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Callus Induction and Plant Regeneration of *Chrysanthemum morifolium* and *C. coccineum* via Direct and Indirect Organogenesis and Genetic Fidelity Analysis Using IRAP, ISSR and SCoT Molecular Markers

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Abstract

In vitro propagation of *C. morifolium* cv. 'Homa' and cv. 'Delkash' and wild *C. coccineum* via direct and indirect organogenesis and somatic embryogenesis were investigated. BAP at 0, 1, 2 and 3 mg l^{-1} or NAA at 0, 0.05, 0.1 and 0.2 mg l^{-1} concentrations were used to induce direct and indirect organogenesis of shoot tip explants. To study the callus induction and somatic embryogenesis, the young leaf explants were cultured on MS medium containing BAP (0, 1, 2 or 3 mg l^{-1}) and 2,4-D (0, 1, 2 or 3 mg l^{-1}). Direct shoot regeneration was achieved from shoot tip explants of 'Homa' and 'Delkash' as well as *C. coccineum*. The highest number of shoots through direct regeneration (13.78 and 8.89 shoots per explant for *C. coccineum* and *C. morifoilum* 'Homa', respectively) were observed in the treatment with 2 mg l^{-1} BAP and 0.05 mg l^{-1} NAA. In both species, the highest frequency of callus formation and embryogenesis were obtained on medium containing 2.0 mg l^{-1} 2,4-D and 2 mg l^{-1} BAP. Genetic fidelity of 10 acclimatized plants derived from direct regeneration of each species was confirmed using six inter-retrotransposon amplified polymorphism (IRAP), inter-simple sequence repeat (ISSR) and start codon targeted (SCoT) primers. A total of 56, 56 and 39 fragments were amplified for IRAP, ISSR, and SCoT, respectively. In general, our results showed that finding a better response of explants to embryogenesis or organogenesis in a specific cultivar and with special PGRs combinations and concentrations play an important role in the *in vitro* propagation efficiency of chrysanthemum species.

Keywords: Callus induction, Cultivar response, Direct regeneration, Embryogenesis, Genetic fidelity.

Abbreviations:
2,4-D 2,4- dichlorophenoxy acetic acid;
BAP N6-benzylaminopurine;
IRAP Inter Retrotransposon Amplified Polymorphism;
ISSR Inter Simple Sequence Repeat;
MS Murashige and Skoog;
NAA a-Naphthalene acetic acid;
PGRs Plant growth regulators;
SCoT Start Codon Targeted Polymorphism.

INTRODUCTION

Chrysanthemum is an important genus within the Asteraceae family which is comprised of about 40 species, including economically valuable species, mostly used as ornamentals and insecticides. Among these species, *C. morifolium* is one of the most valuable commercial cut flowers with the second highest demand in the global flower market just one position behind the top-ranked rose (Kumar *et al*., 2006). The elegant appearance of leaves and flowers along with a wide color range of petals make chrysanthemum one of the growers' first choices. Besides, long vase life accounts for its extensive use as cut flowers in floral arrangements. On the other hand, Persian pyrethrum (*C. coccineum*) is a perennial and rosette plant whose dried flowers are commercially used to produce a natural plant insecticide known as pyrethrum or Persian Insect Powder (Wandahwa *et al*., 1996).

Chrysanthemums propagation is carried out by conventional methods such as the use of root suckers or terminal cuttings, but these are slow processes. Furthermore, they are inadequate to support large-scale production for commercial purposes. In addition, the transmission of the virus and other diseases to propagated plant is still a challenge for the propagation of disease-free plant materials in chrysanthemum (Teixeira da Silva *et al*., 2013).

Pyrethrums are propagated by division or shoot cuttings, but their multiplication by division usually leads to a low rate of multiplication, and the propagation efficiency is only 5-10 daughter plants per stock plant (Hitmi *et al*., 1998). Another problem is the high sensitivity of Persian pyrethrum to the root-knot nematode (*Meloidogyne hapla*), which is usually transmitted from infected stock plants to propagated clones (Florence and Rangan, 1981).

The modern methods of plant propagation based on tissue culture techniques can improve the efficiency of plant propagation processes and provide rapid reproduction of clonal and superior genotypes (Waseem *et al*., 2009; Teixeira da Silva, 2014; Shahzad *et al*., 2017). As an alternative approach to traditional vegetative propagation systems, in direct organogenesis, plants are regenerated directly from existing meristems or non-meristematic centers such as internodes, leaves, and roots (Satish *et al*., 2015) to produce true-to-type daughter plants. The production of regenerated plants through indirect organogenesis is not only applicable to propagate plant material, but it is also a possible method to improve the genetic diversity of chrysanthemum species to introduce useful traits or produce new cultivars (Kengkarj *et al*., 2008; Miler and Zalewska, 2014). Today, the use of molecular markers is the most important part of a set of tools for assessing the genetic diversity of plants and the populations of endangered species, and their use in evaluations and genetic fidelity analysis is very important in plants (Petersen and Seberg, 1998). The evaluation of genetic uniformity and somaclonal variations of *in vitro* derived plants via more than one marker system has been recommended to be beneficial as each marker system can cover and target different regions of a plant species genome (Thakur *et al*., 2016).

There are numerous reports on adventitious shoot regeneration from various explants of chrysanthemum, which have been mainly taken place via the callus phase (Teixeira da Silva, 2004; Song *et al*., 2011; Miler and Zalewska, 2014). However, the efficiency of plant propagation for direct regeneration is relatively low, propagated plants are usually true-to-type and there is a lack of any mutation or somaclonal variation. On the other hand, indirect regeneration methods represent higher regeneration ability to produce multiple shoots within a short time course than direct regeneration. In the previous studies, the effects of different types of explants such as leaf (Himstedt *et al*., 2001; Miler and Zalewska, 2014), pedicle (Petty *et al*., 2003), protoplast (Sauvadet *et al*., 1990), shoot bud (Waseem *et al*., 2009), and stem (Jevremovic and Radojevic, 2004) have been evaluated for the generation of the callus and regeneration of shoots.

Although the micropropagation of chrysanthemum has been already well-established and studied (Teixeira da Silva 2004, 2014; Teixeira da Silva *et al*., 2013), there are still uncovered items regarding its regeneration responses to several important *in vitro* factors that need further investigation. Establishment of a high-performance regeneration system in the *in vitro* culture is a

prerequisite and important step before starting any selection under *in vitro* conditions. On the other hand, the introduced protocols for a cultivar are not necessarily consistent with other cultivars because each cultivar has its own reaction to *in vitro* conditions. In this regard, the key issue for chrysanthemum breeders is to quickly respond to recent market demands in order to create new and attractive cultivars every year, so multiplication protocols should be considered for each new cultivar. In addition, the establishment of an effective and efficient *in vitro* protocol for generation and discrimination of true-to-type micropropagated chrysanthemum and Persian pyrethrum plants without any somaclonal variations is crucial to supply homogenous stock propagules.

Therefore, the present study was designed to: i) investigate the individual and interactive effects of N6-benzylaminopurine (BAP) and naphthalene acetic acid (NAA) on direct and indirect organogenesis; ii) to study the influence of 2,4- dichlorophenoxy acetic acid (2,4-D) and BAP and their interaction on embryogenic callus induction and proliferation of three newly introduced chrysanthemum cultivars (*C. morifolium*) as well as Persian pyrethrum (*C. coccineum*), and iii) to analyze clonal genetic identity of *in vitro* micropropagated plants derived from direct regeneration using IRAP, ISSR and SCoT marker systems.

MATERIALS AND METHODS

Plant materials

The cuttings of three newly-released cultivars of *Chrysanthemum morifolium* ('Homa' and 'Delkash') were received from National Institute of Ornamental Plants (NIOP), Mahallat, Iran. The seeds of *C. coccineum* wild species were gathered from northern regions of Guilan Province, Iran (1420 m altitude, 35°16'51.4"N 46°59'46.5"E). Prior to seed sowing and explant preparation, *C. coccineum* was taxonomically verified by comparison to a voucher specimen at the Agriculture and Natural Resources Research Centre of Kurdistan, Iran. The *C. morifolium* cuttings and *C. coccineum* seeds were then transferred to the research lab and research greenhouse of the University of Kurdistan for further experimentation.

Explant source and sterilization

Shoot and leaves of chrysanthemum cultivars were taken from rooted cuttings under greenhouse conditions during April and May 2016 (temperature 22-28°C, RH 80-90% and PAR 850–1000 µmol $m^{-2}s^{-1}$). The shoot tips and leaves were first washed with distilled water containing tween 20 (0.15 w/v) for 10 min and they were then surface sterilized by immersion 1% sodium hypochlorite solution for 12 min. Explants were rinsed three times (each 30 s) in sterilized distilled water. The fragments of the shoot tips (0.7-1 cm) and leaf disc (1×1 cm) were cultured on the prepared media. The explants were incubated at 25±3°C under a 16/8h light-dark photoperiod at an irradiance intensity of 35-40 µmol m^{-2} s^{-1}. Base MS medium containing 30 g sucrose and 0.6% agar with pH 5.8 was used for all experiments.

Direct and indirect organogenesis

For the induction of direct or indirect organogenesis in *C. morifolium* and *C. coccineum*, the shoot tip explants were cultured on the MS medium supplemented with 0, 1, 2 and 3 mg l^{-1} BAP and 0, 0.05, 0.1 and 0.3 mg l^{-1} NAA, alone or in combination. The explants were weekly subcultured on fresh media with the respective PGRs and concentrations. After 9 weeks, the number of adventitious shoots, regeneration percentage, shoot length (cm), and days required to develop regenerated shoots were recorded.

Embryogenic callus induction

To induce embryogenic calli in both species, leaf explants were incubated on MS medium containing different concentrations of 2,4-D (0, 1, 2 and 3 mg l^{-1}) and BAP (0, 1, 2 and 3 mg l^{-1})

individually or in combination. After 4-6 weeks of incubation, the callus formation (%), the number of somatic embryos, and somatic embryogenesis percentage were recorded. Increased callus mass was recorded as weight gain after 8 weeks of incubation.

Callus proliferation and shoot regeneration

To study the simple and interactive effects of BAP (0 and 2 mg l^{-1}) and NAA (0, 0.05 and 0.1 mg l^{-1}) on callus proliferation and shoot regeneration, the calli with somatic embryos (yellowish and compact calli differentiated into various stages of embryogenesis) on their surfaces derived from the leaf explants were divided into small pieces of about 0.5 - 0.75 cm and were then cultured on media supplemented with above-mentioned PGRs.

Root induction and acclimatization

All plantlets obtained from direct or indirect organogenesis of shoot tip and leaf explants, as well as plantlet produced by embryogenic calli, were transferred to half-strength MS medium for further growth and development. To initiate rooting in *C. morifolium*, regenerated plantlets with a mean length of 2 cm were separated from multiple shoot clumps and were then directly transferred to pots containing perlite and coco peat in a volume ratio of 1:1. The pots were covered with a transparent coating to prevent the plants from dehydration. The pots were gradually uncovered 12-14 days after the establishment of plants in the culture medium mixture. After 3-4 weeks, the survived plantlets were successively transferred to the greenhouse.

In connection with *C. coccineum*, root formation was induced by transfer of shoots (with a mean length of 4-6 cm and with one or two adventitious buds) into a hormone-free MS medium. On the other hand, we were able to produce plants within 8-9 weeks after the cultivation of explants.

DNA isolation and genetic fidelity screening

Due to possible somaclonal variations in indirectly derived plants (passed from callus phase), we considered 10 representative fully-developed and hardened plants regenerated via direct organogenesis for genetic fidelity analysis. To this end, total genomic DNA was extracted from young leaves of randomly selected plants according to CTAB-based method of Doyle and Doyle (1990). DNA quantity was evaluated spectrophotometrically at 260 and 280 nm (UV-DU 520 spectrophotometer, Beckman, USA) and its quality was checked by loading in 1% (w/v) agarose gel. A selection of six primers was used for IRAP (Kalendar *et al*., 1999), ISSR (Meyer *et al*., 1993) and SCoT (Collard and Mackill, 2009) markers (Table 6). PCR amplification was carried out in a 10 µl reaction mixture consisted of 10 ng template DNA using *Taq* DNA Polymerase Mix Red (Ampliqon, Odense, Denmark) according to the manufacturer's manual. Visualization of PCR products was performed under UV light using a gel documentation system (Bio-Rad, Hercules, USA) after separation on 1.2% agarose [w /v] in 1× TAE buffer at 70 V for 70 min. Band scoring and size estimation of amplified fragments for IRAP, ISSR and SCoT markers were carried out as described by Rahmani *et al*. (2015) and Emami *et al*. (2018).

Statistical analyses

Experiments were carried out with 6 replications (defined as petri dish or test container), each replication with at least 10 explants. Thus, each treatment included 60 explants. The data collected from various treatments were subjected to analysis of variance (ANOVA). Mean separations were determined by Duncan's multiple range test at a probability level of 5% ($P< 0.05$). Analyses were performed using SAS version 9.1.

RESULTS AND DISCUSSION

Direct and indirect organogenesis

In both studied species, the initiation of shoot primordia was observed within 4-8 weeks on the surface or from the cut edges of shoot tips (Fig. 1b-c; Fig. 2c-d). The development of shoots took place three weeks after their initiation. The regeneration of adventitious shoots from shoot tip explants in the studied cultivars of *C. morifolium* as well as in *C. coccineum* is shown in Fig. 1 and 2. There have been observed differences in the ability of adventitious shoot regeneration from shoot tip explants among evaluated cultivars. In the present study, direct shoot regeneration was obtained from shoot tip explants of all *C. morifolium* cultivars and from *C. coccineum* plants without intervening callus phase. Regeneration via indirect organogenesis was achieved in all three *C. morifolium* cultivars and in *C. coccineum* plants where the number of regenerated shoots per explant was higher than direct shoot regeneration (Tables 1 and 2).

In both studied species, it was observed that in a medium free from any growth regulator, only 1.17-2.67 shoots per explant were developed. *In vitro* adventitious shoot regeneration in chrysanthemum is highly affected by the PGRs interaction, plant genotype, and types of explant (Nahid *et al*., 2007; Song *et al*., 2011; Lim *et al*., 2012; Teixeira da Silva, 2014). In *C. morifolium*, the highest average shoot number (8.89) and shoot induction percentage (34.33%) via direct organogenesis were observed in cultivar 'Homa' from shoot tip explants treated with 2 mg l^{-1} BAP and 0.05 mg l^{-1} NAA followed by 'Delkash' (27% shoot induction and 6.01 shoots per explant). The results of Table 1 show that cv. 'Delkash' had a lower mean shoot length than 'Homa'. Also, 'Delkash' showed a poor induction of shoots through indirect and direct regeneration compared to 'Homa'. Diversity in adventitious shoot formation of various cultivars has been already reported in other chrysanthemum cultivars (Teixeira da Silva, 2004; Lim *et al*., 2012; Song *et al*., 2011).

Regarding indirect organogenesis of *C.coccineum*, the maximum shoot regeneration (93%), number of shoots per explant (19.66), and the longest shoots (7.47 cm) were observed in plants treated with 2 mg l^{-1} BAP and 0.1 mg l^{-1} NAA followed by those treated with 2 mg l^{-1} BAP and 0.05 mg l^{-1} NAA with 79.04% shoot regeneration, 14.33 shoots per explant and 4.30 cm long shoots (Tables 1 and 2).

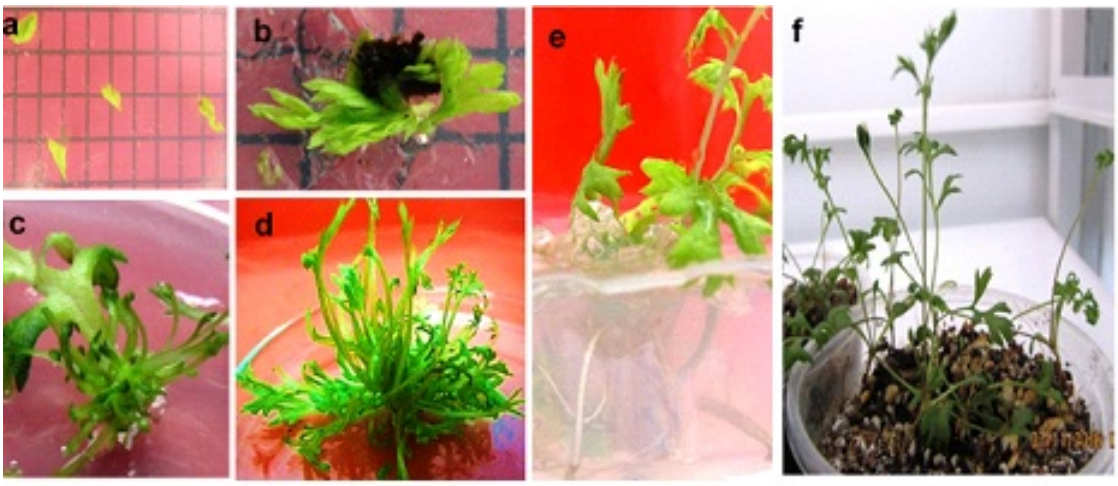

Fig. 1. Different stages of direct organogenesis from shoot tip explant of *C. coccineum*. a: Shoot tips three days after culture; b and c: Buds and shoots regenerated from the cut ends of shoot tips after 2-3 weeks on MS medium containing 2 mg l^{-1} BAP and 0.05 mg l^{-1} NAA; d: Shoot multiplication after 5-6 weeks on MS+2 mg l^{-1} BAP and 0.05 mg l^{-1} NAA; e: Rooting of shoots on hormone-free MS medium; f: Plantlets acclimatized in greenhouse.

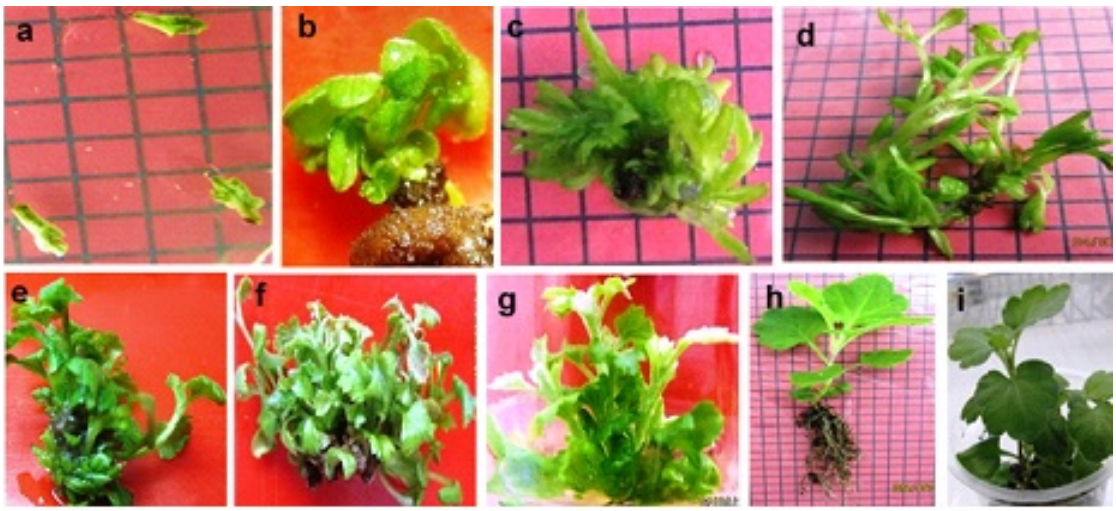

Fig. 2. Different stages of direct organogenesis from shoot tip explant of *Chrysanthemum morifolium*. a: Shoot tips three days after culture b: Shoot tips 10-12 days after culture; c and d: Buds and shoots regenerated from the cut ends of shoot tips after 3-4 weeks on MS + with 2 mg l^{-1} BAP and 0.05 mg l^{-1} NAA; e, f and g: Shoot multiplication after 4-6 weeks on MS+2 mg l^{-1} BAP and 0.05 mg l^{-1} NAA; h: Rooting of shoots in the culture medium containing perlite and coco peat mixture (1:1, v/v); i: Plantlets acclimatized in greenhouse.

With respect to direct regeneration, individual application of BAP on the media resulted in relatively poor shoot induction while its combination with NAA (particularly 2 mg l^{-1} BAP and 0.5 mg l^{-1} NAA which provided the greatest potential for shoot regeneration and shoot elongation) increased the number of shoots in all evaluated cultivars of *C. morifolium* as well as in *C. coccineum*. Therefore, the addition of exogenous auxins or other plant regulators can lead to altering and synthesis of enzymes (Gaspar *et al*., 2003), which in turn results in adjusting the concentration of endogenous hormone and the formation of shoots. In this regards, Chaudhury and Rongda (2000) reported similar results.

However, there was a significant difference in the rate of regeneration among various chrysanthemum cultivars in response to PGRs. The balance of cytokinin and auxin is obviously fundamental for efficient shoot organogenesis (Waseem *et al*., 2009) and higher levels of cytokinin than auxin is more effective for direct organogenesis on leaf explants in chrysanthemum (Gao *et al*., 2001). Our results were also in line with previous studies (Gao *et al*., 2001; Waseem *et al*., 2009; Teixeira da Silva, 2014). It seems that the content of endogenous cytokinin in chrysanthemum leaves is insufficient to induce *in vitro* shoot regeneration.

In our study, the presence of BAP in the culture medium was essential for shoot regeneration, although the increase in BAP concentration from 2 to 3 mg l^{-1} resulted in a decrease in shoot regeneration frequency average, the number of shoots, and shoot length in both *C. morifolium* and *C. coccineum* species. This disincentive effect has been also reported in other chrysanthemum cultivars, and it has been attributed to an undesirable influence of BAP on protein synthesis (Staden and Crounch, 1996).

In both chrysanthemum and Persian pyrethrum, 0.2 mg l^{-1} NAA in combination with BAP could induce indirect organogenesis, while lower concentrations of NAA (0.05-0.1 mg l^{-1}) led to direct organogenesis (Table 1). These results are in agreement with previous studies (Ali *et al*., 2005; Waseem *et al*., 2008; Teixeira da Silva, 2014) that have reported that the lower concentration of auxin is suitable for shoot regeneration in chrysanthemum. Shoot formation requires the re-initiation of cell division by adding a proper concentration of plant growth regulators to the culture medium (Benson, 2000). This can suggest that the level of endogenous hormones or their responsiveness might be variable among plant organs (Zia *et al*., 2010).

Table 1. Effect of NAA and BAP and their interaction on the shoot regeneration parameters from shoot tip explants of *C. morifolium* and *C. coccineum*.

BAP×NAA (mg l^{-1})		Shoot number (indirect)			Shoot length (cm)			Shoot number (direct)		
		C. coccineum	Homa	Delkash	*C. coccineum*	Homa	Delkash	*C. coccineum*	Homa	Delkash
0	0	2.67±0.33^{o-s}	1.67±0.57^{q-s}	1.17±0.55^{ss}	4.25±0.41^{n-q}	6.07±0.07^{e-i}	5.60±0.17^{g-l}	-	-	
0	0.05	6.00±1^{h-k}	3.00±1^{n-s}	3.00±1^{n-t}	4.71±1^{k-p}	6.91±0.30^{c-f}	6.16±0.99^{e-h}	-	-	
0	0.1	4.00±1^{k-p}	3.00±1^{n-s}	2.11±0.11^{p-st}	3.81±1^{q}	5.50±1^{g-m}	5.31±1^{g-n}	-	-	
0	0.2	3.67±0.48^{l-q}	2.65±0.15^{0-s}	1.310.52^{r-s}	3.27±1^{q-r}	3.25±0.15^{q-r}	4.11±1^{n-q}	-	-	
1	0	10.66±2 ^{cd}	3.67±0.57^{l-q}	3.33±0.57^{m-r}	4.47±0.35^{l-p}	4.71±0.64^{k-p}	5.74±0.15^{f-k}	-	-	
1	0.05	12.00±2.64^{c}	5.67±0.57^{h-k}	4.24±0.67^{k-p}	6.10±0.32^{e-i}	7.41±1^{b-d}	5.97±0.51^{e-j}	-	1.79±0.43^{g}	0.71±0.017^{h}
1	0.1	9.00±1d-f	4.00±1^{k-p}	3.77±0.49^{l-q}	4.96±0.32^{h-o}	6.91±1^{c-f}	5.86±0.14^{e-k}	-	1.69±0.35^{g}	0.68±0.04^{h}
1	0.2	8.33±1.15^{e-g}	2.66±0.57^{o-s}	3.04±0.16^{n-s}	4.8±0.45^{j-o}	6.09±1.27^{e-i}	5.50±0.16^{g-m}	-	-	-
2	0	11.33±2^{c}	7.33±1.52^{e-i}	4.49±0.51^{k-o}	3.67±0.44^{p-q}	8.51±0.58^{b}	6.13±0.98^{e-i}	-	-	-
2	0.05	14.33±2 ^{b}	8.67±1.52^{e-g}	5.67±0.57^{h-l}	4.30±0.34^{m-q}	8.31±0.40^{b}	6.41±0.51^{d-g}	13.78±0.78^{a}	8.89±0.83^{b}	6.01±0.23^{d}
2	0.1	19.66±1.52^{a}	12.09±1^{c}	6.67±0.57^{g-j}	7.47±1^{b-d}	9.72±0.39^{a}	8.31±0.43^{b}	7.89±0.17^{c}	5.41±0.52^{e}	3.71±0.49^{f}
2	0.2	11.33±1.15^{c}	7.67±1.52^{e-h}	4.67±0.57^{j-o}	4.70±0.86^{k-p}	7.88±0.68^{bc}	6.17±0.29^{e-h}	-	-	-
3	0	9.33±1.52^{de}	7.00±0.0^{e-i}	3.67±1.15^{l-q}	4.17±0.50^{n-q}	7.47±0.54^{b-d}	5.76±0.51^{f-k}	-	-	-
3	0.05	11.33±2^{c}	7.54±0.51^{e-h}	4.90±0.17^{j-n}	4.92±0.20^{i-o}	7.77±0.44^{bc}	5.99±0.39^{e-j}	5.09±0.11^{e}	1.56±0.37^{g}	0.71±0.11^{h}
3	0.1	7.67±1.52^{e-h}	5.33±0.57^{i-m}	4.30±1.20^{k-o}	4.13±50^{n-q}	7.01±0.34^{c-e}	5.66±0.57^{g-k}	3.33±0.57^{f}	0.68±0.15^{h}	0.42±0.07^{hi}
3	0.2	4.67±0.57^{j-o}	5.33±0.57^{i-m}	4.69±0.46^{j-o}	2.50±0.20^{r}	6.33±0.32^{d-g}	5.59±0.37^{g-l}	-	-	-

* In each column, means with the similar letter (s) are not significantly different at 5% level of probability using Duncan's test.

Table 2. Effect of NAA and BAP and their interaction on the shoot regeneration parameters from shoot tip explants of *C. morifolium* and *C. coccineum*.

BAP×NAA (mg l^{-1})		Indirect regeneration (%)			Days to generation of shoots			Direct regeneration (%)		
		C. coccineum	Homa	Delkash	*C. coccineum*	Homa	Delkash	*C. coccineum*	Homa	Delkash
0	0	16.00±1^{s}	6.67±0.21^{t}	3.67±0.23^{t}	40.33±3.05^{mn}	47.67±1.54^{p}	44±2^{o}			
0	0.05	19.00±1^{rs}	7.16±0.15^{t}	5.12±0.12^{t}	37.33±1^{kl}	42.00±1no	42.00±1no			
0	0.1	17.00±1^{s}	4.91±1^{t}	5.71±0.44^{t}	39.00±1^{lm}	44.00±1^{o}	43.00±1.73^{no}			
0	0.2	9.00±1t	3.00±0.21^{t}	3.21±0.21^{t}	47.00±1^{p}	49.00±1^{p}	49.00±1.73^{p}			
1	0	54.67±3.27^{f-h}	28.67±1.52^{pq}	18.33±1.52^{s}	27.00±2^{cd}	37.33±1.52^{kl}	35.66±0.87^{i-k}	-	-	-
1	0.05	62.33±2.30^{d-e}	40.33±3.03^{l-m}	33.67±2.30^{n-p}	24.33±2^{abc}	30.33±2.14^{ef}	33.66±0.57^{g-j}	-	13.67±1.52^{ef}	7.81±0.78^{h}
1	0.1	67.67±3.11^{cd}	38.66±2.08^{l-o}	29.33±2.48^{pq}	31.00±1^{e-g}	32.67±2^{e-h}	33.33±1.15^{f-j}	-	12.33±0.57^{fg}	3.33±0.57^{j}
1	0.2	62.33±2.51^{de}	35.67±1.15^{m-o}	25.00±2.11^{q}	30.33±2^{ef}	36.33±2.30^{j-l}	36.33±0.577^{j-l}	-	-	-
2	0	48.95±2.43^{h-j}	41.67±3.05^{k-m}	50.33±3.87^{g-i}	24.00±1^{ab}	33.00±1^{f-i}	31.66±1.52^{e-h}	-	-	-
2	0.05	79.04±1.19^{b}	61.672.51±^{e}	51.00±1^{g-i}	30.33±2^{ef}	29.00±1de	30.33±0.57^{ef}	50.33±3.05^{a}	34.33±2.78^{b}	27.00±3^{c}
2	0.1	93.00±1.32^{a}	80.05±3.11^{b}	57.33±3.11^{e-f}	22.00±2^{ab}	25.67±1.51^{bc}	28.66±1.52^{de}	6.00±1^{i}	16.00±1.73^{d}	14.67±1.52^{e}
2	0.2	24.00±2.13^{q-r}	52.00±1^{f-i}	44±2.64^{j-l}	30.33±1.52^{ef}	30.33±0.577^{ef}	31.00±1.52^{efg}	-	-	-
3	0	69.33±3.21^{cd}	38.67±2.41^{l-0}	39.66±2.5^{l-n}	29.00±2de	34.66±0.77^{h-k}	33.33±1.52$^{f1.52-i}$	-	-	-
3	0.05	74.67^{bc}	47.33±$^{i-k}$	42.00±1.31^{k-l}	33.33±2^{f-j}	32.33±1.52^{f-h}	32.33±1.52^{f-h}	27.00±2^{c}	11.00±1^{g}	5.83±0.76^{i}
3	0.1	56.00±^{fg}	46.33±$^{i-k}$	38.67±2.13^{l-o}	35.66±2.45^{i-k}	33.33±1.52^{f-j}	32.67±2^{f-i}	8.00±1^{h}	5.00±1^{ij}	3.71±0.61^{j}
3	0.2	29.00±^{pq}	41.67±$^{k-m}$	33.33±2.76^{o-p}	41.00±1^{mn}	34.66±1.57^{h-k}	35.66±0.57^{i-k}	-	-	-

* In each column, means with the similar letter (s) are not significantly different at 5% level of probability using Duncan's test.

Although several different methods for *in vitro* shoot regeneration of chrysanthemums have been successfully developed, they mainly depended on indirect organogenesis pathways. Direct shoot regeneration in *C. coccineum* has not been reported so far. Here, the best media for inducing direct organogenesis in both *C. morifolium* and *C. coccineum* species were observed in MS medium enriched with 0.05 mg l^{-1} NAA and 2 mg l^{-1} BAP (Fig. 2 and 4).

Embryogenic callus induction and proliferation

As is shown in Table 3, in both *C. morifolium* and *C. coccineum* species, callus induction was significantly influenced by the cultivar and plant growth regulator. *C. morifolium* cv. 'Homa' and *C. coccineum* showed the earliest signs of callus formation 10-15 days after culturing of explants, but cv. 'Delkash' started to initiate callus from leaf cut surfaces 20-25 days later. After 28-31 days, the roots were unexpectedly produced on some calluses, and in several cases, shoots were produced from calluses after about 40-43 days.

The calluses were observed on leaf explants of both species only when MS medium was supplemented with 2,4-D alone or in combination with BAP (Table 3). Thus, the addition of 2,4-D to the medium was required for callus induction. Compared to *C. morifolium*, *C. coccineum* showed a higher ability to induce calli and in this term, both BAP and 2,4-D were effective in inducing calli. The percentage of produced calli on leaf explants was significantly improved by increasing 2,4-D concentration (from 1 to 2 mg l^{-1}). In both species, the highest frequency of callus formation was obtained on MS medium containing 2 mg l^{-1} 2,4-D and 2 mg l^{-1} BAP. Consistent with our results, Obukosia *et al*. (2004) also reported that MS medium supplemented with 2.0 mg l^{-1} 2,4-D was optimal for callus induction in chrysanthemum.

Between cv. 'Homa' and cv. 'Delkash' of chrysanthemum, 'Homa' had the highest callus induction rate (96.95%) on MS medium supplemented with 2 mg l^{-1} BAP and 2 mg l^{-1} 2,4-D followed by 'Delkash' (83.66%) while the highest callus induction rate during the experiment was obtained for *C. coccineum* (98.16%). We found that adding 1-2 mg l^{-1} BAP into callus induction medium was more efficient than medium without BAP, but further increasing of 2,4-D concentration resulted in lower callus induction and the formation of browned calli (Table 3). This effect is also reported with other chrysanthemum cultivars where BAP has induced callus induction (Teixeira da Silva *et al*., 2013; Teixeira da Silva, 2014).

The morphology of the obtained calli varied with cultivar as well as the nature and concentrations of PGRs used in the medium. Two types of calli were observed in the present study; type A was friable, white, whitish green, green, and greenish calli which were easy to break into small parts (Fig. 3a and 4b); type B was compact and yellow calli with smooth surfaces and spherical structures (Fig. 3b and 4c). In the medium containing different concentrations of 2,4-D (1-3 mg l^{-1}) without BAP, type A calli were predominantly produced which failed to enter embryogenesis phase (Fig. 3a and 4b) while at different concentrations of 2,4-D + BAP (particularly 2 mg l^{-1} 2,4-D in presence of 2 mg l^{-1} BAP), type B calli were often developed as typical of embryogenic calli (Fig 3b and 4c, about 55-60%, data not shown). The explants or calli browning were common phenomena in chrysanthemum cv. 'Delkash', and when the friable and brownish calli were produced at high concentrations of 2,4-D (3 mg l^{-1}), they turned dark brown after 2 subcultures and finally they died.

Overall, our results showed that the presence of 1-2 mg l^{-1} 2,4-D in culture medium is critical for embryogenic callus formation. Thomas and Maseena (2006) also reported that a given range of 2,4-D concentrations (0.1-2.0 mg l^{-1}) is necessary for embryogenic callus formation from leaf and nodal explants in *Cardiospermum halicacabum*. Regarding the effects of BAP, the lower concentrations generally induced green, granular and compact calli, while higher concentrations led to much larger number of soft, watery, and friable calli. Due to the increased sensitivity of tissue to auxin than cytokinin, cytokinins can lead to inducing cell dedifferentiation and finally differentiation was needed to transfer from a somatic cell status to an embryogenic one. It has been

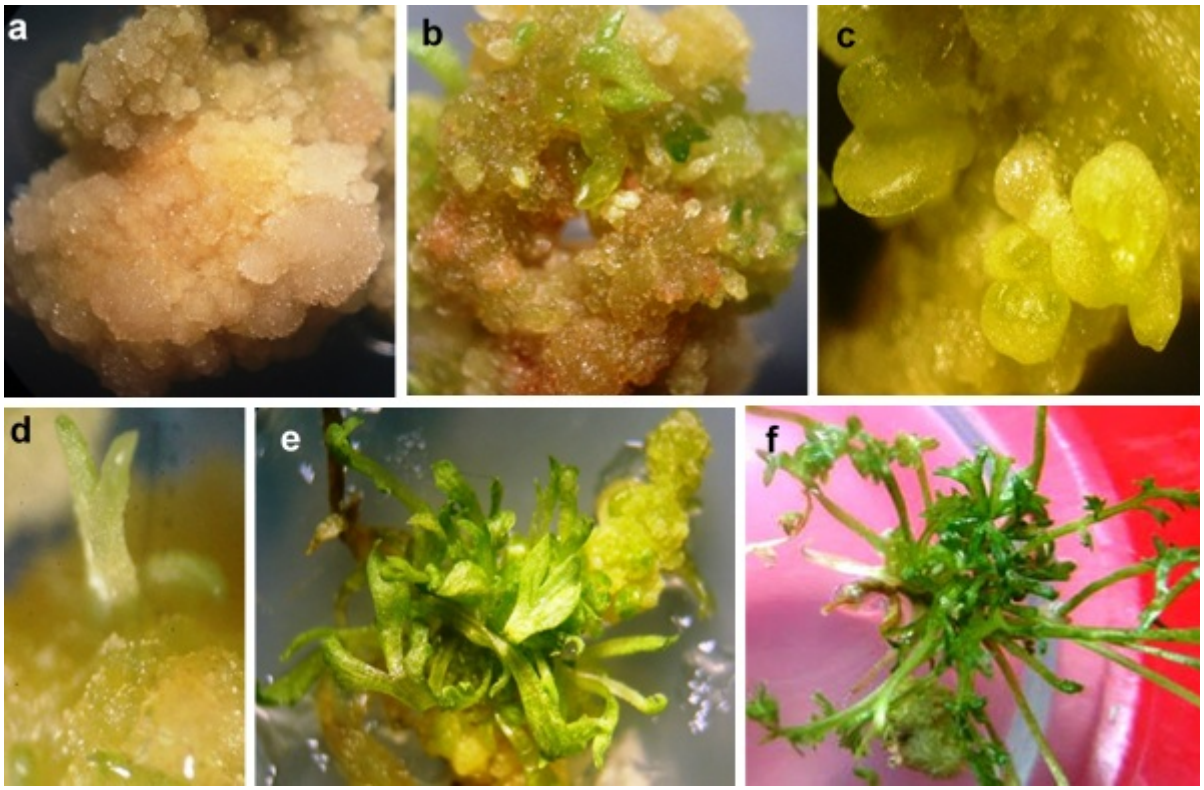

Fig. 3. Different stages of callus induction, somatic embryos and plant regeneration from leaves explants of C. coccineum. a: white and friable sections of callus; b: compact bright yellow embryogenic sections of callus with embryos in globular stage after four weeks of culture (2 mg l^{-1} 2,4-D and 2 mg l^{-1} BAP); c: heart-shaped stage; d: torpedo stage; e and f: cotyledonous stage along shoot-primordia and shoots multiplication after 6-8 weeks of culture (2 mg l^{-1} BAP and 0.05 mg l^{-1} NAA).

concluded that endogenous levels of auxin during the initiation stages of embryogenesis must be considered (Feher *et al*., 2003). Therefore, above-mentioned medium proved to be the best for obtaining the maximum proliferation of totipotent embryogenic calli.

Callus weight

The growth and proliferation rate of calli were highly dependent on the type of cultivar, PGRs and their levels used in the medium. The maximum callus weight was obtained on MS medium containing 2 mg l^{-1} 2,4-D and 2 mg l^{-1} BAP where cv. 'Homa' produced the highest callus weight (0.62 g/explant) and cv. 'Delkash' had the lowest callus weight (0.44 g/explant). Similar results were obtained in the case of chrysanthemum by Kumar *et al*. (2005). They reported that the presence of BAP in suspension cultures was desirable for callus growth in chrysanthemum. Also, 2,4-D was an effective synthetic auxin that could induce and maintain highly regenerative callus growth (Rashmi and Trivedi, 2014). These results showed that the weight rate of the callus was dependent on the genotypes and the plant growth regulators employed. Auxins are included in a culture medium to induce callus formation and cell growth, while cytokinins are used in micropropagation to induce cell division and organization (Gamborg *et al*., 1976; Turrea, 1989). This means that both 2,4-D and BAP are necessary for callus formation and morphogenesis. Variation in growth and weight callus of various cultivars has been already reported in other chrysanthemum cultivars (Song *et al*., 2011; Lim *et al*., 2012).

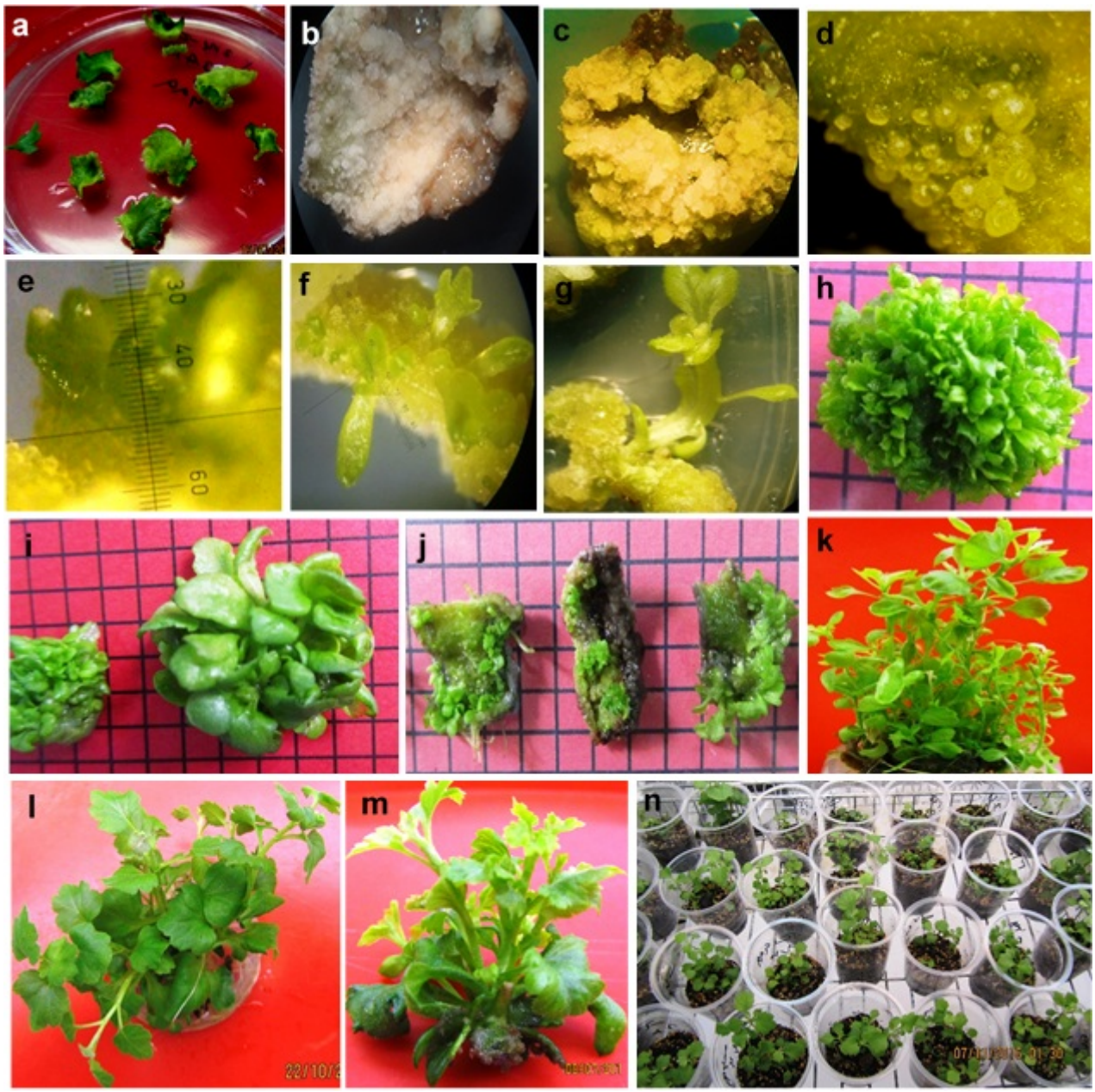

Fig. 4. Different stages of callus induction, somatic embryos and plant regeneration from leaves explants of *C.morifolium*. a: leaf explants 10-12 days after culture; b: white and friable sections of callus; c: compact bright yellow embryogenic sections of callus; d: embryos in globular stage after four weeks of culture (2 mg l^{-1} 2,4-D and 2 mg l^{-1} BAP); e: heart-shaped stage; f: torpedo stage; g: cotyledonous stage; h, i and j: shoot-primordia and shoots multiplication after 6-8 weeks of culture in c.v. Homa and Delkash, respectively (2 mg l^{-1} BAP and 0.05 mg l^{-1} NAA). k, l and m: transfer to 1/2 MS medium for further growth and development (k: 'Homa' and l and m: 'Delkash'); n: Acclimatized plants to preparation for transfer to greenhouse.

Table 3. Effect of 2,4-D and BAP their interaction on callus induction, callus weight and embryogenesis from leaf explants of *C. morifolium* and *C. coccineum*.

2,4-D×BAP (mg l^{-1})		Callus (%)			Embryogenesis (%)			Callus weight (g/explant)		
		Homa	Delkash	*C. coccineum*	Homa	Delkash	*C. coccineum*	Homa	Delkash	*C. coccineum*
0	0	-								
0	1	-								
0	2	-								
0	3	-								
1	0	71.67±2.55^{j-l}	60.33±0.78^{0}	75.00±3.21^{i-k}				0.20±0.09^{no}	0.18±0.02^{o}	0.25±0.02^{m}
1	1	79.67±0.87^{f-h}	71.33±2^{k-m}	91.00±1^{b}	12.33±1.52^{e}	4.67±0.57^{ij}	7.00±2^{g-i}	0.25±0.01^{m}	0.22±0.01^{n}	0.33±0.02^{j}
1	2	76.33±2.30^{h-i}	63.33±3.21^{n}	89.00±1.73^{bc}	17.33±1.52^{d}	10.00±1^{ef}	9.15±1.72^{fg}	0.28±0.01^{l}	0.25±0.04^{m}	0.37±0.01^{i}
1	3	65.33±2.76^{n}	51.67±1.52^{p}	78.67±0.57^{g-i}	10.67±0.57^{ef}	9.33±1.15^{fg}	7.06±1^{g-i}	0.19±0.01^{o}	0.15±0.01^{p}	0.29±0.02^{l}
2	0	82.67±2^{e-g}	71.00±1^{k-m}	86.33±3^{c-e}	-	-	-	0.34±0.01^{j}	0.32±0.01^{jk}	0.38±0.07^{hi}
2	1	87.33±2.51^{b-d}	79.33±0.57^{g-h}	88.67±2.21^{bc}	28.33±1.15^{b}	11.00±1^{ef}	17.33±2^{d}	0.46±0.04^{cd}	0.40±0.05^{f-h}	0.42±0.01^{ef}
2	2	96.95±1^{a}	83.66±1.52^{d-f}	98.16±1.76^{a}	44.33±3.12^{a}	23.67±3.11^{c}	29.00±2.44^{b}	0.62±0.01^{a}	0.44±0.02^{de}	0.56±0.02^{b}
2	3	79.67±1.52^{f-h}	76.33±2.30^{h-i}	83.67±0.57^{d-f}	22.00±1^{c}	12.33±1.52^{e}	17.67±2^{d}	0.37±0.02^{i}	0.33±0.01^{j}	0.48±0.01^{c}
3	0	71.00±4^{km}	65.33±1.15^{n}	77.67±3.21^{h-i}	-	-	-	0.34±0.01^{j}	0.33±0.01^{j}	0.40±0.05^{eh}
3	1	76.33±3.58^{h-i}	74.66±1.52^{i-l}	86.00±1^{c-e}	1.33±0.57^{lm}	1.72±0.47^{klm}	5.33±0.57^{h-j}	0.33±0.05^{j}	0.34±0.06^{j}	0.41±0.04^{fg}
3	2	74.67±3.51^{i-l}	70.67±0.78^{lm}	77.66±3.54^{h-i}	5.00±1^{h-j}	4.00±1^{jk}	7.37±0.64^{gh}	0.39±g-0.01^{i}	0.38±0.01hi	0.44±0.02^{de}
3	3	67.33±2.51^{m-n}	66.33±1.15^{n}	75.66±3.78^{h-j}	4.33±0.57^{j}	3.03±0.15^{j-l}	1.70±0.51^{k-m}	0.30±0.05^{l}	0.27±0.04^{l}	0.38±0.03^{h}i

* In each column, means with the similar letter(s) are not significantly different at 5% level of probability using Duncan's test.

Induction of somatic embryos

The process of somatic embryogenesis is a favorable way of plant regeneration with stable regeneration ability and is also a prerequisite for high-performance micropropagation and genetic improvement in chrysanthemums. Somatic embryogenesis can be initiated in *C. morifolium* directly from the epidermal cells of explant tissues (Mandal and Datta, 2005) or indirectly through the intervention of callus cells (Shinoyama *et al*., 2004; Teixeira da Silva, 2014). In the present research, somatic embryogenesis was induced from produced calli on the leaf segments in both studied species. Different developmental stages of somatic embryos were detected at the surface of explants 30-35 days from the start of cultivation. Depending on the different concentrations of BAP and 2,4-D, globular stage (Fig. 3b and 4d), heart-shaped stage (Fig. 3c and 4e), torpedo-stage (Fig. 3d, 4f), and cotyledonary stage (Fig. 3e, and 4g) of somatic embryos were simultaneously observed at the callus surface. Somatic embryos were developed with the addition of BAP in the presence of 2,4-D while no somatic embryogenesis took place on the media containing 2,4-D without cytokinin. Similar results have been reported by Tanaka *et al*. (2000) for chrysanthemum. Furthermore, the ratio of 2,4-D/BAP significantly affected the percentage and number of somatic embryos. In this investigation, the highest embryogenesis response with the maximum number of somatic embryos per responding explant was observed with 2.0 mg l^{-1} BAP and 2.0 mg l^{-1} 2,4-D in both evaluated species (Tables 3 and 4). Lower or higher BAP/2,4-D ratios distinctly reduced the embryogenesis frequency and the number of somatic embryos per explant. Similarly, a combination of 2 mg l^{-1} 2,4-D and 2 mg l^{-1} kinetin has been reported to be optimal for induction of somatic embryos in chrysanthemum (Shinoyama *et al*., 2004). The effects of 2,4-D and BAP combinations on somatic embryogenesis of chrysanthemum have been also reported by Shinoyama *et al*. (2004), Mandal and Datta (2005), and Naing *et al*. (2013).

According to the results, the best embryogenesis response was found in 'Homa' with a maximum 44.33% embryogenesis and 12 somatic embryos per explant while in *C. morifolium* cv. 'Delkash' and *C. coccineum*, only 23.67% and 29.00% embryogenesis and 8.76 and 7.87 somatic embryos per explant were obtained, respectively. Such genotypic influences on somatic embryogenesis have also been well documented in other cultivars of chrysanthemum (Tanaka *et al*., 2000; Shinoyama *et al*., 2004; Mandal *et al*., 2005; Miler and Zalewska, 2014).

Table 4. Effect of 2,4-D and BAP and their interaction on the number of somatic embryos from leaf explants of *C. morifolium* and *C. coccineum*.

2,4-D×BAP (mg l^{-1})		Number of somatic embryos		
		Homa	Delkash	*C. coccineum*
0	0	-	-	-
0	1	-	-	-
0	2	-	-	-
0	3	-	-	-
1	0	-	-	-
1	1	$3.33\pm0.57^{i-k}$	$4.33\pm0.57^{f-i}$	$3.79\pm0.68^{g-j}$
1	2	5.33 ± 0.57^{de}	5.00 ± 1^{ef}	$4.30\pm0.60^{f-i}$
1	3	$3.67\pm0.57^{g-k}$	4.57 ± 0.74^{eg}	$3.43\pm0.51^{h-k}$
2	0	-	-	-
2	1	6.00 ± 1^{d}	7.33 ± 1.15^{c}	6.00 ± 1^{d}
2	2	12.00 ± 1^{a}	8.76 ± 0.56^{b}	7.87 ± 0.81^{bc}
2	3	8.00 ± 1^{bc}	6.00 ± 1^{d}	$4.36\pm0.57^{e-h}$
3	0	-	-	-
3	1	1.00 ± 0.33^{no}	1.33 ± 0.57^{mn}	1.59 ± 0.52^{mn}
3	2	$2.80\pm0.7^{2j-k}$	2.23 ± 0.40^{lm}	1.11 ± 0.11^{n}
3	3	2.66 ± 0.57^{kl}	$3.11\pm0.11^{j-l}$	1.20 ± 0.35^{n}

* In each column, means with the similar letter(s) are not significantly different at 5% level of probability using Duncan's test.

Regeneration of embryogenic callus

The regeneration of the embryos was observed in the shoot induction medium during 15 to 20 days after development of embryos. The majority of embryos were turned into visible shoots on the callus surface (Fig. 3f and Fig. 4h-i). The color of the calli was changed to dark green and it seems that embryos had more capability to initiate leaf primordia and subsequent emergence of the shoot in this stage (Figs. 3 and 4), especially in the media containing 2 mg l^{-1} BAP+ 0.05 mg l^{-1} NAA.

BAP plays a key role in the regeneration of shoots from callus under *in vitro* conditions (Waseem *et al*., 2009). In the present experiment, individual application of BAP (2 mg l^{-1}) could lead to the adventitious shoot induction at all cultivars (Table 5). These results were in line with other reports on chrysanthemum (Jevremovic and Radojevic, 2004; Waseem *et al*., 2009; Teixeira da Silva, 2014). The results showed that 2 mg l^{-1} BAP with 0.05 mg l^{-1} NAA was the optimal combination to achieve the highest frequency of regeneration in 'Homa' (49.99%) and 'Delkash' (73.57%) (Table 5). In *C. coccineum*, about 77.11% of the calli successfully produced shoots in MS medium with 2 mg l^{-1} BAP and 0.05 mg l^{-1} NAA followed by 47% in medium supplemented with 1 mg l^{-1} BAP and 0.1 mg l^{-1} NAA. A mean number of 19 shoots per callus was obtained with 2 mg l^{-1} BAP and 0.05 mg l^{-1} NAA. In general, higher concentrations of NAA (0.1 mg l^{-1}) prevented the formation of shoot and promoted callus formation. Auxins and cytokinins are important factors in embryogenic responses due to inclusive and effective interventions in the cell cycle regulation and cell division (Francis and Sorrell, 2001). In this regard, Song *et al*. (2011) reported that the rate of shoot multiplication is a genotype-dependent phenomenon in *C. morifolium*. For genetic modification purposes, particular plant cultivars with high regeneration ability are usually needed. Therefore, the induction of embryogenic calli and regeneration of shoots are significantly genotype-dependent processes (Deyi *et al*., 2011). Eventually, the ability to regenerate shoots from the calli and somatic embryos is essential for the success of most biotechnological techniques such as *in vitro* mutagenesis and gene transferring (Shahzad *et al*., 2017).

Table 5. Effect of NAA and BAP and their interaction on the regeneration of embryogenic calli in two species chrysanthemums (*C. morifolium* and *C. coccineum*).

BAP×NAA (mg l^{-1})		Shoot number			Regeneration (%)		
		Homa	Delkash	*C. coccineum*	Homa	Delkash	*C. coccineum*
0	0	1.80±0.34h	1.29±0.33h	1.33±0.15h	7.66±1.15h	2.37±0.47ij	5.29±0.61hi
0	0.05	-	-	-	-	-	-
0	0.1	-	-	-	-	-	-
2	0	12.66±1.52d	6.55±0.77g	10.00±1e	41.63±3.11d	26.33±2.52g	37.38±2.41e
2	0.05	22.89±2.16a	14.33±0.57c	19.00±2b	89.49±2.32a	73.57±1.50b	77.11±3.54b
2	0.1	10.03±0.95e	7.66±0.57fg	9.00±1.73ef	30.55±2.51f	27.66±2.31fg	47.00±2.98c

* In each column, means with the similar letter(s) are not significantly different at 5% level of probability using Duncan's test.

Acclimatization and rooting

Since most micro-shoots originated from calli were very small and tiny (1 or 2 cm) and were not suitable for rooting (Fig. 4 h-i), they were isolated and transferred into a culture medium (half-strength MS) free from any plant growth regulator for further growth and development. The rooting stage in individual shoots was performed without using any external growth regulators in the in vivo media (perlite and cocopeat mixture) or in the *in vitro* culture conditions. After 2 weeks, profuse rooting was observed in all plantlets. With respect to the *C. morifolium* species, from 778 micro-shoots produced by direct and indirect organogenesis as well as somatic embryogenesis, 734.55 plantlets successfully formed root.

In our study, more than 94.33% of the regenerated plants of the cultivars of *C. morifolium*

survived and they reached the maturity stage. In the case of *C. coccineum*, after 3 weeks, rooting was observed in most plantlets. About 165.75 plantlets from a total of 221 plantlets were rooted under *in vitro* conditions. In general, after an acclimatization period of one month, the survival rate was 75% (Table 6).

Table 6. The number of hardened plants and rooting percentage of two chrysanthemums species (*C. morifolium* and *C. coccineum*).

Shoot number			Regeneration (%)		
Homa	**Delkash**	***C. coccineum***	**Homa**	**Delkash**	***C. coccineum***
274	257	221	96.95	93.84	75

Molecular marker analysis

Although we previously used RAPD markers to analysis clonal fidelity of micropropagated *Satureja avromanica* Maroofi plants (Mozafari *et al*., 2015) due to known disadvantages of RAPD marker especially its weak reproducibility, in the present study we took the advantage of IRAP, ISSR and SCoT markers to check whether chrysanthemum and Persian pyrethrum *in vitro* miro-propagated plants were true-to-type or not. SCoT is an easy, gene-targeted, and inexpensive marker in which short conserved nucleotide sequences that flank the ATG start codons are targeted. It has been confirmed that polymorphisms generated by SCoTs are efficient and reproducible for the analysis of genetic fidelity of *in vitro* raised clonal plant material (e.g. *Albizia julibrissin*, Rahmani *et al*., 2015; *Dendrobium nobile*, Bhattacharyya *et al*., 2014; *Pittosporum eriocarpum*, Thakur *et al*., 2016). On the other hand, ISSRs are repeated sequences (0.1-3 kb) located among adjacent SSR regions with opposite orientation (Meyer *et al*., 1993) and the retrotransposon-based IRAP markers discriminate insertional polymorphisms of retrotransposon sequences. Both marker systems have also been proven to be appropriate markers. For example, ISSR has been used for genetic homogeneity evaluation in finger millet (Atul Babu *et al*., 2018), *Swertia chirayita* (Joshi and Dhawan, 2007), silk tree (Rahmani *et al*., 2016) and *Gerbera* (Bhatia *et al*., 2011).

In the present study, six primers were analyzed for each marker system, and a total of 56, 56 and 39 fragments were amplified for IRAP, ISSR, and SCoT, respectively. Information of the applied primers and comparative summary of the data collected with the IRAP, ISSR and SCoT markers are given in Table 7. The highest (13) and the lowest (3) band number were obtained in *C. coccineum* with ISSR (LTR6149 primer) and SCoT (SCoT23 primer), respectively. No polymorphism was observed among all analyzed micropropagated plants and mother plant of each species which show their genetic fidelity and confirm that they are true to type (Fig. 5). In most analyzed primers, the banding pattern of *C. morifolium* micropropagated plants was different from *C. coccineum* plants (Fig. 5b-c) which can be attributed to their genetic background as they are two independent species. Taken together, our results showed that the direct regeneration from shoot tips can be used as one of the safest methods for *in vitro* propagation of many horticultural plant species with a low risk of somaclonal variation and genetic instability.

CONCLUSIONS

In the present study, we introduced ways to achieve both direct shoot formation through shoot tips explants and the somatic embryogenesis and proliferation through leaf explants of *C. morifolium* and *C. coccineum*. We can consider two important aspects of our findings. First, our protocol for embryogenesis from calli can be not only used to optimize genetic transformation of *C. morifolium* and *C. coccineum* and *in vitro* mutagenesis studies but it can be also useful to study embryogenesis process, particularly in *C. coccineum* wild species. Second, we proposed a reliable direct regeneration from shoot tips along with a platform introduced to confirm genetic homogeneity, which can be applied to establish clonally uniform progeny.

Table 7. List of IRAP, ISSR and SCoT primers and their sequences, number and size range of scorable fragments, and total number of amplified fragments in *C. morifolium* and *C. coccineum in vitro*-regenerated plants.

Primer name	Sequence	Reference	Size (bp)	No. of scorable bands per primer		Total no. of amplified bands		Similarity (%)
				C.M	C.C	C.M	C.C	
IRAP								
Hana	CACGATTCACCTTAATATCTGACA	Kalendar *et al*. (1999)	400-1500	8	9	80	90	100
Gaga	GGGAACCAACCGTCACA	Kalendar *et al*. (1999)	500-1500	7	7	70	70	100
Nikita	CGCATTTGTTCAAGCCTAAACC	Kalendar *et al*. (1999)	300-1400	12	10	120	100	100
5′LTR1	TTGCCTCTAGGGCATATTTCCAACA	Kalendar *et al*. (1999)	500-1300	7	6	70	60	100
LTR6149	CTCGCTCGCCCACTACATCAACCGCGTTTATT	Kalendar *et al*. (1999)	300-1600	12	13	120	130	100
LTR6150	CTGGTTCGGCCCATGTCTATGTATCCACACATGTA	Kalendar *et al*. (1999)	350-1000	8	8	80	80	100
ISSR								
UBC-807	AGAGAGAGAGAGAGAGT	Meyer *et al.* (1993)	250-1200	7	8	70	80	100
UBC-811	GAGAGAGAGAGAGAGAC	Meyer *et al.* (1993)	200-1100	10	7	100	70	100
UBC-818	CACACACACACACACAG	Meyer *et al.* (1993)	300-900	7	6	70	60	100
UBC-820	GTGTGTGTGTGTGTGTC	Meyer *et al.* (1993)	400-900	7	7	70	70	100
UBC-840	GAGAGAGAGAGAGAGAYT	Meyer *et al.* (1993)	200-1300	12	12	120	120	100
UBC-841	GAGAGAGAGAGAGAGYC	Meyer *et al.* (1993)	300-1400	12	6	120	60	100
SCoT								
SCoT7	CAACAATGGCTACCACGG	Collard and Mackill (2009)	800-1200	5	4	50	40	100
SCoT11	AAGCAATGGCTACCACCA	Collard and Mackill (2009)	400-900	6	6	60	60	100
SCoT16	ACCATGGCTACCACCGAC	Collard and Mackill (2009)	400-1400	7	4	70	40	100
SCOT21	ACGACATGGCGACCCACA	Collard and Mackill (2009)	300-1200	9	9	90	90	100
SCoT22	AACCATGGCTACCACCAC	Collard and Mackill (2009)	500-1700	7	7	70	70	100
SCoT23	CACCATGGCTACCACCAG	Collard and Mackill (2009)	400-1400	5	3	50	30	100

*C.M: *Chrysanthemum morifoulim*, C.C: *Chrysanthemum coccineum*

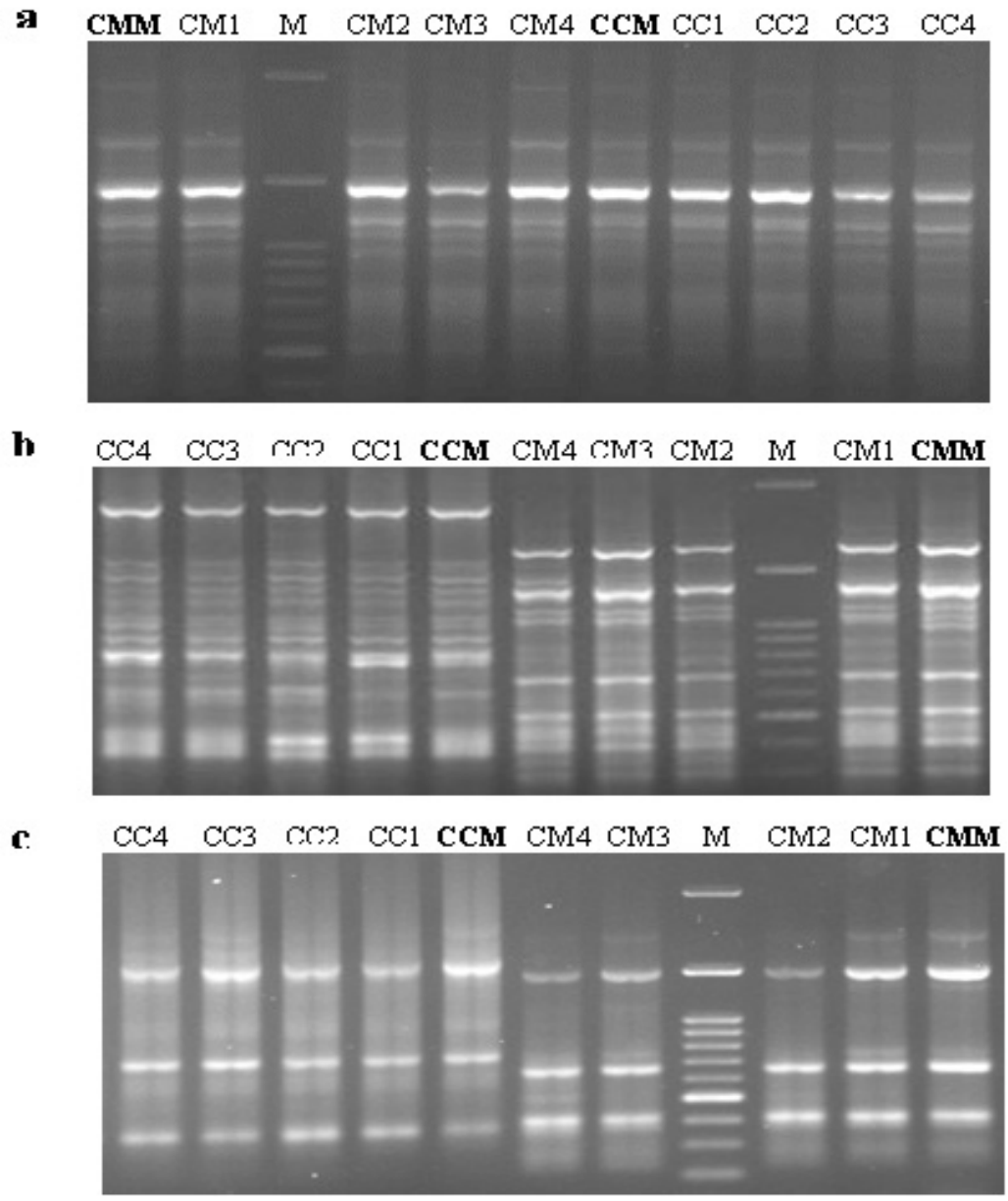

Fig. 5. Polymerase chain reaction (PCR) amplification products obtained with (A) Inter Retrotransposon Amplified Polymorphism IRAP)Gaga((B) Inter Simple Sequence Repeat ISSR (UBC-840) (C) Start Codon Targeted Sequence SCoT (SCoT7) markers, M 1 kb molecular weight marker; CMM and CCM *Chrysanthemum morifolium* and *Chrysanthemum coccineum* mother plant; CM1-CM4 *in-vitro* raised hardened *C. morifoilum* plants. CC1-CC4 *in vitro* raised hardened *C. coccineum* plants.

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