**ORIGINAL RESEARCH**

# Developing an efficient in vitro callusing and regeneration protocol in *Dendranthema × grandiflorum* Kitam.

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## Abstract

To standardize the methodology for in vitro callusing and regeneration of two chrysanthemum cultivars, 'Candid' and 'Flirt,' an experiment was carried out. The petals and leaf segments were used as explants. In both cultivars, petals were shown to be the most effective for callusing and regeneration. In both cultivars, MS medium enriched with 1.00 mg L$^{-1}$ BAP + 1.50 mg L$^{-1}$ NAA resulted in the best callusing of petal explants in terms of minimal days to callus initiation, maximal callus induction, and callus weight per explant. When using petal explants from both cultivars, the shortest time (13.25 and 16.00 days) to callus initiation, the highest callus induction (91.66 and 83.33%) and the highest mean callus weight (2.43 and 2.31 g per explant) were all found in media augmented with 1.00 mg L$^{-1}$ BAP + 1.50 mg L$^{-1}$ NAA. In both cultivars, MS media supplied with 1.00 mg L$^{-1}$ Kinetin + 0.50 mg L$^{-1}$ IAA resulted in the best regeneration of petal explants in calli-producing shoots and shoot number per explant. Maximum calli-producing shoots 89.58 and 87.49% and significantly maximum shoot number 3.25 and 2.75 per explant were noticed in media fortified with Kinetin 1.00 mg L$^{-1}$ + IAA 0.50 mg L$^{-1}$ in case of petal explants of both the cultivars. Nevertheless, the finest callusing of leaf explants in terms of minimum days to callus initiation, highest callus induction in both cultivars was achieved using MS media supplied with 1.00 mg L$^{-1}$ BAP + 1.50 mg L$^{-1}$ NAA, whereas the highest callus weight per explant was acquired using MS media supplied with 0.50 mg L$^{-1}$ BAP and 2.50 mg L$^{-1}$ NAA. Explants from both cultivars showed a minimum callus initiation period of 15.25 and 16.75 days, callus induction rates of 89.57 and 81.24%, and maximum mean callus weights of 0.94 and 0.89 g, correspondingly.

**Keywords** Chrysanthemum · Callus · Cultivars · Growth regulators · Protocol · Regeneration · Tissue culture

## Abbreviations

| | |
|---|---|
| Mm | Micro molar |
| PGRs | Plant growth regulators |
| NAA | Naphthalene acetic acid |
| MS | Murashige and Skoog medium |
| IAA | Indole-3-acetic acid |
| IBA | Indole-3-butyric acid |
| GA$_3$ | Gibberellic acid |
| G.R | Growth regulator |
| 2; 4-D | 2,4 Dichloro-phenoxyacetic acid |
| FW | Fresh weight |
| BAP | 6-Benzyl amino purine |
| BA | 6-Benzyladenine |

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## Introduction

All across the world, chrysanthemums are sought for use in flower arrangements, potted plants, and herbaceous perennials (Anderson 2007). Most chrysanthemum varieties are propagated through stem cuttings or lateral branches, called suckers (Teixeira da Silva 2003; Levin et al. 1988). Although the conventional method of shoot cuttings is a long and painstaking process thus, we will need efficient tissue culture methods (Kaul et al. 1990; Levin et al. 1988). Chrysanthemum biotechnology relies heavily on tissue culture, a vital part of the process (Teixeira da Silva and Kulus 2014). With the advancement of in vitro regeneration technology, it is now a viable alternative to traditional

propagation methods. One of the prerequisites for an economically effective micropropagation technique is the development of healthy shoots and increased multiplication rates. Through the use of in vitro propagation, it is now possible to produce an enormous number of plants from a single explant (Bajaj 1992). Chrysanthemums have turned out to be one of the primary commercial goals for tissue culture due to their high demand and appeal (Levin et al. 1988). The interaction of PGRs, the type of explant, and the plant genotype in chrysanthemum influence the induction of adventitious shoots leading micro-shoot regeneration. (Zalewska et al. 2011; Nahid et al. 2007). A developing mass of disorganized plant parenchyma cells is referred to as a callus. Callus cells are the cells that cover a wound in live plants. After surface sterilization and plating onto tissue culture medium in vitro, callus development is generated from plant tissue explants. The culture medium is supplied with plant growth regulators such as auxins, cytokinins, and gibberellins to induce callus formation or somatic embryogenesis. Specific auxin–cytokinin ratios in plant tissue culture medium result in an unorganized mass of callus cells that grows and divides. Callus cultures are frequently characterized as compact or friable. Friable calluses are readily broken apart and can create cell suspension cultures. Callus can undergo direct organogenesis and/or embryogenesis, which results in forming a whole new plant. This process is referred to as callus culture (Qadri et al. 2018). Despite somaclonal variation in the vegetative tissues, tissue generated from the culture of Chrysanthemum ray florets may be separated as solid mutants. During induced mutagenesis, the direct regeneration methodology successfully isolates the chimeric mutant tissues formed through sports and develops unique floral color/shape mutants (Datta et al. 2005). Flower regeneration provides more potential for developing new chrysanthemum cultivars than other explants such as leaves and stems. Numerous spontaneous or induced inflorescence color alterations are seen on flowers as little spots or streaks. Due to the small size of tissues, such mutations are lost because no currently accessible method for separating chimeras can maintain them. However, when shoots or somatic embryos are produced from floral explants, this possibility exists, providing a chance to obtain stable mutants (Chakrabarty and Datta 2010). Chimerism may also be a significant factor in generating genetic variation within a breeding program, such as the emergence of new inflorescence colors in chrysanthemum as a result of propagation via adventitious buds derived from periclinal chimeras because chimeral components can be separated (Zalewska et al. 2007). In vitro propagation of chrysanthemum, an ornamental plant, using flower tissue provides a unique and valuable explant type for organogenesis (regeneration of adventitious shoots/roots) or somatic embryogenesis. This is critical for the reproduction of this species. In vitro culture of flower, tissue can result in the disassembly of chimera components and the generation of stable mutants. Additionally, regenerants descended from ray (ligulate) or disk (tubular) florets exhibit somaclonal variation. The ability to regenerate such plant material can produce plants with novel flower colors, altered architecture, or other beneficial characteristics, even more so after physical mutagens such as gamma radiation are applied (Teixeira da Silva et al. 2015). Although in vitro flowering is a rare occurrence in chrysanthemum, the possibility exists for the creation of floral material that might serve as in vitro-disinfected material without the need to introduce explants from the ex vivo milieu and risk contamination. Regeneration protocols from flower tissue would enable advancements in breeding with such tissues and a better understanding of the process of in vitro flowering. Chrysanthemum petals have been suggested as a possible source of organogenesis. (Nahid et al. 2007). Numerous researchers have demonstrated chrysanthemum petal regeneration using a variety of cultivars and diverse PGR's (plant growth regulator) conjugations or concentrations (Verma et al. 2012a; Mani and Senthil 2011; Song et al. 2011; Barakat et al. 2010; Park et al. 2007; Nahid et al. 2007; Manal and Datta 2005; Datta et al. 2005). Plant growth regulators offer a proportionate stimulus that enables the cell cycle to be regulated to induce cell division during plant development (Hodson de Jaramillo et al. 2008). It was found that growing petal explants on the MS fortified medium (IAA 57.0 μM, BAP 44.0 μM, and Kinetin 0.4 μM) resulted in the maximum adventitious shoot regeneration frequency (Park et al. 2007). In another experiment Song et al. (2011) discovered substantial changes in the rejuvenation rate and observed maximum occurrence of shoot organogenesis along with maximum number of shootlets per explant when various cultivars were cultivated on MS fortified media with different PGR's. Creating shoots or adventitious organogenesis is desirable because it preserves clonal fidelity, important for floricultural crops. After all, many cultivars are bred for specific characteristics (Park et al. 2007). The current goal is to examine the influence of various plant growth regulator combinations on shoot organogenesis in two chrysanthemum cultivars using distinct explants to establish a more efficient system for high-frequency regeneration rate.

## Materials and methods

### Collection of explants

Actively growing shoots (7–10 cm) under polyhouse were collected from the healthy plants of chrysanthemum cvs. 'Candid' and 'Flirt'. The shoots were excised from the mother plants with the help of a sharp blade and placed in a beaker containing distilled water, and brought to the

laboratory for isolation of explants. Different types of explants used during the present investigation were shoot tips, nodal segments, leaf segments, and petals. Shoot tips were mainly collected from the secondary branches of the mother plant. The older leaves were removed from shoot tips and cut to 2–3 cm. The explants were placed in cleanly washed flasks containing distilled water for further processing in the laboratory. Explants were extracted from the plants growing in the field. The explants were excised from the first upper node than from the second or third node and were cut approximately 5 cm before final processing. The young leaves (5–7 cm in length) just below the apical meristem were separated from the shoots with the help of scissors. They were cut into small pieces and placed in flasks containing distilled water for further processing in the laboratory. After removing a few outer leaves, the explants were placed in clean flasks containing distilled water before further processing.

## Explant surface sterilization and inoculation

Explant surface sterilization is the first vital step for the initiation of cultures in vitro. Final steps in surface sterilization were performed under the aseptic conditions of laminar flow hood. Knives and forceps were flame sterilized before use. While making incisions, knives and forceps were flame sterilized as and when thought necessary to avoid the spread of contamination between different parts of the explants. Proper disinfection procedures for chrysanthemum flower tissue explants for in vitro regeneration studies mainly include two agents: sodium hypochloride (NaOCl) and mercuric chloride (HgCl$_2$). The main differences usually concern the agent's concentration and incubation period. NaOCl concentration for disinfection of ray florets ranged from 0.1% in Tanaka et al. (2000), 0.2% in Lee et al. (2013) and Sung et al. (2013) to 0.5–1% in De Jong and Custers (1986), Miler and Muszczyk (2013), Tymoszuk and Zalewska (2014a, b) and Matsumura et al. (2010). Frequently, pure NaOCl is replaced by domestic chlorox bleach solution containing NaOCl in the composition. Khalid et al. (1989) disinfected ray flowers in 8% (v/v) ''Domestos'', Thangmanee and Kanchanapoom (2011) in 5% (v/v) ''CloroxTM'', but Vilasini and Latipah (2000) and Latado et al. (2004) used 15% (v/v) of domestic chlorox bleach solution with a 5.25% concentration of NaOCl. Other kinds of explants, such as flower buds could be disinfected at a concentration of 40% ''Chlorox'' (Dwimahyani and Widiarsih 2010). Disinfection period in solutions of NaOCl ranges from 10 to 20 min. Sometimes explants are immersed additionally in a 70% solution of ethanol for a few seconds (Matsumura et al. 2010; Lee et al. 2013; Sung et al. 2013) or for 1 min (Thangmanee and Kanchanapoom 2011). HgCl$_2$ is another agent often used to sterilize explants derived from chrysanthemum inflorescences.

Misra and Datta (2007) and Barakat et al. (2010) applied 0.1% HgCl$_2$ for 2 or 4 min, combining a follow-up disinfection step with 70% ethanol, but Kengkarj et al. (2008) used 0.1% HgCl$_2$ for 10 min without ethanol. Teixeira da Silva and Kulus (2014) used a higher concentration of HgCl$_2$ (0.2%) for 2 min applied to immature buds first dipped in 70% ethanol for 10 s. This latter method of disinfection resulted in 84% explant survival and 0% explant contamination; other ''softer'' disinfection protocols resulted in higher explant survival, but conversely, much higher explant contamination. Among various sterilants, 2 min HgCl$_2$ at 0.1% dip marginally enhanced culture asepsis of chrysanthemum explants as compared to 4 min dip. However, mercuric chloride 0.1% irrespective of time duration in combination with higher concentration of carbendazim (200 ppm) for 30 min dip followed by ethyl alcohol 70% dip for 10 s significantly enhanced the culture asepsis (Din et al. 2018). Details of surface sterilization procedures for different explants are given in the following sections. The details regarding the composition of various sterilant formulations and duration of application are given in Table 1.

## Shoot tips

Shoot tips brought to the laboratory were washed with running tap water several times to remove any adhering dirt or dust particles. The outer leaves, except few young, were removed to reduce shoot tips to a manageable size and thereafter given a vigorous shake for 30 min in Tween-20 surfactant solution (few drops) fortified with required concentration of fungicide concentration. The surfactant and the fungicide were washed off under running tap water, followed by a final washing with single distilled water. After the initial cleaning, the explants were later brought to laminar flow hood for treatment with mercuric chloride. The shoot tips were given final 3–5 rinses with sterile water. Then, all the leaves were removed from shoot tips and were placed on the

**Table 1** Composition of sterilant formulations and duration of application for surface sterilization of various explants

| | Details of the treatment |
|---|---|
| $T_1$ | Mercuric chloride 0.1% for 2 and 4 min |
| $T_2$ | Sodium hypochlorite 0.1% for 2 and 4 min |
| $T_3$ | Carbendazim 100 and 200 ppm for 30 min followed by mercuric chloride 0.1% for 2 and 4 min followed by ethyl alcohol 70% for 10 s |
| $T_4$ | Carbendazim 100 and 200 ppm for 30 min followed by sodium hypochlorite 0.1% for 2 and 4 min followed by ethyl alcohol 70% for 10 s |

medium for aseptic inoculation conforming to their original polarity.

### Nodal segments and leaf segments

Explants on arrival to the laboratory were run through the same surface sterilization procedure as followed in the shoot tips. In case of nodal segments, two keeled leaves were removed and finally cut to the size of 1.0, 2.0 and 3.0 cm by giving both top and basal recut to the segments to remove the damaged sterilant tissues, while as leaf explants were reduced to the uniform size of 1 cm segments.

### Petals

Petals (7 mm) from both the cultivars were cut and placed in distilled water. Finally, a horizontal cut was given at the terminal end before placing them on the inoculation medium. Observations with regard to aseptic cultures (without infection) and explant survival (surviving explants) were made up to 4 weeks of inoculation. Data for the following parameters were recorded during the investigation.

## Callusing

For callus induction, leaf and nodal segment explants of two cultivars, 'Candid' and 'Flirt' were used in standardization experiments. Explants after sterilization were placed on media containing different auxin and cytokinin combinations (Table 2). Each treatment combination was assigned 12 explants per test tube and replicated four times. Data for callusing was recorded under the following parameters.

### Days to callus initiation

Measured from inoculation to the date when callus became visible to the naked eye.

### Callus induction (%)

Data was recorded after 4 weeks of culture.

**Table 2** PGR supplements used for standardization of callus induction and growth

| S. no. | Growth regulators | Conc. (mg L$^{-1}$) |
| --- | --- | --- |
| 1 | BAP+NAA | 0.50+1.00 |
| 2 | BAP+NAA | 0.50+1.50 |
| 3 | BAP+NAA | 0.50+2.00 |
| 4 | BAP+NAA | 1.00+1.00 |
| 5 | BAP+NAA | 1.00+1.50 |
| 6 | BAP+NAA | 1.00+2.00 |

### Callus weight (g per explant)

Callus weight was recorded at the end of 6 weeks of culture. Test tubes/flasks with fresh media were weighed individually. Callus from growing cultures was separated from the explants and transferred to pre-weighed test tubes/flasks with fresh media. The test tubes/flasks were weighed again. The difference between the two measurements was recorded as the callus weight per explant.

### Callus type

Callus type in each case was visually assessed, and observations recorded accordingly.

## Regeneration

Various strategies were employed to regenerate shoots from calluses derived from two different morphological structures like leaf, petals, and nodal segments. Callus pieces of uniform size and age originating from different morphological structures were placed on various regeneration media (Table 3). Data for regeneration studies were recorded under the following parameters.

### Regeneration (%)

Data were recorded after 8 weeks of culture.

## Statistical analysis:

Four replicated trials were set up in a CRD using the SPSS statistical tool for analysis of variance (ANOVA). 'DMRT' was used to distinguish between treatment means that differ. The percentage data were square root transformed to ensure the model assumptions for analysis of variance to be met.

**Table 3** Media composition for regeneration studies from callus

| S. no. | Growth regulators (mg L$^{-1}$) | | | |
| --- | --- | --- | --- | --- |
| | BAP | Kin | NAA | IAA |
| 1 | 1.00 | – | 0.50 | – |
| 2 | 2.00 | – | 0.50 | – |
| 3 | – | 1.00 | 0.50 | – |
| 4 | – | 2.00 | 0.50 | – |
| 5 | 1.00 | – | – | 0.50 |
| 6 | 2.00 | – | – | 0.50 |
| 7 | – | 1.00 | – | 0.50 |
| 8 | – | 2.00 | – | 0.50 |

## Results

Three distinct explants were used in this investigation (leaf, petals and nodal segments) of cv "Candid" and two explants (leaf and petals) of cv. "Flirt" was employed to generate callus. However, nodal segments of cv. "Candid" botched to spawn any callus and consequently were not incorporated as an explant in the final experimentation.

### Callusing of petals of cv. "Candid" and "Flirt"

Data related to minimum days to callus initiation, callus induction percentage, callus type, and callus weight due to the interaction of many growth regulator concentrations in petal explant of chrysanthemum (*Dendranthema grandiflorum* Kitam) cv. "Candid" and "Flirt" are presented in Table 4. Petal explants inoculated on medium 0.5 or 1.0 mg $L^{-1}$ BAP blended with 1.0, 1.5, and 2.0 mg $L^{-1}$ NAA, recorded significant variation in means for minimum days to callus initiation, callus induction percentage, callus type and callus weight in cv. "Candid". Significantly minimum days 13.25 to callus initiation was recorded on media 1.0 mg $L^{-1}$ BAP blend with 1.5 mg $L^{-1}$ NAA· followed by 14.75, 16.00 and 17.75 days on media 1.0 mg $L^{-1}$ BAP + 2.0 mg $L^{-1}$ NAA, 1.0 mg $L^{-1}$ BAP + 1.0 mg $L^{-1}$ NAA and 0.5 mg

$L^{-1}$ BAP + 2.0 mg $L^{-1}$ NAA, correspondingly. However, maximum number of days 22.50 followed by 20.00 to callus initiation was recorded by 0.5 mg $L^{-1}$ BAP + 1.0 mg $L^{-1}$ NAA and 0.5 mg $L^{-1}$ BAP + 1.5 mg $L^{-1}$ NAA, respectively. Pronounced callus initiation 91.66 % was noted with 1.0 mg $L^{-1}$ BAP + 1.5 mg $L^{-1}$ NAA G.R amalgamation (Fig. 1a, b). This was a significant improvement over the means of 89.58, 87.50, 83.33, 81.25 and 79.17%, respectively, recorded with 1.0 mg $L^{-1}$ BAP + 2.0 mg $L^{-1}$ NAA, 1.0 mg $L^{-1}$ BAP + 1.0 NAA mg $L^{-1}$, 0.5 mg $L^{-1}$ BAP + 2.0 NAA mg $L^{-1}$, 0.5 mg $L^{-1}$ BAP + 1.5 NAA mg $L^{-1}$ and 0.5 mg $L^{-1}$ BAP + 1.0 mg $L^{-1}$ NAA. Elevated concentrations of 1.0 mg $L^{-1}$ (BAP) yielded a radically better callus induction percentage than lower concentrations (0.5 mg $L^{-1}$ BAP). Following 6 weeks of culture, the fresh weight of callus in petal explants incubated with NAA-based growth regulator combinations was considerably higher. Notably, the highest weight of callus (2.43 g per explant) was measured under 1.0 mg $L^{-1}$ BAP + 1.5 mg $L^{-1}$ NAA, followed by 2.00 g per explant under 1.0 mg $L^{-1}$ BAP + 2.0 mg $L^{-1}$ NAA and 1.74 gram per explant under BAP 1.0 + NAA 1.0 mg $L^{-1}$. These treatments also differed significantly from each other. A minimum callus fresh weight of 1.45 g per explant was noted with 0.5 mg $L^{-1}$ BAP + 1.00 mg $L^{-1}$ NAA. The other NAA treatment combinations varied between 1.50 and 1.57 g per explant callus fresh weight. BAP significantly improved the fresh

**Table 4** The impact of plant growth regulators on callusing in petal explant of Chrysanthemum cvs. "Candid" and "Flirt"

| MS + PGRs | Days to callus initiation | | Callus induction (%)[A] | | Callus weight (per explant)[B] | | Callus type | |
|---|---|---|---|---|---|---|---|---|
| | Candid | Flirt | Candid | Flirt | Candid | Flirt | Candid | Flirt |
| BAP (0.50 mg $L^{-1}$) + NAA (1.00 mg $L^{-1}$) | 22.50[a] | 23.45[a] | 79.17[c] (8.89) | 66.66[d] (8.16) | 1.45[e] | 1.42[d] | Friable fleshy creamish | Friable fleshy creamish |
| BAP (0.50 mg $L^{-1}$) + NAA (1.50 mg $L^{-1}$) | 20.00[b] | 22.11[a] | 81.25[c] (9.01) | 70.83[d] (8.41) | 1.50[de] | 1.46[d] | Friable fleshy creamish | Friable fleshy creamish |
| BAP (0.50 mg $L^{-1}$) + NAA (2.00 mg $L^{-1}$) | 17.75[c] | 18.32[b] | 83.33[bc] (9.13) | 79.16[c] (8.89) | 1.57[d] | 1.53[d] | Friable fleshy creamish | Friable fleshy creamish |
| BAP (1.00 mg $L^{-1}$) + NAA (1.00 mg $L^{-1}$) | 16.00[de] | 17.75[bc] | 87.50[ab] (9.35) | 83.33[bc] (9.13) | 1.74[c] | 1.70[c] | Friable fleshy light green | Friable fleshy green |
| BAP (1.00 mg $L^{-1}$) + NAA (1.50 mg $L^{-1}$) | 13.25[fg] | 15.25[c] | 91.66[a] (9.57) | 89.57[a] (9.46) | 2.43[a] | 2.31[a] | Friable fleshy light green | Friable fleshy compact green |
| BAP (1.00 mg $L^{-1}$) + NAA (2.00 mg $L^{-1}$) | 14.75[h] | 16.00[bc] | 89.58[a] (9.46) | 87.49[ab] (9.35) | 2.00[b] | 1.93[b] | Friable fleshy light green | Friable fleshy green |

Figures in the parenthesis are square root transformed value of the percentage data

Means with different alphabets differ significantly

[A]Data recorded after 4 weeks of culture

[B]Callus weight recorded after 6 weeks of culture
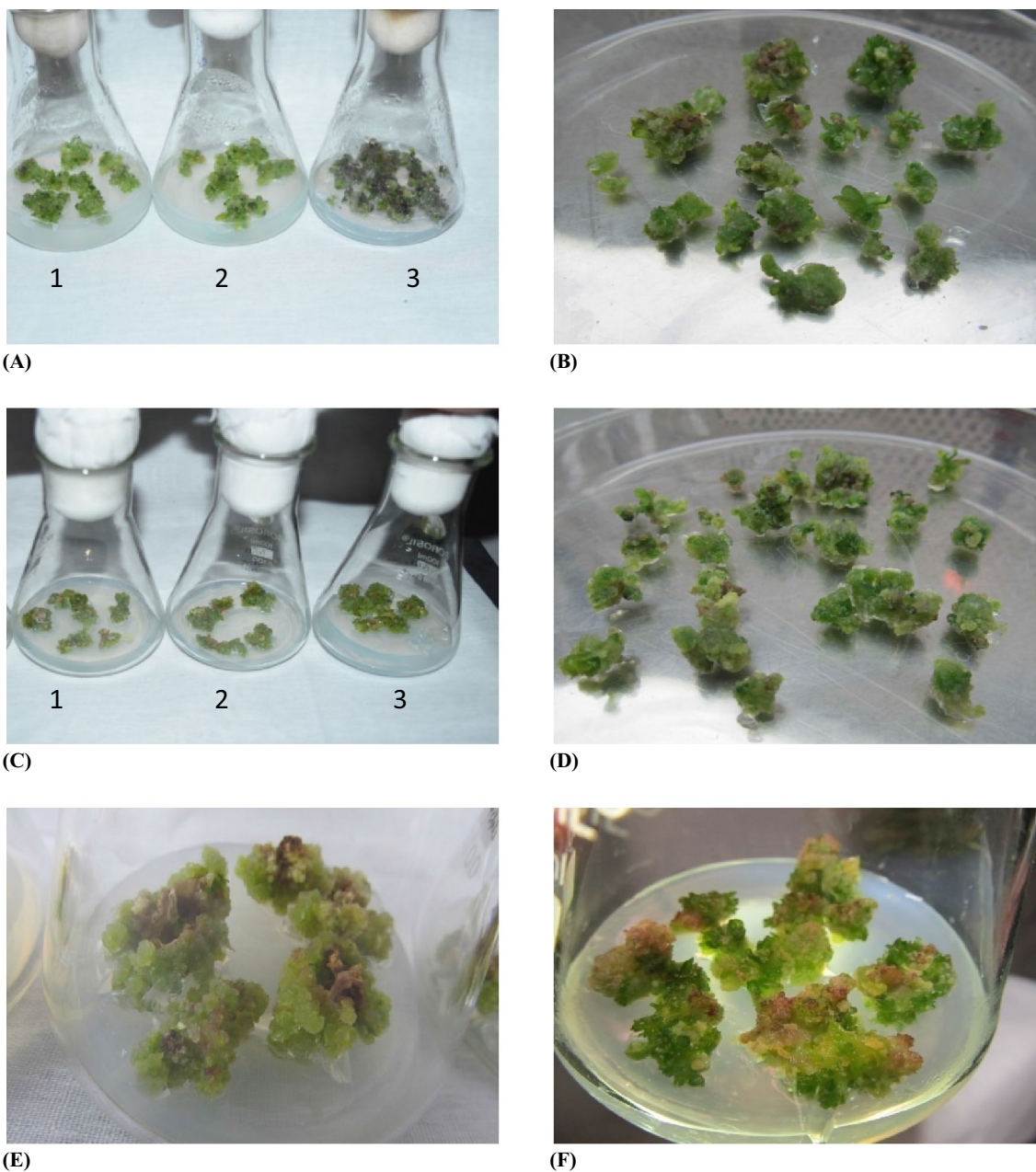
**Fig. 1 a** Callusing in petal explant cv. "Candid" (1) BAP + NAA = 1.00 + 1.50 mg L$^{-1}$ (2) BAP + NAA = 1.00 + 2.00 mg L$^{-1}$ (3) BAP + NAA = 1.00 + 1.00 mg L$^{-1}$. **b** Callusing in petal explant cv. "Candid", BAP + NAA = 1.00 + 1.50 mg L$^{-1}$. **c** Callusing in petal explant cv. "Flirt" (1) BAP + NAA = 1.00 + 1.50 mg L$^{-1}$ (2) BAP + NAA = 1.00 + 2.00 mg L$^{-1}$ (3) BAP + NAA = 1.00 + 1.00 mg L$^{-1}$. **d** Callusing in petal explant cv. "Flirt" BAP + NAA = 1.00 + 1.50 mg L$^{-1}$. **e** Callus type-I (friable fleshy, light green). **f** Callus type-II (friable fleshy, compact green)

callus weight when used in combination with auxins. There was a momentous raise in the fresh weight of callus when the BAP application was enhanced as of 0.5–1.0 mg L$^{-1}$. Callus type in cv. "Candid" was friable fleshy light green to friable fleshy creamish in color when NAA was used in combination with BAP (Fig. 1e).

Petal explants in medium 0.5 or 1.0 mg L$^{-1}$ BAP blend with 1.0, 1.5 and 2.0 mg L$^{-1}$ NAA recorded significant differences in means for callusing percentage, callusing weight (g per explant) and callus type in cv. "Flirt". Significantly minimum 15.25 days to callus initiation was recorded on medium containing 1.0 mg L$^{-1}$ BAP + 1.5 mg L$^{-1}$ NAA followed by 16.00, 17.75 and 18.32 days under treatment combinations 1.0 mg L$^{-1}$ BAP + 2.0 mg L$^{-1}$ NAA, 1.0 mg L$^{-1}$ BAP + 1.0 mg L$^{-1}$ NAA and 0.5 mg L$^{-1}$ BAP + 2.0 mg L$^{-1}$ NAA, correspondingly. However, maximum time

period to callusing (23.45) was noted with 0.5 mg L$^{-1}$ BAP + 1.0 mg L$^{-1}$ NAA, followed by 0.5 mg L$^{-1}$ BAP + 1.5 mg L$^{-1}$ NAA (22.11 days). A marked callus induction of 89.57 % was witnessed with G.R combination of 1.0 mg L$^{-1}$ BAP + 1.5 mg L$^{-1}$ NAA (Fig. 1c, d). This was a significant improvement compared to the means of 87.49, 83.33, 79.16, 70.83 and 66.66%, respectively, recorded by treatment combinations 1.0 mg L$^{-1}$ BAP + 2.0 mg L$^{-1}$ NAA, 1.0 mg L$^{-1}$ BAP + 1.0 mg L$^{-1}$ NAA, 0.5 mg L$^{-1}$ BAP + 2.0 mg L$^{-1}$ NAA, 0.5 mg L$^{-1}$ BAP + 1.5 mg L$^{-1}$ NAA and 0.5 mg L$^{-1}$ BAP + 1.0 mg L$^{-1}$ NAA. Higher amounts of 1.0 mg L$^{-1}$ BAP yielded considerably better percentage of callusing compared to lower concentrations (0.5 mg L$^{-1}$). FW of callus was considerably higher after 6 weeks of petal culture when incubated on NAA-based G.R combinations. Maximum mean callus weight (2.31 g per explant) was significantly greater under 1.0 mg L$^{-1}$ BAP + 1.5 mg L$^{-1}$ NAA, supervened by 1.93 g per explant with 1.0 mg L$^{-1}$ BAP + 2.0 mg L$^{-1}$ NAA and 1.70 g under 1.0 mg L$^{-1}$ BAP + 1.0 mg L$^{-1}$ NAA. These treatments also differed significantly from each other. Least callus fresh weight of 1.42 g explant was produced with 0.5 mg L$^{-1}$ BAP + 1.0 mg L$^{-1}$ NAA. The other NAA treatment combinations varied between 1.46 and 1.53 g per explant callus fresh weight. BAP significantly improved the callus fresh weight when used in combination with auxins. Significant enhancement in fresh weight of callus was recorded with elevated concentrations of BAP from 0.5 to 1.0 mg L$^{-1}$. Callus type in cv. "Flirt" was friable fleshy compact green to friable fleshy creamish in color when NAA was used in combination with BAP (Fig. 1f).

## Callusing of leaf segments of cv. "Candid" and "Flirt"

Data pertaining minimum days to callus initiation, callus induction percentage, callus type and callus weight as influenced by different G.R concentrations in leaf explant of chrysanthemum (*Dendranthemum grandiflorum* Kitam) 'Candid' and 'Flirt' have been presented in Table 5. In cv. "Candid" perusal of data reveal that significantly minimum 16.00 days to callus initiation was recorded on media fortified by 1.0 mg L$^{-1}$ BAP + 1.5 mg L$^{-1}$ NAA· followed by 17.10, 18.25 and 19.00 days under the treatment combinations 1.0 mg L$^{-1}$ BAP + 2.0 mg L$^{-1}$ NAA, 1.0 mg L$^{-1}$ BAP + 1.0 mg L$^{-1}$ NAA and 0.5 mg L$^{-1}$ BAP + 2.0 mg L$^{-1}$ NAA, respectively. However, treatment mixture 0.5 mg L$^{-1}$ BAP + 1.0 NAA mg L$^{-1}$ depicted highest 24.00 days to callusing. This treatment was followed by 0.5 mg L$^{-1}$ BAP + 1.5 mg L$^{-1}$ NAA recording 22.65 days to callusing. The callusing percentage following 4 weeks of culture was noticed significantly highest 87.50% in medium fortified by

**Table 5** The impact of plant growth regulators on callusing in leaf explant of Chrysanthemum "Candid" and "Flirt"

| MS + PGRs | Days to callus initiation | | Callus induction (%)[A] | | Callus weight (g per explant)[B] | | Type of callus | |
|---|---|---|---|---|---|---|---|---|
| | Candid | Flirt | Candid | Flirt | Candid | Flirt | Candid | Flirt |
| BAP (0.50 mg L$^{-1}$) + NAA (1.00 mg L$^{-1}$) | 24.00[a] | 24.45[a] | 65.00[e] (8.21) | 64.57[c] (8.03) | 0.51[e] | 0.50[a] | Friable whitish light green | Friable whitish light cream |
| BAP (0.50 mg L$^{-1}$) + NAA (1.50 mg L$^{-1}$) | 22.65[a] | 23.00[a] | 69.92[d] (8.33) | 66.66[bc] (8.16) | 0.55[de] | 0.54[a] | Friable whitish light green | Friable whitish light cream |
| BAP (0.50 mg L$^{-1}$) + NAA (2.00 mg L$^{-1}$) | 19.00[b] | 19.46[b] | 79.00[b] (9.15) | 83.33[a] (8.13) | 0.94[a] | 0.89[c] | Friable whitish light green | Friable whitish light cream |
| BAP (1.00 mg L$^{-1}$) + NAA (1.00 mg L$^{-1}$) | 18.25[b] | 19.00[b] | 75.00[c] (8.66) | 70.83[b] (8.41) | 0.60[d] | 0.54[a] | Friable whitish light green | Friable whitish light cream |
| BAP (1.00 mg L$^{-1}$) + NAA (1.50 mg L$^{-1}$) | 16.00[c] | 16.75[c] | 83.33[a] (9.13) | 81.24[a] (9.01) | 0.79[b] | 0.69[b] | Friable whitish light green | Friable whitish light cream |
| BAP (1.00 mg L$^{-1}$) + NAA (2.00 mg L$^{-1}$) | 17.10[bc] | 17.77[bc] | 81.25[ab] (9.01) | 79.16[a] (8.89) | 0.68[c] | 0.58[a] | Friable whitish light green | Friable whitish light cream |

Figures in the parenthesis are square root transformed value of the percentage data

Means with different alphabets differ significantly

[A]Data recorded after 4 weeks of culture

[B]Callus weight recorded after 6 weeks of culture

0.5 mg L$^{-1}$ BAP + 2.0 NAA mg L$^{-1}$. This treatment combination was followed by 1.0 mg L$^{-1}$ BAP + 1.5 NAA mg L$^{-1}$ (83.33%), 1.0 mg L$^{-1}$ BAP + 2.0 mg L$^{-1}$ NAA (81.25%), 1.0 mg L$^{-1}$ BAP + 1.0 mg L$^{-1}$ NAA (75.00%) and BAP 0.5 + NAA 1.5 mg L$^{-1}$ (72.92%). The lowest 70.83% callusing was observed with 0.5 mg L$^{-1}$ BAP + 1.0 mg L$^{-1}$ NAA. Significantly maximum mean callus weight 0.94 g per explant was measured by the treatment combination 0.5 mg L$^{-1}$ BAP + 2.0 mg L$^{-1}$ NAA, followed by 0.79 g per explant under 1.0 mg L$^{-1}$ BAP + 1.5 mg L$^{-1}$ NAA and 0.68 g per explant under 1.0 mg L$^{-1}$ BAP + 2.0 NAA mg L$^{-1}$. These treatment combinations also differed significantly from each other. However, a least callus fresh weight of 0.51 g per explant was produced with BAP 0.5 + NAA 1.0 mg L$^{-1}$. Friable whitish light green callus type was recorded in all the treatment combinations using NAA in conjugation with BAP.

In cv. "Flirt" perusal of data reveal that significantly minimum days 16.75 to callus initiation was recorded in medium fortified by 1.0 mg L$^{-1}$ BAP + 1.5 mg L$^{-1}$ NAA· followed by 17.77, 19.00 and 19.46 days with 1.0 mg L$^{-1}$ BAP + 2.0 mg L$^{-1}$ NAA, 1.0 mg L$^{-1}$ BAP + 1.0 NAA mg L$^{-1}$ and 0.5 mg L$^{-1}$ BAP + 2.0 NAA mg L$^{-1}$, respectively. However, maximum number of days 24.45, followed by 23.00 to callus initiation was recorded by 0.5 mg L$^{-1}$ BAP + 1.0 mg L$^{-1}$ NAA and 0.5 mg L$^{-1}$ BAP + 1.5 NAA mg L$^{-1}$, respectively. Callus induction following 4 weeks of culture was found significantly maximum 83.33% in MS media fortified with 0.5 mg L$^{-1}$ BAP + 2.0 mg L$^{-1}$ NAA. This treatment combination was followed by 1.0 mg L$^{-1}$ BAP + 1.5 mg L$^{-1}$ NAA (81.24%), 1.0 mg L$^{-1}$ BAP + 2.0 mg L$^{-1}$ NAA (79.16%), 1.0 mg L$^{-1}$ BAP + 1.0 NAA mg L$^{-1}$ (70.83%), and 0.5 mg L$^{-1}$ BAP + 1.5 NAA mg L$^{-1}$ (66.66%). The lowest 64.57% callusing was observed with 0.5 mg L$^{-1}$ BAP + 1.0 NAA mg L$^{-1}$. Significantly maximum mean callus weight 0.89 g per explant was measured under 0.5 mg L$^{-1}$ BAP + 2.0 NAA mg

L$^{-1}$, followed by 0.69 g per explant under 1.0 mg L$^{-1}$ BAP + 1.5 mg L$^{-1}$ NAA and 0.85 g per explants under 1.0 mg L$^{-1}$ BAP + 2.0 mg L$^{-1}$ NAA. These treatments also differed significantly from each other. The treatment mixture of 0.5 mg L$^{-1}$ BAP + 1.0 mg L$^{-1}$ NAA resulted in a significantly lower FW of callus 0.50 g per explant. Callus type in cv. "Flirt" was noticed similar to cv. "Candid" having friable whitish light cream colored callus in NAA containing treatments in conjunction with BAP.

## Regeneration and organogenesis

In the current study, two explants (petals and leaf segments) of cv. "Candid" and "Flirt" were employed to regenerate. However, in exploratory trials, leaf segments of both cultivars failed to regenerate, so they were omitted from the final experiment as explant. In the present investigation, various growth regulators were used to study the influence on the callus' regeneration potential obtained from both the cultivar petal explant.

## Shoot regeneration from petal-derived callus of cv. "Candid" and "Flirt"

The data in the Table 6 show the percentage of petal-derived calli arising shoots and the quantity of shoots per callus in chrysanthemum (*Dendranthemum grandiflorum* Kitam) cv. "Candid." Significantly superior 89.58% calli-producing shoots were noticed under the PGR combination 1.00 mg L$^{-1}$ kinetin + 0.50 mg L$^{-1}$ IAA, preceded by 87.50, 83.33, 81.25, 79.17 and 75.00% under the treatment combinations 1.0 mg L$^{-1}$ BAP + 0.5 NAA mg L$^{-1}$, 2.0 mg L$^{-1}$ BAP + 0.5 NAA mg L$^{-1}$, 2.0 mg L$^{-1}$ Kinetin + 0.5 NAA mg L$^{-1}$, 2.0 mg L$^{-1}$ BAP + 0.5 IAA mg L$^{-1}$, and 1.0 mg L$^{-1}$ BAP + 0.5 IAA mg L$^{-1}$, correspondingly (Fig. 2a, b, e). All these treatments differed significantly among themselves. Whereas,

**Table 6** The impact of combinations of plant growth regulators on shoot regeneration in petal-derived callus of Chrysanthemum "Candid" and Flirt

| MS + PGRS | Calli-producing shoots (%)[A] | | Shoot number per callus | |
|---|---|---|---|---|
| | Candid | Flirt | Candid | Flirt |
| BAP (1.00 mg L$^{-1}$) + NAA (0.50 mg L$^{-1}$) | 87.50$^{ab}$ (9.35) | 83.33$^{ab}$ (9.13) | 2.75$^{ab}$ | 2.25$^{ab}$ |
| BAP (2.00 mg L$^{-1}$) + NAA (0.50 mg L$^{-1}$) | 83.33$^{bc}$ (9.13) | 81.24$^{b}$ (9.01) | 2.50$^{abc}$ | 1.75$^{bc}$ |
| Kinetin (1.00 mg L$^{-1}$) + NAA (0.50 mg L$^{-1}$) | 70.83$^{e}$ (8.41) | 66.66$^{e}$ (8.16) | 1.25$^{e}$ | 0.25$^{e}$ |
| Kinetin (2.00 mg L$^{-1}$) + NAA (0.50 mg L$^{-1}$) | 72.92$^{e}$ (8.53) | 70.83$^{de}$ (8.41) | 1.50$^{de}$ | 0.50$^{de}$ |
| BAP (1.00 mg L$^{-1}$) + IAA (0.50 mg L$^{-1}$) | 75.00$^{de}$ (8.66) | 72.91$^{d}$ (8.53) | 1.75$^{cde}$ | 0.75$^{de}$ |
| BAP (2.00 mg L$^{-1}$) + IAA (0.50 mg L$^{-1}$) | 79.17$^{cd}$ (8.89) | 75.00$^{cd}$ (8.66) | 2.00$^{bcde}$ | 1.00$^{de}$ |
| Kinetin (1.00 mg L$^{-1}$) + IAA (0.50 mg L$^{-1}$) | 89.58$^{a}$ (9.46) | 87.49$^{a}$ (9.35) | 3.25$^{a}$ | 2.75$^{a}$ |
| Kinetin (2.00 mg L$^{-1}$) + IAA (0.50 mg L$^{-1}$) | 81.25$^{c}$ (9.01) | 79.16$^{bc}$ (8.89) | 2.25$^{bcd}$ | 1.25$^{cd}$ |

Figures in the parenthesis are square root transformed value of the percentage data

Means with different alphabets differ significantly

[A]Data recorded after 8 weeks of culture

**(A)**

**(B)**

**(C)**

**(D)**

**(E)**

**(F)**
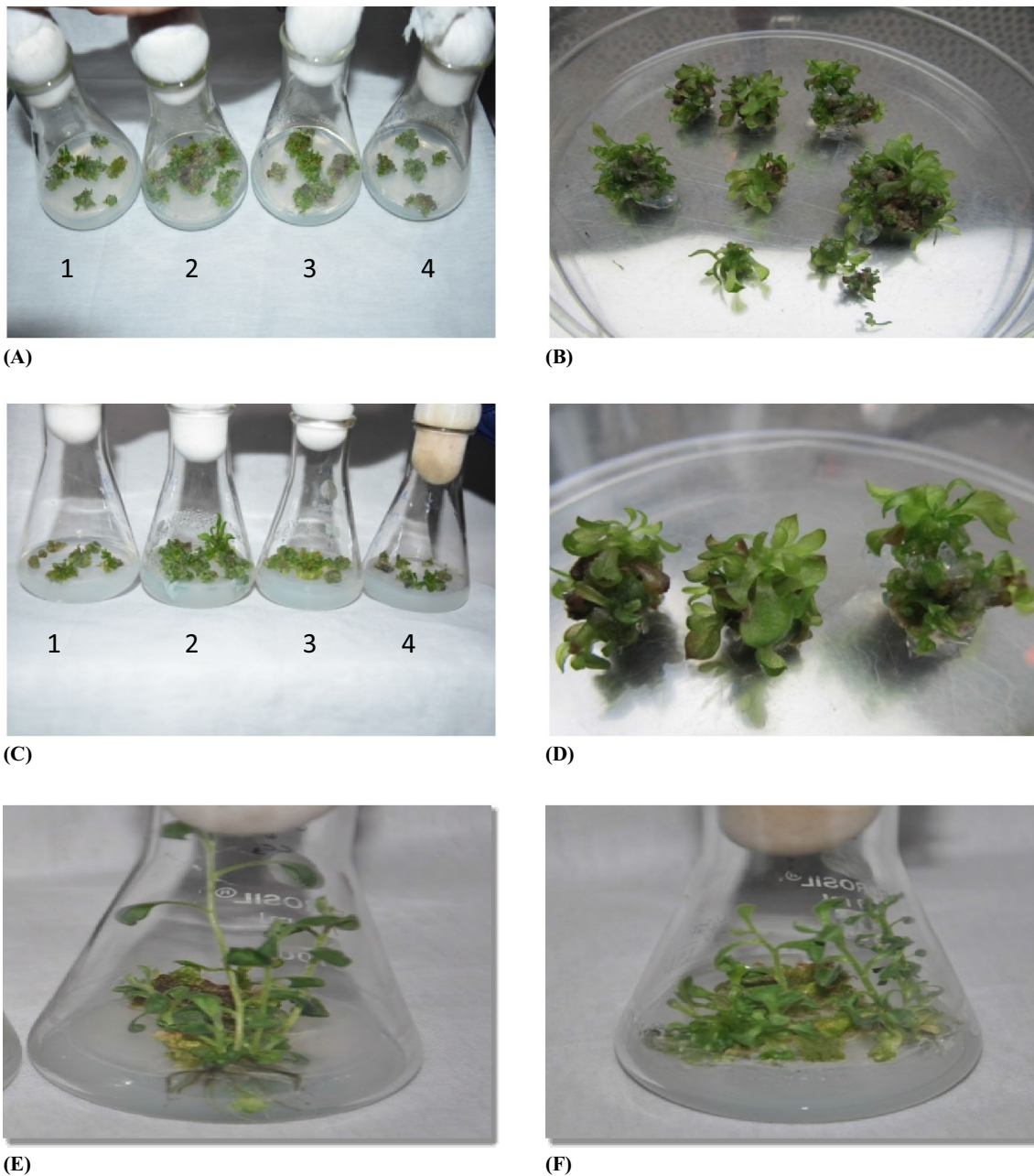
**Fig. 2 a** Regeneration in petal explant cv. "Candid" (1) Kinetin 1.00+IAA 0.50 mg L$^{-1}$ (2) BAP 1.00+NAA 0.50 mg L$^{-1}$ (3) BAP+NAA=2.00+0.50 mg L$^{-1}$ (4) kinetin 2.00+NAA 0.50 mg L$^{-1}$. **b** Regeneration in petal explant cv. "Candid", Kinetin 1.00+IAA 0.50 mg L$^{-1}$. **c** Regeneration in petal explant cv. "Flirt" (1) Kinetin 1.00+IAA 0.50 mg L$^{-1}$ (2) BAP 1.00+NAA 0.50 mg L$^{-1}$ (3) BAP+NAA=2.00+0.50 mg L$^{-1}$ (4) Kinetin 2.00+NAA 0.50 mg L$^{-1}$. **d** Regeneration in petal explant cv. "Flirt", Kinetin 1.00+IAA 0.50 mg L$^{-1}$. **e** Regenerated plantlet in petal explant cv. "Candid", Kinetin 1.00+IAA 0.50 mg L$^{-1}$. **f** Regenerated plantlet in petal explant cv. "Flirt", Kinetin 1.00+IAA 0.50 mg L$^{-1}$

treatment combination 1.0 mg L$^{-1}$ Kinetin + 0.5 NAA mg L$^{-1}$ recorded significantly lowest 70.83% calli produced shoots, followed by 72.92% under treatment combination 2.0 mg L$^{-1}$ Kinetin + 0.5 NAA mg L$^{-1}$. Regarding the shootlet number per callus, significantly highest mean 3.25 shoots per callus were depicted in cultures inoculated in medium supplied by 1.0 mg L$^{-1}$ kinetin + 0.5 IAA mg L$^{-1}$ followed by 1.0 mg L$^{-1}$ BAP + 0.5 mg L$^{-1}$ NAA, 2.0 mg L$^{-1}$ BAP + 0.5 mg L$^{-1}$ NAA, 2.0 mg L$^{-1}$ Kinetin + 0.5 mg L$^{-1}$ NAA, 2.0 mg L$^{-1}$ BAP + 0.5 mg L$^{-1}$ IAA, and 1.0 mg L$^{-1}$ BAP + 0.5 mg L$^{-1}$ IAA recording 2.75, 2.50, 2.25, 2.00 and 1.75 shoots per callus, respectively. Whereas, treatment combination 1.0 mg L$^{-1}$ Kinetin + 0.5 NAA mg L$^{-1}$ recorded significantly lowest 1.25 shoots callus$^{-1}$, followed by 1.50

shoots under the treatment combination 2.0 mg L$^{-1}$ Kinetin + 0.5 mg L$^{-1}$ NAA.

Regeneration from petal-derived callus in cv. "Flirt" revealed the similar trend as that of cv. "Candid". Significantly superior 87.49% calli-producing shoots were noticed under the treatment combination 1.0 mg L$^{-1}$ kinetin + 0.5 mg L$^{-1}$ IAA, preceded by the conjugations of 1.0 mg L$^{-1}$ BAP + 0.5 mg L$^{-1}$ NAA, 2.0 mg L$^{-1}$ BAP + 0.5 mg L$^{-1}$ NAA, 2.0 mg L$^{-1}$ Kinetin + 0.5 mg L$^{-1}$ NAA, 2.0 mg L$^{-1}$ BAP + 0.5 mg L$^{-1}$ IAA, and 1.0 mg L$^{-1}$ BAP + 0.5 mg L$^{-1}$ NAA registering 83.33, 81.24, 79.16, 75.00 and 72.91%, respectively (Fig. 2c, d, f). All these treatments differed significantly among themselves. Whereas, treatment combination 1.0 mg L$^{-1}$ Kinetin + 0.5 mg L$^{-1}$ NAA recorded significantly lowest 66.66% calli produced shoots, followed by 70.83% under the treatment combination 2.0 mg L$^{-1}$ Kinetin + 0.5 mg L$^{-1}$ NAA. Regarding shoot number per callus, significantly maximum shootlets 2.75 callus $^{-1}$were recorded in cultures inoculated in medium supplied by 1.0 mg L$^{-1}$ Kinetin + 0.5 mg L$^{-1}$ IAA. This treatment combinations was preceded by 1.0 mg L$^{-1}$ BAP + 0.5 mg L$^{-1}$ NAA, 2.0 mg L$^{-1}$ BAP + 0.5 mg L$^{-1}$ NAA, 2.0 mg L$^{-1}$ Kinetin + 0.5 mg L$^{-1}$ NAA, 2.0 mg L$^{-1}$ BAP + 0.5 mg L$^{-1}$ IAA, and 1.0 mg L$^{-1}$ BAP + 0.5 mg L$^{-1}$ IAA , recording 2.25, 1.75, 1.25, 1.00 and 0.75 shoots per callus, respectively. Whereas, treatment combination 1.0 mg L$^{-1}$ Kinetin + 0.5 mg L$^{-1}$ NAA recorded significantly lowest 0.25 shoots per callus, followed by 0.50 shoots under the treatment combination 2.0 mg L$^{-1}$ Kinetin + 0.5 mg L$^{-1}$ NAA.

## Discussion

### Callusing of petals of cv. "Candid" and "Flirt"

All of the G.R. combinations tested using petal explants resulted in callus formation in both cultivars. As shown in the data (Table 4) there were substantial changes in callusing parameters, such as days to callus initiation, callus induction percentage, and callus weight per explant, under the impact of different G.R combinations. Callus induction was significantly better in petal explants than other explants in both cultivars. BAP at 1.00 mg L$^{-1}$ in conjugation with NAA at 1.50, 2.00, or 1.00 mg L$^{-1}$ recorded significantly minimum days to callus initiation and highest callus per cent compared to BAP at 0.50 mg L$^{-1}$ in combination with NAA at all the three concentrations in both the cultivars. Callusing is universally acclaimed as pre-determined uncontrolled multiplication and de-differentiations of cells. This behavior is triggered by a high auxin/cytokinin ratio; the magnitude and effectiveness depend on the plant species and growing conditions. The observations are in compliance with those of Mandal et al. (2000) who found that 4.0 mg L$^{-1}$ BAP and 1.0 mg L$^{-1}$

NAA enriched MS media induced the most callus formation. The most effective callus production was observed in MS media supplied with 13.3 μM BA and 0.5 μM 2, 4-D, after many attempts with various combinations of BA and 2, 4-D were made (Thangmanee and Kanchanapoom 2011). Similarly, Verma et al. (2012b) observed the maximum callus induction on MS media supplied with BAP 4.0 mg L$^{-1}$ and NAA 1.0 mg L$^{-1}$. Explant of Chrysanthemum with high frequency of shoot bud production on MS media supplemented with 23.23 μM Kinetin and 5.37 μM NAA showed callus induction, as reported by Mandal et al. (2000). When node and young leaf explants of two cultivars of chrysanthemum "Snow Ball" and "Miss Universe" were cultured on MS basal media supplied with varying concentrations of IAA, NAA, 2,4-D, BAP, and kinetin, alone or in combination, an improved result of callus induction and growth was observed when compared to intermodal explants. While as highest callus induction was recorded in capitulum explants when supplemented with 2 mg L$^{-1}$ BAP + at 0.2 mg L$^{-1}$ NAA (Kumari et al. 2001, 2003). Lakshmi et al. (2006) recorded the highest callusing in petal segment and flower bud explants of chrysanthemum cv. "PKV Shubhra" on MS media under treatment combination 0.1 mg L$^{-1}$ BAP + 0.1 mg L$^{-1}$ NAA.

Maximum callus production was observed on media having 1.50 mg L$^{-1}$ NAA in conjunction with 1.00 mg L$^{-1}$ BAP. Auxins that aids in cell division are also known to enhance cell volume through a process termed "acid growth," in which the cell wall loosens due to acidification, authorizing for volume expansion via increased water uptake. Increased cell volume results in an increase in individual cell weight, which increases the callus's weight. Lee et al. (1979) reported the highest production of callus fresh weight when shoot apices (1.0 × 0.5 mm) of chrysanthemum cv. "Shin Dong Ah" were cultured on MS medium fortified with 0.5 ppm Kinetin + 1.0 ppm NAA.

### Callusing of leaf segments of cv. "Candid" and "Flirt"

Compared to petal explant, the time it took for a callus to develop and emerge was shorter (Table 5). The eventual callus growth and inductions in callus weight per explants were comparatively subdued. Due to the NAA's potential to form conjugates with other compounds found in plant systems (Mohr and Schopfer 1995), it becomes less effective, which may account for its decreased efficacy demonstrated in the current study. The results regarding callusing in leaf segments were in conformity with Widiastoety (1987) who observed only 50% callus formation in leaf explant of chrysanthemum within 13–14 days. Kumari et al. (2001) obtained a better response of callus induction and growth from young leaves of 2 cultivars of chrysanthemum on MS media fortified with varying mixtures (0.5–2.0 mg L$^{-1}$) of

G.Rs (IAA, NAA, 2,4-D, BAP and kinetin). Lakshmi et al. (2006) identified MS + 0.1 mg $L^{-1}$ BAP + 0.2 mg $L^{-1}$ NAA as desirable treatment for callus induction in leaf segment explant of chrysanthemum cv. "PKV Shubhra".

## Regeneration and organogenesis

### Shoot regeneration from petal-derived callus of cv. "Candid" and "Flirt"

In petal explants of cultivar "Candid" and "Flirt", variation in means noted in terms of percent calli initiating shoots and number of shoots per callus under different G.R combinations were significant. It is quite evident from the data that petal-derived callus incubated on media combination kinetin 1.00 + IAA 0.50 mg $L^{-1}$ produced significantly highest per cent and number of shoots per callus in both the cultivars (Table 6). The reason may be benzyladenine (synthetic cytokinin) in the presence of IAA synergistically improves ethylene production as observed in mungbeans (Yoshii and Imaseki 1982). Etiolated seedlings of many species were prompted to stimulate ethylene production with kinetin alone while the notable synergistic result of Kinetin on IAA-induced ethylene production has been recorded (Fuches and Lieberman 1968). Based on the preceding arguments, it can be inferred that Kinetin is crucial for regeneration in terms of ethylene production. As a result, Kinetin exerted a beneficial effect on chrysanthemum callus regeneration. Additionally, Tymoszuk and Zalewska (2014a, b) demonstrated *in vitro* regeneration of adventitious shoots from *Chrysanthemum grandiflorum* ligulate florets on MS media enriched with BAP or Kinetin and NAA. Verma et al. (2012a, b) recorded the maximum regeneration of microshoots (95.56%) from the ray floret induced callus on MS media fortified with BAP 4.0 mg $L^{-1}$ and NAA 0.1 mg $L^{-1}$. Kulpa (2011) documented highest number of regenerated plants from inflorescence parts of "Erica" variety of chrysanthemum on MS media fortified with 2 mg.dm$^{-3}$ BAP and an auxin 5 mg.dm$^{-3}$ NAA. The maximum incidence of shoot organogenesis and the mean number of shoots per explant were found when petals were given 0.00–6.66 μM BA alone or in conjunction with 2.85–8.56 μM IAA, and 0.46 μM kinetin, as observed by Song et al. (2011) in different explants (leaves, petals, petioles and intermodal stems) of six chrysanthemum cultivars. Additionally, Nalini (2012) found that Chrysanthemum shoot tip callus regeneration on MS media supplied with Kinetin 3.0 mg $L^{-1}$ and IAA 2.0 mg $L^{-1}$ led to multiple shoot regeneration. Using *Dendranthemum grandiflorum* nodal segments grown on MS media supplied with 0.1 mg $L^{-1}$ and 0.2 mg $L^{-1}$ IAA and intermediate concentrations of 2.0 mg $L^{-1}$ and 1.0 mg $L^{-1}$ BAP, Waseem et al. (2011) created an effective plant regeneration system. Maheshwaramma et al. (2008) documented the highest regeneration frequency 61.3% in Ratlam selection with shoot tip explant culture on MS media supplied with 1.5 mg $L^{-1}$ IAA and 3.0 mg $L^{-1}$ BAP. Kashif et al. (2007), observed the best response in regeneration parameters with least concentrations of 0.1 mg $L^{-1}$ IAA, recording the highest shoot initiation, maximum shoots per explant, shoot length and number of leaves per shoot. Chitra et al. (2006) reported that when chrysanthemum nodal segments and shoot tips were cultivated on media containing MS + 2.0 mg $L^{-1}$ BAP + 1.0 mg $L^{-1}$ IBA, the occurrence of multiple shoot regeneration response was 95% and 80%, respectively. Park et al. (2005), observed maximum number of shoots per explant in chrysanthemum cultivar "Orlando", when inoculated on MS media fortified with IAA 57.1 μM, BAP 44.2 μM and kinetin 0.4 μM. Mandal et al. (2000) recorded the maximum regeneration of micro-shoots from the ray floret induced callus on MS medium fortified with 4.0 mg $L^{-1}$ BAP and 0.1 mg $L^{-1}$ NAA.

## Conclusion

Finally, by analyzing various combinations and concentrations of PGRs, an efficient technique for in vitro organogenesis of two cultivars of chrysanthemum from the explants of petals was devised in this work. The protocol is anticipated to be used for genetic transformations and even genome editing with modern tools like CRISPR/CAS9. Additionally, the multiplicity of genes involved in the precise response to variable tissue culture conditions should be explored to understand the phenomenon better.

## Declarations

**Conflict of interest** The authors have no conflict of interest.

## References

Anderson NO (2007) Flower breeding and genetics: issues, challenges and opportunities for the 21st century. Springer, Dordrecht, pp 389–437

Bajaj YPS (1992) A suggested method for in vitro long-term storage at40C of chrysanthemum and petunia germplasm. Plant Tissue Cult 3:57–58

Barakat MN, AbdelFattah RS, Badr M, EI-Torky MG (2010) In vitro culture and plant regeneration derived from ray florets of *Chrysanthemum morifolium*. Afr J Biotechnol 9(8):1151–1158

Chakrabarty D, Datta SK (2010) Management of chimera and in vitro mutagenesis for development of new flower colour/shape and chlorophyll variegated mutants in chrysanthemum. In: Datta SK, Chakrabarty D (eds) Floriculture: role of tissue culture and molecular techniques. Pointer Publishers, Jaipur, pp 157–164

Chitra R, Arulmozhiyan R, Jawaharlal M, Indhumathi K, Vadivel E (2006) Rapid multiplication of chrysanthemum (*Dendranthema grandiflora* Tzvelev) through in vitro culture. J Ornam Hortic 9(1):58–60

da Teixeira Silva JAT, Lema-Rumińska J, Tymoszuk A, Kulpa D (2015) Regeneration from chrysanthemum flowers: a review. Acta Physiol Plant 37:36. https://doi.org/10.1007/s11738-015-1773-3

Datta SK, Misra P, Mandal AKA (2005) In vitro mutagenesis—a quick method for establishment of solid mutant in chrysanthemum. Curr Sci 88(1):155–158

De Jong J, Custers JBM (1986) Induced changes in growth and flowering of chrysanthemum after irradiation and in vitro culture of pedicels and petal epidermis. Euphytica 35:37–148

Din A, Qadri ZA, Rather ZA, Mir MS, Murtaza I, Khan FA, Neelofar K, Wani MA (2018) In vitro sterilisation of different explants of chrysanthemum (*Dendranthemum morifolium* L.) cvs. "Candid" and "Flirt." Curr J Appl Sci Technol 31(5):1–14

Dwimahyani I, Widiarsih S (2010) The effects of gamma irradiation on the growth and propagation of in vitro chrysanthemum shoot explants (cv. Yellow Puma). At Indones 36(2):45–49

Fuches Y, Lieberman M (1968) Effects of Kinetin, IAA and gibberellins on ethylene production and their interactions in growth of seedlings. Physiology 43:2029–2036

Hodson de Jaramillo E, Forero A, Cancino G, Moreno AM, Monsalve LE, Acero W (2008) In vitro regeneration of three chrysanthemum (*Dendrathema grandiflora*) varieties "via" organogenesis and somatic embryogenesis. Univ Sci 13(2):118–127

Kashif W, Khan MQ, Jaffar J, Khan MS (2007) Impact of different auxins on the regeneration of chrysanthemum (*Dendranthema morifolium*) through in vitro shoot tip culture. Pak J Agric Res 20(1/2):51–57

Kaul V, Miller RM, Hutchinson JF, Richards D (1990) Shoot regeneration from stem and leaf explants of *Dendranthema grandiflora* Tzvelev (syn *Chrysanthemum morifolium* Ramat.). Plant Cell Tissue Organ Cult 21:21–30

Kengkarj P, Smitamana P, Fujime Y (2008) Assessment of somaclonal variation in chrysanthemum (*Dendranthema grandiflora* Kitam.) using RAPD and morphological analysis. Plant Tissue Cult Biotechnol 18(2):139–149

Khalid N, Davey MR, Power JB (1989) An assessment of somaclonal variation in *Chrysanthemum morifolium*: the generation of plants of potential commercial value. Sci Hortic 38:287–294

Kulpa D (2011) Plant regeneration in inflorescence culture of chrysanthemum (*Dendranthema × grandiflora* (Ramat.) Kitamura). J Food Agric Environ 9(1):715–718

Kumari M, Varghese TM, Mehta PK, Kumari M (2001) In vitro plant regeneration of chrysanthemum through callus culture. Ann Agric Res 22(4):514–519

Kumari M, Varghese TM, Kumari M (2003) Efficient in vitro regeneration of plantlets from capitulum explant in chrysanthemum cultivars Miss Universe and Snow Ball. J Ornam Hortic New Ser 6(4):316–321

Lakshmi MK, Patil SR, Chakrapani K, Kalamkar VB, Lende SR (2006) Studies on callus induction and differentiation in chrysanthemum (*Dendranthema grandiflora*). J Soils Crops 16(2):324–330

Latado RR, Adames AH, Neto AT (2004) In vitro mutation of chrysanthemum (*Dendranthema grandiflora* Tzvelev) with ethylmethanesulphonate (EMS) in immature floral pedicels. Plant Cell Tissue Organ Cult 77:103–106

Lee JK, Paek KY, Chun CK (1979) In vitro propagation of chrysanthemum through shoot apical meristem culture. J Korean Soc Hortic Sci 20(2):192–199

Lee YM, Kang EJ, Sung SY, Kim SH, Ha BK, Kim DS, Kim JB, Kang SY (2013) The effects of plant growth regulators on plant regeneration and direct shoots formation of petal explants of chrysanthemum flower colour mutants varieties, 'ARTI-purple' and 'ARTI-queen.' Korean J Hortic Sci Technol 31(3):359–365 (**in Korean with English abstract**)

Levin R, Gaha V, Tal B, Hirsh S, Denola D, Vasil I (1988) Automated plant tissue culture for mass propagation. Biotechnology 6:1035–1040

Maheshwaramma S, Reddy SS, Reddy DL (2008) Studies on effect of hormonal concentration and type of explant for in vitro regeneration of chrysanthemum cultivar Ratlam selection. Progress Res 3(1):19–21

Mandal AKA, Datta SK (2005) Direct somatic embryogenesis and plant regeneration from ray florets of chrysanthemum. Biol Plant 49(1):29–33. https://doi.org/10.1007/s10535-005-0033-6

Mandal AKA, Chakrabarty D, Datta SK (2000) In-vitro isolation of solid novel flower colour mutants from induced chimeric ray florets of chrysanthemum. Euphytica 114:9–12

Mani T, Senthil K (2011) Multiplication of *Chrysanthemum* through somatic embryogenesis. Asian J Pharm Sci 1(1):13–16

Matsumura A, Nomizu T, Furutani N, Hayashi K, Minamiyama Y, Hase Y (2010) Ray florets color and shape mutants induced by 12C5? Ion beam irradiation in chrysanthemum. Sci Hortic 123(4):558–561

Miler N, Muszczyk P (2013) Regeneration of callus and shoots from ovules and ovaries of chrysanthemum in vitro. In: 8th international symposium on in vitro culture and horticultural breeding, Coimbra, Portugal, book of abstracts: 58, June 2–7

Misra P, Datta SK (2007) Standardization of in vitro protocol in *Chrysanthemum* cv. Madam E Roger for development of quality planting material and to induce genetic variability using radiation. Indian J Biotechnol 6:121–124

Mohr H, Schopfer D (1995) Plant physiology. Springer, Berlin, p 386

Nahid JS, Shyamali S, Kazumi H (2007) High frequency shoot regeneration from petal explants of *Chrysanthemum morifolium* Ramat. In vitro. Pak J Biol Sci 10:3356–3361

Nalini R (2012) Micropropagation of chrysanthemum (*Chrysanthemum morifolium*) using shoot tip as explant. Int J Food Agric Vet Sci 2(2):62–66

Park S, Kim G, Jeong BR (2005) Adventitious shoot regeneration in chrysanthemum as affected by plant growth regulators, sucrose and dark period. J Korean Soc Hortic Sci 46(5):335–340

Park HP, Kim GH, Jeong BR (2007) Adventitious shoot regeneration from cultured petal explants of Chrysanthemum. Hortic Environ Biotechnol 48(6):387–392

Qadri ZA, Masoodi NH, Din A, Wani MA (2018) In vitro Callussing of Carnation (Dianthus caryophyllus L.) cv. Scania and Indios. Curr J Appl Sci Technol 27(1):1–10. https://doi.org/10.9734/CJAST/2018/39618

Song JY, Mattson NS, Jeong BR (2011) Efficiency of shoot regeneration from leaf, stem, petiole and petal explants of six cultivars of *Chrysanthemum morifolium*. Plant Cell Tissue Organ Cult 107:295–304

Sung SY, Lee YM, Kim SH, Ha BG, Kang SY, Kim JB, Kim DS (2013) Comparative analysis of growth antioxidant enzyme activities from two chrysanthemum varieties, 'ARTI-purple'and 'ARTI-queen' by chronic irradiation of gamma-ray. Kor J Hortic Sci Technol 31(4):490–495 (**in Korean with English abstract**)

Tanaka K, Kanno Y, Kudo S, Suzuki M (2000) Somatic embryogenesis and plant regeneration in chrysanthemum [Dendranthema grandiflorum (Ramat.) Kitamura]. Plant Cell Rep 19:946–953

Teixeira da Silva JA (2003) Chrysanthemum: advances in tissue culture, cryopreservation, postharvest technology, genetics and transgenic biotechnology. Biotechnol Adv 21:715–766

Teixeira da Silva JA, Kulus D (2014) Chrysanthemum biotechnology: discoveries from the recent literature. Folia Hortic 26(2):67–77

Thangmanee C, Kanchanapoom K (2011) Regeneration of Chrysanthemum plants (Chrysanthemum × grandiflorum (Ramat.) Kitam.) by callus derived from ray floret explants. Propag Ornam Plants 11(4):204–209

Tymoszuk A, Zalewska M (2014a) In-vitro adventitious shoots regeneration from ligulate florets in the aspect of application in *Chrysanthemum* breeding. Acta Scientiarum Polonorum Hortorum Cultus 13(2):45–58

Tymoszuk A, Zalewska M (2014b) Biological factors affecting regeneration of adventitious shoots from in vitro isolated ligulate florets of chrysanthemum. Acta Sci Pol Hortorum Cultus 13(3):155–165

Verma AK, Prasad KV, Janakiram T, Kumar S (2012a) Standardization of protocol for pre-treatment, surface sterilization, regeneration, elongation and acclimatization of *Chrysanthemum morifolium* Ramat. Int J Hortic 2(3):7–12

Verma AK, Prasad KV, Singh SK, Kumar S (2012b) In vitro isolation of red coloured mutant from chimeric ray florets of chrysanthemum induced by gamma-ray. Indian J Hortic 69(4):562–567

Vilasini P, Latipah Z (2000) Somaclonal variation in *Chrysanthemum morifolium* generated through petal cultures. J Trop Agric Food Sci 28(2):115–120

Waseem K, Jilani MS, Jaskani MJ, Khan MS, Kiran M, Khan GU (2011) Significance of different plant growth regulators on the regeneration of chrysanthemum plantlets (*Dendranthema morifolium* L.) through shoot tip culture. Pak J Bot 43(4):1843–1848

Widiastoety D (1987) Preliminary experiment on tissue culture of *Chrysanthemum morifolium*. Buletin Penelitian Hortikultura 15:231–236

Yoshii H, Imaseki H (1982) Regulation of auxin-induced ethylene biosynthesis. Repression of inductive formation of 1-amino-cyclopropane-1-carboxylate synthase by ethylene. Plant Cell Physiol 23:639–649

Zalewska M, Lema-Rumińska J, Miler N (2007) In vitro propagation using adventitious buds technique as a source of new variability in chrysanthemum. Sci Hortic 113:70–73

Zalewska M, Lema-Rumińska J, Miler N, Gruszka M, Dąbal W (2011) Induction of adventitious shoot regeneration in chrysanthemum as affected by the season. In Vitro Cell Dev Biol Plant 47:375–378. https://doi.org/10.1007/s11627-010-9330-7