialization.

Introduction

Chrysanthemum morifolium is an important floriculture crop, belongs to the family Asteraceae (Miler and Jendrzejczyk 2018). Low amount production of ethylene during its growth phase, prolongs its shelf life and makes it suitable for cut flower production (Zafarullah et al. 2013). Most of the countries are earning foreign exchange by exporting this aesthetic beauty (Chica Toro and Garzón-González 2018; Roh et al. 2019). Pakistan can also have great export potential by setting its own ornamental plants industry. (Anjum et al. 2007). However, poor number of efforts have been made regarding the cultivar improvement and production.

Conventional stem cuttings method is being used for the propagation of *C. morifolium* plants in Pakistan. This conventional method produces low-quality plants that are prone to carry infections which are already present in the mother plant (Mehedi et al. 2020). These plants are also slow growing and not able to fulfil the increasing demand of the floriculture industry (Panicker et al. 2016). Rapid initiation of flowering in *C. morifolium* requires low temperature (10–20°C) for 3–4 weeks, above this defined range anthesis is delayed (Cho and Kim 2020). Some exotic varieties of *C. morifolium* also fail to adapt under hot weather conditions of Pakistan.

C. morifolium is a top commercial target for tissue culture because of its demand and to overcome the problems of conventional propagation methods (Hesami 2020). Production of *in vitro* plantlets through somatic embryogenesis offers a rapid and large-scale regeneration system (Natarajan et al. 2020). Moreover, somatic embryo may induce genetic and/or epigenetic changes leads to somaclonal variation that would be exploited for improvement of ornamental plant (Bednarek and Orłowska 2020). Such type

of somaclonal variation has been reported in *Gentiana decumbens* (Tomiczak et al. 2015), *Lilium distichum* and *Lilium cernuum* (Fu et al. 2019). Previously, somatic embryogenesis in *C. morifolium* has been successfully induced from various explants such as young leaf, stem and ligulate florets with the help of different plant growth regulators (PGRs) in different concentration and combinations (Xu et al. 2012; Tymoszuk et al. 2014; Ghosh et al. 2018). However, somaclonal variation has not been reported in *in vitro* regenerated plantlets of *C. morifolium*. To the best of our knowledge this is the first report of somaclonal variation in *C. morifolium* without applying any exogenous mutagenic agent.

Furthermore, in present study, the protocol for regeneration of *C. morifolium* from embryogenic callus is easily reproducible in short period of time. For this purpose, the effects of different PGRs were investigated for the induction of embryogenic callus, regeneration and shoot elongation from various explants of *C. morifolium.* Additionally, agronomical important parameters of regenerated plants were also studied to explore the positive variation among somaclones. It is expected that this study will help for future studies regarding *in vitro* multiplication, and establishment of floricultural industry in Pakistan (Datta 2019).

Materials And Methods

Explant used, Embryogenic callus Induction and proliferation

Dante yellow, an exotic variety of ornamental *C. morifolium*, was used as source of explant. Explants were surface sterilized with liquid detergent under running tap water for 5–7 min, then 70% (v/v) of ethanol for 1 min in laminar flow cabinet. Finally, sterilized with 20% (v/v) commercial bleach with 2 drops of tween20 detergent for 5–7 min with gentle stirring. The explant was washed four times with sterile distilled water.

For callus induction of *C. morifolium*, the sterilized explants including young leaves, expanded leaves, roots, nodes and internodes were cultured on LS medium augmented with various concentrations of KT (2.32, 4.64, 6.96, 9.29, 11.61, 13.93, 16.26 and 18.58 µM) and 2,4-D (2.26, 4.52, 6.78, 9.04, 11.31, 13.57, 15.83 and 18.09 µM) alone and in different combinations (2.32 + 9.04, 4.64 + 9.04, 6.96 + 9.04, 9.29 + 9.04, 11.61 + 9.04, 13.93 + 9.04 µM). The formulated media were then supplemented with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar. The pH was adjusted 5.7–5.8 prior to autoclaving at 121°C for 15 min, and cultures were maintained at 24 ± 2°C in dark and 70% – 80% relative humidity.

Induced callus after 2 weeks was then proliferated by using three different auxins

[NAA, Indole-3-butyric acid (IBA) and 2,4-D] and a cytokinin (BAP) each with three different concentrations (Table 1). BAP and NAA were also used in three combinations (0.44 + 5.37, 1.33 + 5.37, 2.219 + 5.37 µM). Cultures were maintained at 24 ± 2°C in dark. The increase in callus fresh weight was recorded after 2 weeks.

Table 1
PGRs concentrations and combination used for the proliferation of embryogenic callus

PGRs	Concentrations and Combinations
Control	LS0
IBA	0.49 µM, 1.47 µM, 2.46 µM
2,4-D	0.45 µM, 1.35 µM, 2.26 µM
BAP	0.44 µM, 1.33 µM, 2.219 µM
BAP + NAA	0.44 µM + 5.37 µM, 1.33 µM + 5.37 µM, 2.219 µM + 5.37 µM

Regeneration of embryogenic callus

Embryogenic callus (0.1 g) was selected according to callus morphology and cultured for regeneration on same media formulations used for callus proliferation (Table 1) at 24 ± 2°C under LED lights (16/8 h photoperiod, 108 µmol m^{-2} s^{-1} light intensity). Frequency of regeneration on LS media augmented with different PGRs was recorded after 4 weeks. Regenerated plantlets were then transferred onto different medium formulations for further development and rooting (Table 2). Cultures were maintained at 24 ± 2°C in dark. Result was recorded after 6 weeks.

Table 2
PGRs concentrations and combination used for the further development and rooting of regenerated plantlets

PGRs	Concentrations and Combinations
Control	LS0
BAP	0.44 µM, 1.33 µM and 2.219 µM
IBA	0.49 µM, 1.47 µM and 2.46 µM
2,4-D	0.45 µM, 1.35 µM and 2.26 µM
IAA	0.57 µM, 1.71 µM, 2.85 µM
BAP + NAA	2.219 µM + 0.53 µM, 4.439 µM + 0.53 µM, 6.659 µM + 0.53 µM & 8.879 µM + 0.53 µM
BAP + NAA + GA3	2.219 µM + 0.53 µM + 0.288 µM, 4.439 µM + 0.53 µM + 0.288 µM, 6.659 + 0.53 µM + 0.288 µM & 8.879 + 0.53 µM + 0.288 µM
BAP + 2,4-D	2.219 µM + 0.45 µM, 4.439 µM + 0.45 µM, 6.659 µM + 0.45 µM & 8.879 µM + 0.45 µM

Three different gelling agents [0.8% (w/v) agar, 0.2% (w/v) gelrite and 0.25% (w/v) phytagel] were also examined for the direct multiplication of regenerated plantlets by using medium for shoot elongation. *In vitro* cultured shoot tips of 2 to 2.5cm in length were used for direct shoot multiplication. Result was recorded after 6 weeks.

Acclimatization of plants

Regenerated plants with well-developed shoots and roots were transferred in three different potting mixtures [T-1: Cocopeat + weekly spray of ½ strength Hoagland's solution, T-2: Garden soil + weekly spray of ½ strength Hoagland's solution, T-3: Cow manure + garden soil (1:3)] to optimize high survival rate of plants. Plantlets were taken out of the vessels one hour before planting after removing dead leaves, they were washed with running tap water to remove agar from their root zone. For T-1, polythene bags with drainage holes were used to give roots more space to expand. Whereas, for T-2 and T-3 germinating trays were used. Garden soil, cocopeat and cow manure were autoclaved to make media safe and contamination-free for tissue cultured plantlets. The experiment was performed under plastic covers to maintain a high amount of moisture. Plantlets were placed under growth room conditions 24 ± 2°C with (16/8 h photoperiod, 108 µmol m^{-2} s^{-1} light intensity). Plantlets were transferred to the nursery after 4 weeks.

Preparation of replicates and screening of somaclones

120 acclimatized (R) plantlets were transferred to the nursery. Plantlets were pinched at two stages; first pinching was performed after 4 weeks and the second pinching was performed after 7 weeks for making more branches for replications as described by Bala (2015). After 11 weeks three replicates were taken from each plantlet and dipped in powdered IBA from the lower end and transferred in germinating trays, placed in the nursery where a regular supply of Hoagland's solution was provided. After 2–3 weeks, three replicated plantlets (R1) with well-developed roots were ready for replicated yield trial by using Randomized Complete Block Design (RCBD). Planting was done in late December and data was collected in late March. Initially, for one week only water was provided then 1g/L of NPK solution was sprayed every week till 6 weeks, then the nursery was covered with a black plastic sheet for flower induction.

In green house three replicated blocks each of 1 m^2 were made. Blocks were distanced from each other by ½ m and each block contain total 120 cloned plants and 10 field-grown plants (control). Plant to plant distance was 2.5 inches and row to row distance was 5 inches. Different important parameters [Stem thickness (mm), Plant height (cm), Flower color, Flower diameter (inches) and Suckers per produced clone] of 3 replicated clones were recorded after approximately 3.5 months of growth period.

Statistical analysis

In present study, callus proliferation, shoot regeneration on different treatments and replicated field trial were evaluated through One-Way Analysis of variance (One-Way ANOVA) and Duncan multiple range test (DMRT) by using SPSS version 16 at $p < 0.05$. For frequency distribution of somaclones histograms were made by using SPSS version 16. Heat-map was constructed through TBtools-master using euclidean distance matrix and average linking.

Results And Discussion

Callus induction

All explants cultured on LS medium augmented with combinations of KT and 2,4-D (9.02 µM of 2,4-D and 11.61 µM of KT) produced callus. However, ample amount of callus was produced from young leaf explant and entire explant turned into callus within 2 weeks. Other explants showed callus only on margins. A heat-map was made by using simultaneous clustering of all varying concentrations and combinations of callus inducing PGRs (Fig. 1). Heat-map shows two broad groups of PGRs (concentrations/combinations) in which group having 4.52 µM 2,4-D alone and combination of 9.02 µM of 2,4-D with 11.61 µM of KT represent most suitable media compositions for callus induction. Cluster of second group is further divided into two sub-groups; first group represents PGR concentrations producing low amount of callus and second sub-group containing PGR concentrations responded poorly for callus induction.

Further microscopic investigations revealed that the callus was embryogenic in nature (Fig. 2A). These observations were contrary with the previous results by Shinoyamo et al. (2004) and Tymoszuk et al. (2014) where, combination of KT and 2,4-D, containing a low concentration of 2,4-D and high concentration of KT were most efficient for inducing embryogenic callus on young leaf explant. In another studies Naing et al. (2013) reported the use of equal quantity of KT with 2,4-D as the best combination for inducing embryogenic callus on leaf explant in 37 days. It was also noticeable in the present study that substantial amount of embryogenic calli were produced using comparatively lower amount of PGRs as reported in Tymoszuk et al. (2014), where 4mg L^{-1} 2,4-D was used along with KT using ligulate florets of *Chrysanthemum grandiflorum*.

The present study revealed a significant amount of callus induction with lower concentrations (2.26, 4.52, 6.78 and 9.04 µM) of 2,4-D alone and from all the lower concentrations of 2,4-D, 4.52 µM gave a relatively higher amount of callus. Whereas, LS medium augmented with high concentrations of 2,4-D (11.31, 13.57, 15.83 and 18.09 µM) induced roots with a significantly lesser amount of callus on the margins of explant. The callus was compact and pale in appearance although non-granulated. Moreover, experiments on the regeneration of such type of callus revealed that it was non-regenerable in nature.

In present study it was observed that LS medium augmented with eight varying concentrations of KT (2.32, 4.64, 6.96, 9.29, 11.61, 13.93, 16.26 and 18.58 µM) did not produce any type of undifferentiated mass of cell or callus with all explants used. This result was also supported by Naing et al. (2013). He reported that somatic embryos (SE) were formed by the addition of cytokinins such as Thidiazuron (TDZ), KT, BA, along 2,4-D. However, cytokinin alone is not useful for embryogenic callus induction.

Callus Morphology

Morphologically three diverse kinds of calli were produced by the combination of 9.02 µM of 2,4-D and 11.61 µM of KT. Young leaf explant, showed the formation of granulated, yellowish-pale coloured callus (Fig. 2B). Further experiment showed that callus produced only from young leaf explant found

regenerable plus showed the increased potential of somatic embryogenesis. Whereas, on fully expanded leaves whitish-pale coloured callus (Fig. 2C) was observed which was present only on the margins and was insufficient in quantity. The third type of callus was mucilaginous callus (Fig. 2D) which was translucent, shiny and soggy in appearance and produced randomly between above-defined calli. This is the first report of mucilaginous callus on *C. morifolium* explant. Previously, it was observed in research work on sugarcane varieties as non-embryogenic, soft and non-regenerable in nature (Uwatoko et al. 2011; Alcantara et al. 2014).

Developmental stages of somatic embryos:

In the present research, 4X magnification of a dissecting microscope and 100X magnification of a compound microscope unveiled five varying development sequence of a somatic embryo (SE). The first structure formed was longitudinal in appearance and termed as pro-embryonic mass (Fig. 2E), was subsequently converted into a globular structure (Fig. 2F). After some time, the globular structure started to transform into a heart shaped structure (Fig. 2G), which was bilaterally symmetrical in appearance. Heart shaped structure was also observed under compound microscope (Fig. 2H). Torpedo embryo was formed as the fourth stage (Fig. 2I). The fifth and final stage observed was a cotyledonal structure (Fig. 2J), which later gave rise to the primary shoot development.

Proliferation of Embryogenic callus

Embryogenic callus was proliferated best on two different formulations, 0.45 µM of 2,4-D (0.27g ± 0.01) (Table 3) and combination of 0.44 µM BAP with 5.37 µM NAA (0.27g ± 0.03 increase fresh weight of callus) (Fig. 3A) compared with other treatments. However, in 0.45 µM of 2,4-D callus started rooting. Thus, result here showed 0.45 µM of 2,4-D as less suitable PGR for callus proliferation. An important finding of this study was the 170% increase of embryogenic callus within 2 weeks only. To the best of our knowledge, it is first time being reported that embryogenic callus of *C. morifolium* was only multiplied when cultured in the form of (0.1 g) cluster onto LS medium, single embryo when cultured on medium turned brown within a few days. This is first report of cluster size having an effect on the multiplication of embryogenic callus of *C. morifolium*. Subculturing of callus was done almost five to six times with 2 weeks.

Table 3
Effect of different concentrations of PGRs on proliferation of callus produced from young leaves after 2 weeks.

Callus FW (g)				
Treatments(mg/L)				Mean & Standard error
BAP	NAA	2,4-D	IBA	
-	-	-	-	$0.16^{ab} \pm 0.01$
0.1	1	-	-	$0.27^{a \pm} 0.03$
0.3	1	-	-	$0.23^{a \pm} 0.02$
0.5	1	-	-	$0.16^{bc} \pm 0.04$
-	-	0.1	-	$0.27^{a \pm} 0.01$
-	-	0.3	-	$0.21^{abc} \pm 0.01$
-	-	0.5	-	$0.14^{c \pm} 0.02$
0.1	-	-	-	$0.18^{bc} \pm 0.01$
0.3	-	-	-	$0.15^{bc} \pm 0.03$
0.5	-	-	-	$0.17^{bc} \pm 0.02$
-	-	-	0.1	$0.17^{bc} \pm 0.04$
-	-	-	0.3	$0.16^{bc} \pm 0.02$
-	-	-	0.5	$0.22^{abc} \pm 0.02$
NOTE. Values with same letter(s) are non-significant at α = 0.05 for using Duncan's multiple range test (DMRT) level				

Conversion of embryogenic callus into plantlets

There are several studies regarding the conversion of embryogenic callus of *C. morifolium* into plantlets on the medium without PGRs (May and Trigiano 1991; Tanaka et al. 2000; Shinoyama et al. 2004; Ilahi et al. 2007; Naing et al. 2013). However, in the present study media without PGRs showed a low frequency of embryogenic callus conversion into plantlets. Thus, the effect of different PGRs was investigated for callus regeneration. In this study, a lower concentration of 2,4-D (0.45 µM) yielded a significant amount of embryogenic callus conversion into plantlets (25 ± 0.8) within 4 weeks (Fig. 3B; Table 4). However, this conflicts with the results of Lema-Rumińska and Niedojadło (2014), who reported that cytokinin as crucial PGR for the regeneration of somatic embryos of chrysanthemum into plantlets.

Table 4
Effect of different concentrations of PGRs on regeneration from callus after 4 weeks.

Number of regenerated shootlets				
Treatments(mg/L)				Mean & Standard error
BAP	NAA	2,4-D	IBA	
-	-	-	-	$7.3^{ef} \pm 1.76$
0.1	1	-	-	$5.3^{fgh} \pm 1.7$
0.3	1	-	-	-
0.5	1	-	-	-
-	-	0.1	-	$25^{a} \pm 0.8$
-	-	0.3	-	$7.3^{ef} \pm 1.45$
-	-	0.5	-	$9.7^{bcde} \pm 2.9$
0.1	-	-	-	$6.3^{fgh} \pm 2.3$
0.3	-	-	-	$3^{gh} \pm 0.57$
0.5	-	-	-	$2.7^{gh} \pm 0.33$
-	-	-	0.1	$9.7^{bcde} \pm 2.9$
-	-	-	0.3	$7^{ef} \pm 1.7$
-	-	-	0.5	$15.7^{bc} \pm 2.02$
-	0.1	-	-	$19^{b} \pm 4.04$
-	0.3	-	-	$13.3^{bcd} \pm 3.28$
-	0.5	-	-	$4.7^{fgh} \pm 1.33$
NOTE. Values with same letter(s) are non-significant at α = 0.05 for using Duncan's multiple range test (DMRT) level				

Moreover, callus was converted into plantlets only when cultured in the form of clusters (0.1 g). Small plantlets when cultured on different concentrations and combinations of PGRs, only lower concentration of IBA (0.49 µM) showed 3.5 folds increase in shoot length along with the induction of roots after 6 weeks (Fig. 3C). Whereas, in other formulations, plantlets started to turn into callus again from the basal region within 4 weeks. Roots were also developed on the same elongation medium augmented with 0.49 µM IBA.

Study on the effect of three different gelling agents (agar, gelrite and phytogel) on direct multiplication revealed that LS medium augmented with 0.49 µM of IBA and solidified with 0.2% (w/v) of gelrite showed highest mean number of shoot proliferation as compare to the other two treatments (Fig. 3D; Table 5). While LS Medium supplemented with 0.49 µM of IBA and solidified with 0.25% (w/v) phytagel (Fig. 3E) and 0.8% (w/v) agar (Fig. 3F) showed increase in shoot multiplication respectively. Lim et al. (2012) reported same results of gelrite superiority over agar for inducing highest number of shoot proliferation in chrysanthemum. Number of shoot proliferation on different gelling agents also varies from species to species (Ivanova and Van Staden 2011). However, further studies regarding the composition of gelling agents can give the clear idea about their role in shoot proliferation. In present study it was also observed that the plantlets developed on medium augmented with gelrite showed brighter green color as compare to the plantlets developed on medium solidified with phytagel or agar. On medium augmented with phytagel or agar plantlets showed darker green color. This difference in color may be the result of high amount of water availability in medium containing gelrite as compare to other medium (Shrivastava and Rajani 1999). However, the average shoot length was very short and lowest mean number of leaves were recorded on medium augmented with gelrite (2.8 ± 0.2cm and 7.4 ± 0.7cm respectively) and there was no root formation. The reason behind this may be the high hyperhydricity present in the medium augmented with gelrite. Previously, it was reported that structure of gelrite highly favoured rapid hyperhydricity in various species by allowing increased absorption of H2O, NH4 and PGR (Ivanova and Van Staden 2011). Highest mean shoot length and number of leaves were recorded in treatment of phytogel with 0.49 µM IBA that was (8.2 ± 0.8cm) and (12.9 ± 0.9) respectively. On agar mean number of shoot length was (5.3 ± 0.7cm) and mean number of leaves was (9.3 ± 1.3). It was reported that agar contains different contaminants as compare to which phytogel is free from phenolic compound (Ramesh and Ramassamy 2014). This may be one of the reasons in the present study that phytogel showed enhanced growth parameters than agar.

Acclimatization of plantlets and survival percentage

The present study showed that out of three different potting mixtures, sterilized cocopeat sprayed with ½ strength of Hoagland's solution weekly (T-1) is the best combination for acclimatization as it gave 100% of survival rate of plantlets. Moreover, plantlets displayed strength, and new leaf also emerged within 4 weeks (Fig. 3G). Contrary to that medium based on sterilized garden soil sprayed weekly with ½ strength of Hoagland's solution gave only 60% survival of plantlets with weak stem development that needs extra support of wooden sticks. Additionally, there was no sign of new leaf emergence even after 4 weeks (Fig. 3H). Garden soil proved to be a compact medium and showed dryness as compared to the other two treatments. Earlier, soil and sand were reported as least desirable media for the acclimatization of *in vitro* developed plantlets of ornamental plants such as *gloxinia*, *saintpaulia* and *Gerbera jamesonii* (Kashyap and Dhiman 2011; Singh et al. 2017). Mixture of cow manure with garden soil (1:3) (T-3) showed only 40% of plant survival and proved to be least suitable for the acclimatization of plantlets. A schematic illustration of the whole protocol developed for *in vitro* multiplication and acclimatization of chrysanthemum cv. Dante yellow is present on Fig. 4.

Screening of somaclones

Among the agronomic characteristics observed during this study stem thickness (mm), plant height (cm), flower color, flower diameter (inches) and suckers produced per clone among somaclones in replicated field trial, it was noticed that substantial variation was observed in flower diameter, flower color and suckers produced per clone. Screening of all somaclones showed that 15 clones have significantly increased in flower diameter as compare to mother plant at $p < 0.05$. However, six clones showed substantial decrease in the flower diameter (Table 6).

Frequency distribution of somaclones for flower diameter was positively skewed with mesokurtosis (Fig. 6A). The values of mean, median and mode of regenerated flower diameter were (0.91, 1 and 1 inches) respectively which were significantly higher than the mean value of flower diameter of field-grown plants (0.83 ± 0.06 inches). This result showed a positive increase in the flower diameter of cloned plants as compared to their field-grown plant.

Flower diameter is a very important parameter for determining the quality of flowers (Singh et al., 2019). In present study, regenerated Clone S84 showed highest (45%) increase in diameter of flower with 1.5 ± 0.5 inches as compare to the mother plant which was 0.83 ± 0.24 inches. Whereas, Clones S18, S19, S21, S23, S26, S29, S30, S35, S41, S44, S59, S66, S67 and S69 were also have slight increase in flower diameter. However, clones S4, S11, S14, S17 and S20 had significant decrease in flower diameter.

Colour of a flower is the primary feature with the highest appeal for consumers, even higher than flower scent (Nasri et al., 2021). In this study, four clones showed phenotypic variation in flower colour as compare to mother plant (Fig. 5A). In clone S4 the petals were devoid of red pigmentation and showed only yellow colour inflorescence (Fig. 5B). In contrast, a clone named S68 showed red inflorescence only (Fig. 5C). However, both showed a significant decrease in the mean diameter of the flower as compared to the mean diameter of the field-grown plants (0.83 ± 0.06 inches). Clone **S4** showed **37.5%** decrease (0.5 ± 00 inches) and clone S68 showed 40% decrease (0.48 ± 0.02 inches) at $p < 0.05$. It was also observed that clone S42 showed less red portion in the centre as compared to the field-grown plant (Fig. 5D). Clone S55 showed some interestingly abnormal petals with flat semi-full inflorescence (Fig. 5E). It showed a 19.27% significant decrease in flower diameter (0.67 ± 0.28 inches) as compared to the flower diameter of field-grown plant (0.83 ± 0.06 inches). Similar to the present result, flat semi-full inflorescence was also observed by Miler and Kulus, 2018. However, in their study chrysanthemum ‘Alchimist’ was first treated with microwaves.

In the present study, frequency distribution for suckers produced per somaclone was positively skewed with leptokurtosis (Fig. 6B). The values of mean, median and mode for the number of suckers produced per somaclone were (4.57, 4, and 4) respectively. All these values were greater than the mean value of suckers produced per field-grown plant (3.57 ± 0.2), which indicates that somaclones showed a positive increase in the production of suckers per plant as compared to field-grown plant. This result representing an increase in multiplication rate of cloned plants.

Frequency distribution for the diameter of the stem was also positively skewed with platykurtosis (Fig. 6C), and the mean value of the stem diameter of field-grown plants was (3.69 ± 0.1mm) identical to

the mean value of the stem diameter of cloned plants (3.7mm). However, the value of mode for cloned plants (4mm) represented that a large number of clones were with improved stem diameter. However, statistical analysis of variance and DMRT showed the variation among somaclones and with that mother plant and non-significant at $p < 0.05$.

Frequency distribution for the height of cloned plants was the only parameter which showed no change (Fig. 6D) as the mean value of field-grown plants was (21.2 ± 1.7cm) identical to the mean value of cloned plants (21.44cm). Most of the parameters represent a positive increase as compared to their field-grown plant, which indicates that plantlets cloned *via* induction of somatic embryogenesis resulted in crop improvement.

A noteworthy result of this study was the easy protocol for plant regeneration *via* embryogenic callus induction. Further experiment for field trial concluded that cloned plantlets showed positive somaclonal variations. Clone S84 showed positive variation in flower diameter as compared to the field-grown plants and is worth pursuing further for direct shoot multiplication as it holds the potential of surviving in local environmental conditions of Pakistan.

Abbreviations

LS: Linsmaier and Skoog

KT: Kinetin

2,4-D: 2,4-dichlorophenoxyacetic acid

BAP: 6-benzylaminopurine

NAA: 1-naphthaleneacetic acid

PGR: Plant growth regulator

Declarations

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Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Code availability

Not applicable

Authors' contributions

Sarah Ali: conducted the experiments, data collection, data analysis and wrote the results and discussion; Saboohi Raza and Saleem Shahzad: conceived and designed the research; Tuba Sharf Batool: collected references and literature; Aasma Abdullah: conducted the experiment and data collection. Naveed Hameed: reviewed the manuscript and contributed to the data analysis. Asad Manzoor: reviewed the manuscript; All authors read and approved the final manuscript.

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Tables

Table 1. PGRs concentrations and combination used for the proliferation of embryogenic callus

PGRs	Concentrations and Combinations
Control	LS0
IBA	0.49 µM, 1.47 µM, 2.46 µM
2,4-D	0.45 µM, 1.35 µM, 2.26 µM
BAP	0.44 µM, 1.33 µM, 2.219 µM
BAP+NAA	0.44 µM + 5.37 µM, 1.33 µM + 5.37 µM, 2.219 µM + 5.37 µM

Table 2. PGRs concentrations and combination used for the further development and rooting of regenerated plantlets

PGRs	Concentrations and Combinations
Control	LS0
BAP	0.44 µM, 1.33 µM and 2.219 µM
IBA	0.49 µM, 1.47 µM and 2.46 µM
2,4-D	0.45 µM, 1.35 µM and 2.26 µM
IAA	0.57 µM, 1.71 µM, 2.85 µM
BAP + NAA	2.219 µM + 0.53 µM, 4.439 µM + 0.53 µM, 6.659 µM + 0.53 µM & 8.879 µM + 0.53 µM
BAP + NAA + GA3	2.219 µM + 0.53 µM + 0.288 µM, 4.439 µM + 0.53 µM + 0.288 µM, 6.659 + 0.53 µM + 0.288 µM & 8.879 + 0.53 µM + 0.288 µM
BAP + 2,4-D	2.219 µM + 0.45 µM, 4.439 µM + 0.45 µM, 6.659 µM + 0.45 µM & 8.879 µM + 0.45 µM

Table 3. Effect of different concentrations of PGRs on proliferation of callus produced from young leaves after 2 weeks.

Callus FW (g)				
Treatments(mg/L)				Mean & Standard error
BAP	NAA	2,4-D	IBA	
-	-	-	-	$0.16^{ab} \pm 0.01$
0.1	1	-	-	$0.27^{a \pm} 0.03$
0.3	1	-	-	$0.23^{a \pm} 0.02$
0.5	1	-	-	$0.16^{bc} \pm 0.04$
-	-	0.1	-	$0.27^{a \pm} 0.01$
-	-	0.3	-	$0.21^{abc} \pm 0.01$
-	-	0.5	-	$0.14^{c \pm} 0.02$
0.1	-	-	-	$0.18^{bc} \pm 0.01$
0.3	-	-	-	$0.15^{bc} \pm 0.03$
0.5	-	-	-	$0.17^{bc} \pm 0.02$
-	-	-	0.1	$0.17^{bc} \pm 0.04$
-	-	-	0.3	$0.16^{bc} \pm 0.02$
-	-	-	0.5	$0.22^{abc} \pm 0.02$

NOTE. Values with same letter(s) are non-significant at α = 0.05 for using Duncan's multiple range test (DMRT) level

Table 4. Effect of different concentrations of PGRs on regeneration from callus after 4 weeks.

Number of regenerated shootlets				
Treatments(mg/L)				Mean & Standard error
BAP	NAA	2,4-D	IBA	
-	-	-	-	$7.3^{ef} \pm 1.76$
0.1	1	-	-	$5.3^{fgh} \pm 1.7$
0.3	1	-	-	-
0.5	1	-	-	-
-	-	0.1	-	$25^{a} \pm 0.8$
-	-	0.3	-	$7.3^{ef} \pm 1.45$
-	-	0.5	-	$9.7^{bcde} \pm 2.9$
0.1	-	-	-	$6.3^{fgh} \pm 2.3$
0.3	-	-	-	$3^{gh} \pm 0.57$
0.5	-	-	-	$2.7^{gh} \pm 0.33$
-	-	-	0.1	$9.7^{bcde} \pm 2.9$
-	-	-	0.3	$7^{ef} \pm 1.7$
-	-	-	0.5	$15.7^{bc} \pm 2.02$
-	0.1	-	-	$19^{b} \pm 4.04$
-	0.3	-	-	$13.3^{bcd} \pm 3.28$
-	0.5	-	-	$4.7^{fgh} \pm 1.33$

NOTE. Values with same letter(s) are non-significant at α = 0.05 for using Duncan's multiple range test (DMRT) level

Table 5. Effect of solidifying agents on shoot proliferation of vertically placed nodal explants on LS medium after 6 weeks.

Table 6. Comparative performance of selected somaclones.

Solidifying agents	Root induction (%)	Survival (%) of explant	Mean no. of shoot proliferation/ explant	Mean of Shoot length (cm)	Mean no. of leaves/plant
Gelrite	0%	80%	$5^{a} \pm 0.9$	$2.8^{c} \pm 0.2$	$7.4^{bc} \pm 0.7$
Agar	84.6%	100%	$1^{bc} \pm 0.3$	$5.3^{b} \pm 0.7$	$9.3^{bc} \pm 1.3$
Phytogel	25.9%	100%	$3^{bc} \pm 0.4$	$8.2^{a} \pm 0.8$	$12.9^{a} \pm 0.9$

S.No.	Clones	Stem diameter (mm)	Flower diameter (inch)	Number of leaves	Plant height (cm)
1	Control	3.69±0.48 n.s	0.83±0.24bcd	18.19±5.06 n.s	21.4±6.1 n.s
2	S4	3.5±0.5 n.s	0.5±00d	19.3±5.5 n.s	19.67±5.85 n.s
3	S11	3.3±0.57 n.s	0.5±00d	16±4 n.s	16.2±3.2 n.s
4	S18	4±0.5 n.s	1±00abc	23.67±7.6 n.s	30±5 n.s
5	S19	3.8±0.76 n.s	0.9±0.17abc	30.33±9.29 n.s	30.67± 5.8 n.s
6	S21	4.3±0.57 n.s	1.33±0.57ab	20.67±8.08 n.s	25±1.2 n.s
7	S23	4±00 n.s	1±00abc	18.67±4.04 n.s	29±3 n.s
8	S26	4±0.5 n.s	1±00abc	24±1 n.s	26±2 n.s
9	S29	4±0.86 n.s	1.17±0.76ab	22.67±2.7 n.s	23±3 n.s
10	S30	4±0.5 n.s	1.17±5.56ab	15±3.6 n.s	20±5.56 n.s
11	S35	4.3±0.28 n.s	1.17±2.8ab	18.33±4.6 n.s	26.8±7 n.s
12	S41	3.17±0.28 n.s	1±00abc	17±4.3 n.s	21.33±9.0 1 n.s
13	S44	3.5±0.5 n.s	1.17±0.28ab	18±2 n.s	21.67±6.8 n.s
14	S51	3.5±0.5 n.s	0.5±00d	19.3±2.3 n.s	19.3±8.14 n.s
15	S55	3.17±0.76 n.s	0.67±0.28cd	10±1 n.s	13.3±5.77 n.s
16	S59	3.5±0.5 n.s	1±00abc	12.3±3.2 n.s	13.8±4.48 n.s
17	S61	3.17±0.28 n.s	0.5±00d	12.67±3.05 n.s	17.5±2.59 n.s
18	S66	3.17±0.28 n.s	0.9±0.17abc	15±2 n.s	22.67±7.5 n.s
19	S67	4.3±0.57 n.s	1.17±0.28ab	17.3±1.5 n.s	20.67±3.05 n.s
20	S68	3.67±0.57 n.s	0.48±0.028d	15.3±8.7 n.s	15±8.18 n.s
21	S69	3.8±0.28 n.s	1±00abc	22±2.5 n.s	29.83±5 n.s
22	S84	4.17±0.76 n.s	1.5±0.5a	18.67±7.6 n.s	23.67±5.5 n.s

Note. Values with same letter(s) are non-significant at α = 0.05 for using Duncan's multiple range test (DMRT) level, n.s= non-significant

Figures

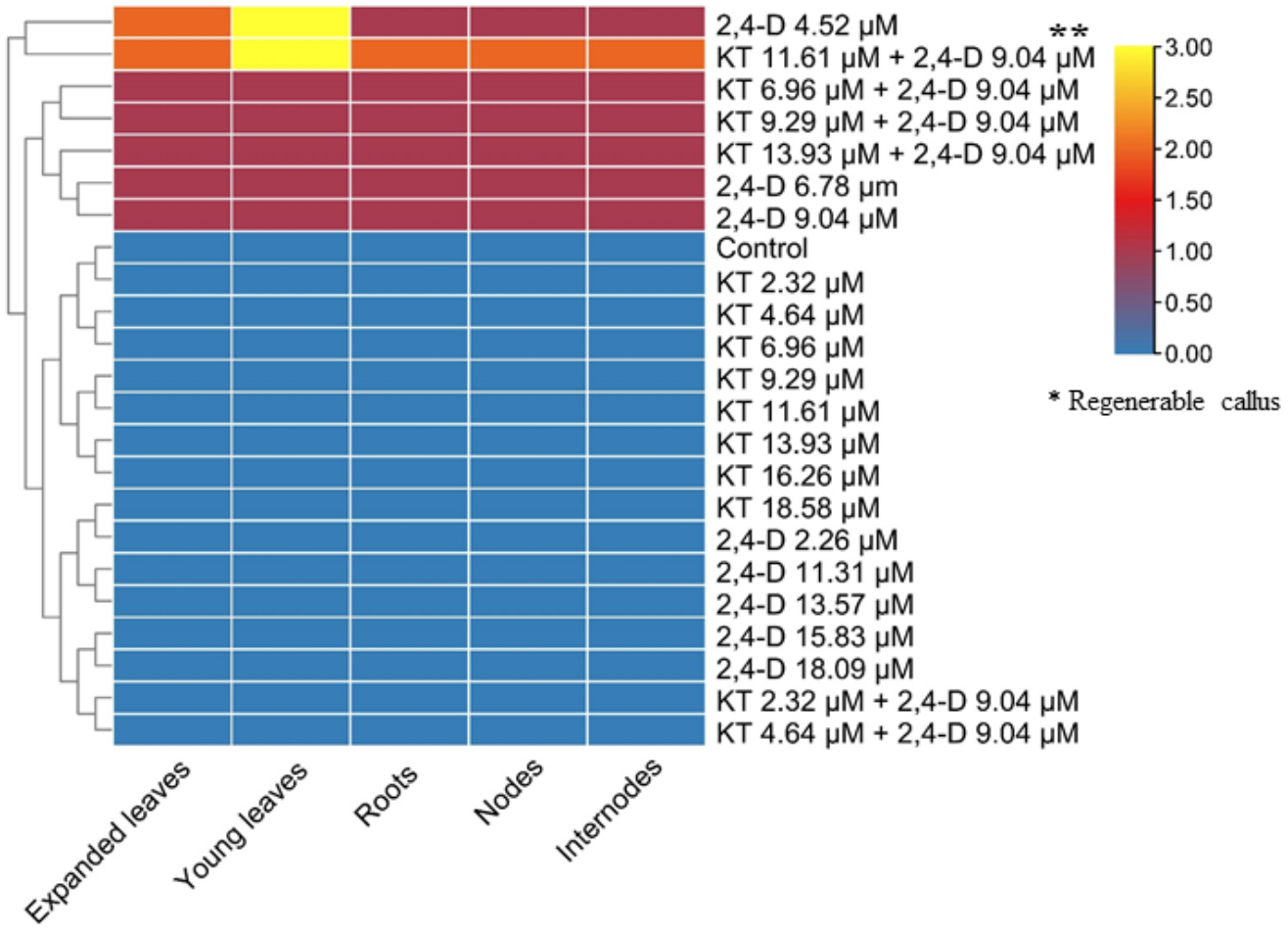


Figure 1

Heat-map representing two broad groups of PGRs (concentrations/combinations) in which group having 4.52 µM 2,4-D alone and combination of 9.02 µM 2,4-D with 11.61 µM KT resulted in most suitable media compositions for callus induction. Cluster of second group is further divided into two sub-groups; first is representing PGR concentrations producing low amount of callus and second sub-group containing PGR concentrations responded poorly for callus induction.

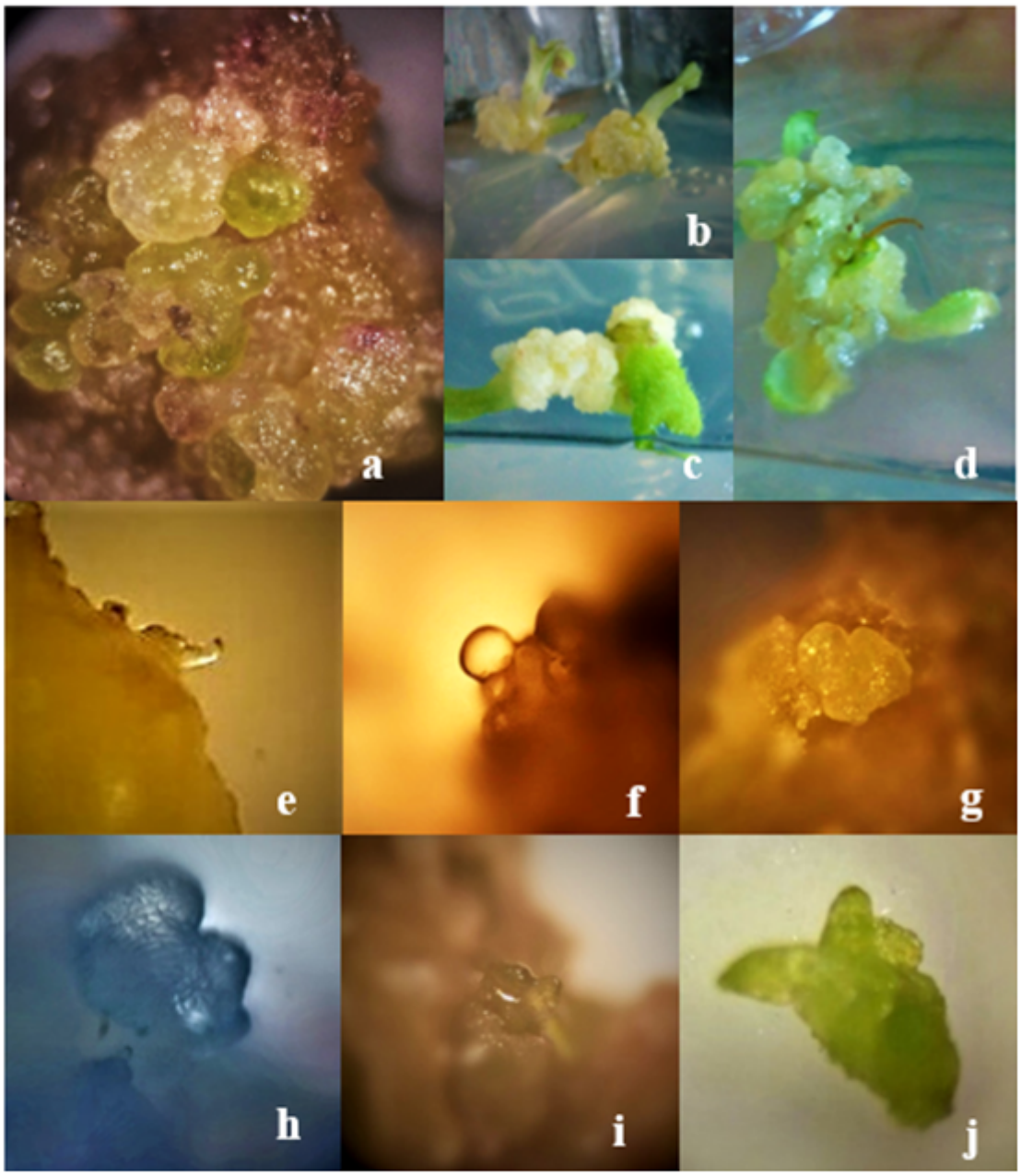


Figure 2

Embryogenic callus induction on medium augmented with the combination of 9.02 µM 2,4-D and 11.61 µM KT within 2 weeks. (a) Embryogenic callus induced on young leaf explant under dissecting microscope. (b) granulated yellowish-pale callus on young leaves, (c) whitish-pale colored callus on expanded leaves, (d) mucilaginous callus, translucent, soggy callus in between different calli. (e-j) *In vitro* developmental stages of somatic embryos of chrysanthemum cv. Dante yellow under 4X dissecting microscope, initial pro-embryonic masses, globular-shaped structure, heart-shaped, heart-shaped structure under 100X compound microscope, torpedo-shaped and cotyledonal stage.



Figure 3

Embryogenic callus proliferation and plant regeneration from embryogenic calli of *C. morifolium*. (a) Proliferation of callus on LS medium augmented with the combination of 0.44 µM BAP with 5.37 µM NAA , (b) Embryogenic callus conversion into plantlets on medium supplemented with 0.45 µM of 2,4-D within 4 weeks, (c) Shoot elongation and root development on medium supplemented with 0.49 µM IBA after 48 day, (d-f) Direct multiplication of regenerated plantlets on three gelling agents [0.8% (w/v) agar, 0.2% (w/v) gelrite and 0.25% (w/v) phytagel] after 6 weeks, (g) Sterilized cocopeat sprayed with ½ strength of

Hoagland's solution weekly (T-1) resulted in 100% of survival rate of plantlets, (h) Sterilized garden soil sprayed weekly with ½ strength of Hoagland's solution gave only 60% survival of plantlets.

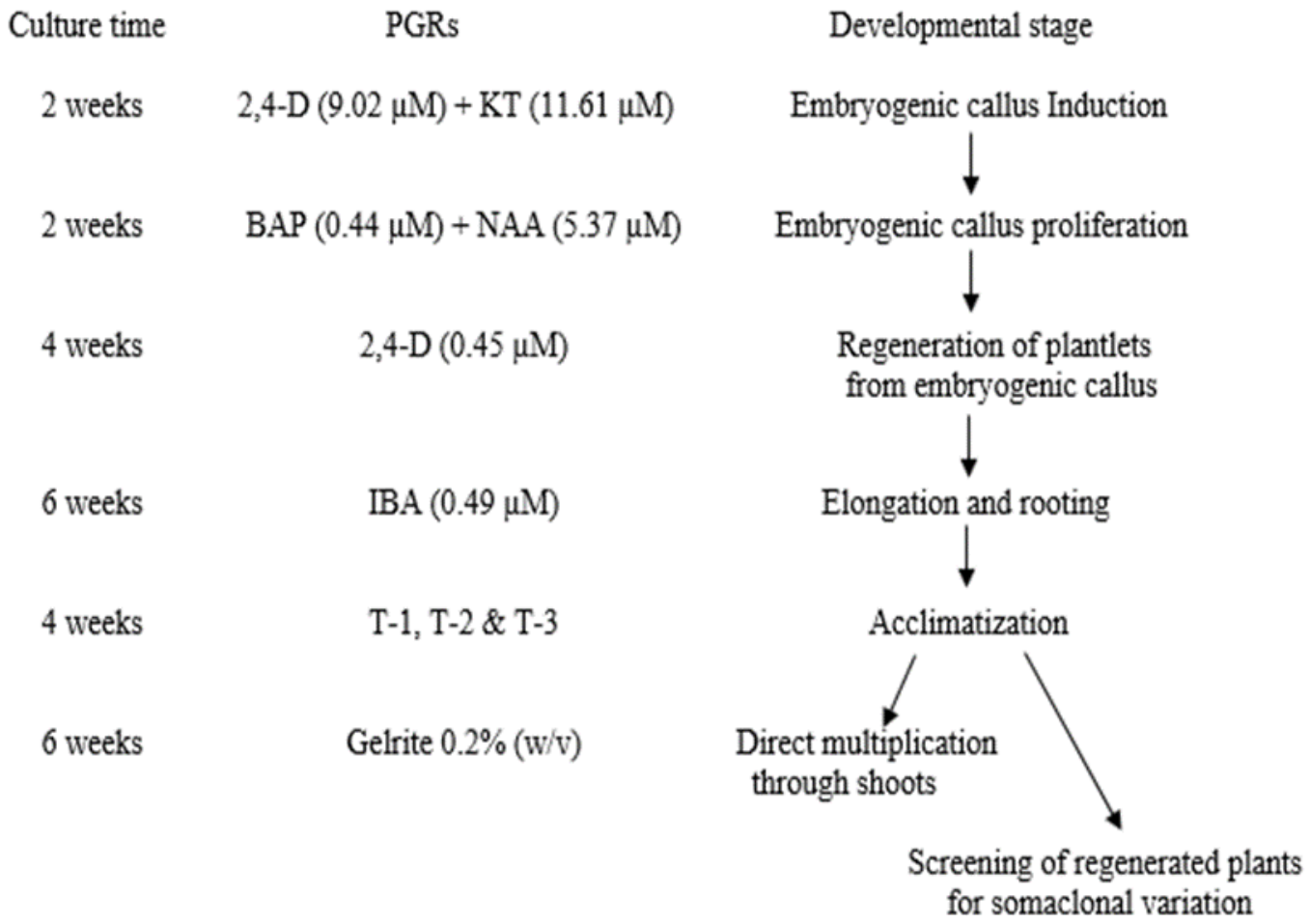


Figure 4

A schematic illustration of the whole protocol developed for *in vitro* multiplication and acclimatization of chrysanthemum cv. Dante yellow. The culture time represents the incubation time of callus sub-cultured on one medium to subculture another medium



Figure 5

(a) Flat full inflorescence of field- grown Dante yellow, (b) Clone S4 the petals were devoid of red pigmentation and showed only yellow colour inflorescence, (c) Clone S68 showed red inflorescence, (d) Clone S42 showed less red portion in the centre and (e) Clone S55 showed some interestingly abnormal petals with flat semi-full inflorescence.

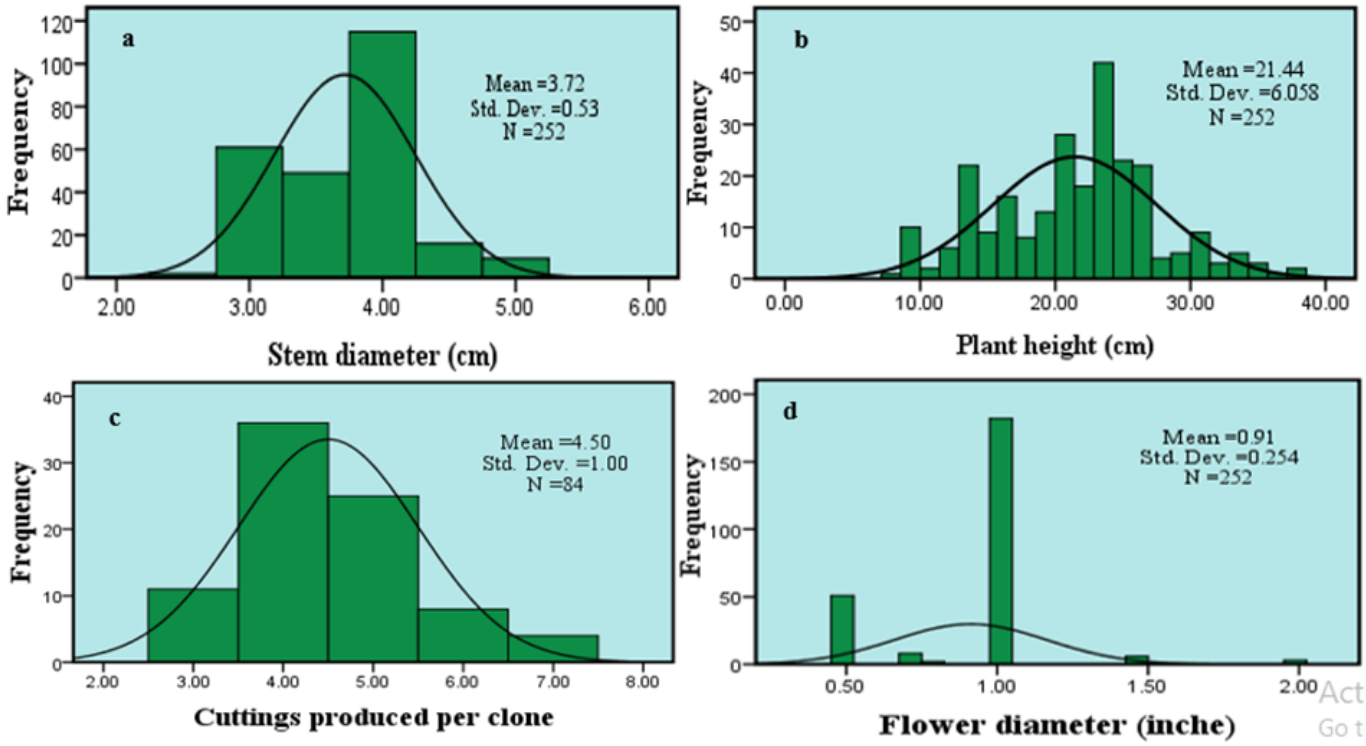


Figure 6

(a) Frequency distribution for diameter of flower (inches) in the regenerated somaclones, (b) Frequency distribution for no. of suckers produced per somaclones, (c) Frequency distribution for diameter of stem (mm) in the regenerated somaclones and (d) Frequency distribution for plant height (cm) in the regenerated somaclones.