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In vitro regeneration of two chrysanthemum (*Chrysanthemum morifolium* Ramat.) cultivars through organogenesis from petal explants

Rezvanolsadat Kazeroonian[1], Amir Mousavi[2*], Sepideh Kalatejari[1], Masoud Tohidfar[3]

[1]*Department of Horticultural Sciences, Science and Research branch, Islamic Azad University, Tehran, Iran*

[2]*National Institute of Genetic Engineering and Biotechnology,Tehran, Iran*

[3]*Seed and Plant Improvement Campus, Agricultural Biotechnology Research Institute of Iran, Karaj, Iran*

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Abstract

Petal explants of two chrysanthemum cultivars were cultured on the Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of plant growth regulators (PGRs). Results indicated the supremacy of "Resomee Splendid" over the other cultivar in regard to the shoot induction percentage with 62.22% vs. 49.63%. Maximum percentages of the shoot induction (93.33% vs. 73.33) were achieved in 3.0 mg l^{-1} benzylaminopurine (BAP) + 0.5 mg l^{-1} 1-naphthaleneacetic acid (NAA) and 4.5 mg l^{-1} BAP +1.0 mg l^{-1} NAA, while, the highest number of shoots per explant (2.73 vs. 3.0) was obtained in 4.5 mg l^{-1} BAP + 1.0 mg l^{-1} NAA in "Resomee Splendid" and "Reagan Elite Salmon", respectively. Considering callogenesis, the later cultivar totally showed a greater response to the PGR treatments. Adventitious shoots were formed indirectly on the "Resomee Splendid" explants, while, considering the other cultivar direct shoot formation was observed on the media fortified with either 3.0 mg l^{-1} BAP + 0.5 mg l^{-1} NAA or 4.5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA. A combination of thidiazuron (TDZ) and NAA resulted in indirect shoot formation of both cultivars. Regenerated shoots successfully elongated and formed roots on MS medium and were finally acclimatized and transplanted into soil. Treatments containing TDZ were totally inferior to the ones comprising of BAP in terms of both quantitative characteristics.

* **Corresponding Author:** Amir Mousavi ✉ m-amir@nigeb.ac.ir

Introduction

Chrysanthemums are one of the most important ornamentals in the cut flower, flowering potted plant and herbaceous perennial markets worldwide (Anderson, 2007). Chrysanthemum cultivars are generally propagated by vegetative cuttings or suckers (Levin *et al*., 1988; Teixeira da Silva, 2003). However, this conventional approach of shoot cutting is very slow (Levin *et al*., 1988). Therefore efficient tissue culture methods for regeneration will need to be developed, especially if genetic transformation via *Agrobacterium* is to be attempted (Kaul *et al*., 1990). *In vitro* shoot regeneration in chrysanthemum through induction of adventitious shoots is affected by the PGRs interaction, the kind of explant and plant genotype (Nahid *et al*., 2007, Zalewska *et al*., 2011).

Chrysanthemum petals are amongst the candidate sources for organogenesis (Nahid *et al*., 2007). Regeneration from petals has been achieved in chrysanthemum by a number of researchers using different cultivars and various plant growth regulator (PGR) combinations or concentrations (Barakat *et al*., 2010; Datta *et al*., 2005; Manal and Datta, 2005; Mani and Senthil, 2011; Nahid *et al*., 2007; Park *et al*., 2007; Song *et al*., 2011; Verma *et al*., 2012; Vilasini and Latipah, 2000). PGRs provide a proportional stimulus that allows regulation of the cell cycle for cell division induction and specialization during plant development (Jaramillo *et al*., 2008).

Park *et al*. (2007) achieved the highest adventitious shoot regeneration frequency by culturing petal explants on the Murashige and Skoog medium (MS) (1962) supplemented with 57.0 µM IAA, 44.0 µM BAP and 0.4 µM kinetin. Song *et al*. (2011) observed significant differences in frequency of regeneration among different cultivars when grown on media supplemented with various plant growth regulators. Among different surveyed explants in their research, petals exhibited the highest frequencies of shoot organogenesis and mean number of shoots per explant. Shoot formation or adventitious organogenesis is preferable due to retaining clonal fidelity as numerous floricultural crops are cultivars that are propagated for unique traits (park *et al*., 2007).

Efficient protocols for a given cultivar may not be suitable for the other ones. Thus, it is necessary to develop appropriate protocols for regeneration of various commercial cultivars (Song *et al*. 2011). This study was implemented to scrutinize the effect of different PGR combinations on shoot organogenesis from petal explants in two chrysanthemum cultivars in order to introduce an efficient protocol for high frequency regeneration rate.

Materials and methods

Plant materials and culture media

Ray florets of two chrysanthemum cultivars, "Reagan Elite Salmon" and "Resomee Splendid" were collected from field-grown plants and rinsed thoroughly under running tap water for 15 min and subsequently were surface-sterilized in 70% ethanol for 30 s and 1% sodium hypochlorite solution plus Tween 20 for 10 min, followed by three rinses with sterile distilled water. Afterwards, ovaries were removed from ray florets and petals were cut into approximately 5 mm long segments. Then explants were cultured on MS medium fortified with different concentrations and combinations of naphthalene acetic acid (NAA) plus benzylaminopurine (BAP) or thidiazuron (TDZ) as a cytokinin (Table 1). All the media contained 6.5 g l^{-1} plant agar, 3% (w/v) sucrose and pH was adjusted to 5.7-5.8 before autoclaving at 121°C for 15 min. cultures were exposed to cool white fluorescent lamps (27 µmol m^{-2} s^{-1}) with 16h photoperiod. Regeneration was conducted without any subculturing. After six weeks of culture, callus induction response, shoot induction percentage (number of explants with visible shoots/ total number of explants) and average number of shoots per explant were recorded for each treatment. During this period, regenerated shoots were separated from petal explants and transferred to MS medium for further elongation and rooting. Thereafter, well-rooted plantlets were washed under running tap water and acclimatized in plastic cups containing sterilized cocopeat and perlite (1:1) within a week.

Experimental design and statistical analysis

Experiment was laid out in a completely randomized design (CRD) with 2 cultivars and 16 plant growth regulator treatments. Each treatment had 3 replications and one replicate consisted of a Petri dish containing 5 explants. Combinations and concentrations of different PGRs applied in the media are presented in Table 1. Means were compared by Duncan's multiple range test and data were analyzed using SPSS version 16.

Results and discussion

Shoot induction percentage

As depicted in Fig. 1, differences in shoot induction percentage from petal explants were significant between cultivars (62.22% for "Resomee Splendid" vs. 49.63% for "Reagan Elite Salmon"). Nahid *et al.* (2007) also observed significant differences in regeneration capacity from petal explants among five genotypes. Based on our results shoot induction ability is strongly influenced by the cultivar. As tabulated in Table 1, by adding auxin or each of the cytokinins to the medium separately, petal explants failed to produce any shoots. In other words, presence of BAP or TDZ in combination with NAA was necessary for shoot regeneration in both of the cultivars. Maximum percentage of the shoot induction (93.33% vs. 73.33%) were obtained in T7 (3.0 mg l^{-1} BAP + 0.5 mg l^{-1} NAA) and T12 (4.5 mg l^{-1} BAP + 1.0 mg l^{-1} NAA), from petal explants of "Resomee Splendid" and "Reagan Elite Salmon", respectively.

Table 1. Effect of different PGR treatments on the shoot induction percentage, average number of shoots regenerated per petal explant and callogenic responses of two chrysanthemum cultivars.

PGR treatment (mg l^{-1})	Shoot Induction (%)		Av. no. shoots explant^{-1}		Callogenesis			
	Resomee Splendid	Reagan Elite Salmon	Resomee Splendid	Reagan Elite Salmon	Resomee Splendid		Reagan Elite Salmon	
T1=0.0BAP+0.0NAA	0.0	0.0	0.0	0.0	-		-	
T2=1.5BAP+0.0NAA	0.0	0.0	0.0	0.0	-		+	
T3=3.0BAP+0.0NAA	0.0	0.0	0.0	0.0	-		+	
T4=4.5BAP+0.0NAA	0.0	0.0	0.0	0.0	+		+	
T5=0.0BAP+0.5NAA	0.0	0.0	0.0	0.0	+		++	
T6=1.5BAP+0.5NAA	60.0±0.08 bcd	40.0±0.00 cd	1.00±0.05 c	0.73±0.07 cd	+	ID	+++	ID
T7=3.0BAP+0.5NAA	93.3±0.03 a	46.6±0.05 bcd	2.53±0.16 ab	0.93±0.03 cd	+	ID	+++	D
T8=4.5BAP+0.5NAA	66.6±0.11 abc	66.6±0.04 ab	1.66±0.20 abc	1.13±0.06 cd	++	ID	+++	D
T9=0.0BAP+1.0NAA	0.0	0.0	0.0	0.0	+		++	
T10=1.5BAP+1.0NAA	60.0±0.08 bcd	53.3±0.05 abc	1.46±0.24 abc	2.50±0.20 ab	+	ID	+++	ID
T11=3.0BAP+1.0NAA	66.6±0.04 abc	66.6±0.04 ab	2.06±0.02 abc	1.60±0.16 bc	+	ID	+++	ID
T12=4.5BAP+1.0NAA	86.6±0.03 ab	73.3±0.04 a	2.73±0.10 a	3.0±0.15 a	+	D	+++	D
T13=0.0TDZ+0.1NAA	0.0	0.0	0.0	0.0	+		-	
T14=0.4TDZ+0.1NAA	53.3±0.05 cd	40.0±0.13 cd	1.13±0.19 c	1.06±0.30 cd	+	ID	++	ID
T15=0.6TDZ+0.1NAA	33.3±0.10 d	33.3±0.10 cd	0.86±0.26 c	0.33±0.06 d	+	ID	+++	ID
T16=0.8TDZ+0.1NAA	40.0±0.00 cd	26.6±0.10 d	1.26±0.11 bc	0.33±0.10 d	+	ID	+++	ID

*Means ± SE within a column followed by different letters are significantly different according to Duncan's Multiple Range Test at P=0.05.

- = No callus formation + = Weak Callus ++ = Good callus +++ = Very good callus

D = Direct organogenesis ID = Indirect organogenesis.

As presented in Table 1, shoot induction percentage was totally higher on media fortified with BAP compared to TDZ. This result is in agreement with the findings of Lindiro *et al.* (2013) who reported the supremacy of BAP to kinetin and TDZ in regard to the microshoot regeneration on nodal explants of *Chrysanthemum cinerariaefolium*. Superiority of BAP for shoot induction may be related to the potential of tissues to metabolize BAP more readily than other synthetic growth regulators or to the production of natural hormones such as zeatin within the plant tissue due to the action of BAP (Malik *et al.*, 2005).

Shoot number per explant

In our experiment, shoot number per explant was not significantly different (1.63 for "Resomee Splendid" vs. 1.29 for 'Reagan Elite Salmon'). The highest number of shoots formed per explant (2.73 vs. 3.0) was achieved in T12 (4.5 mg l^{-1} BAP + 1.0 mg l^{-1} NAA) from petal explants of "Resomee Splendid" and "Reagan Elite Salmon", respectively (Fig. 2). Verma *et al.* (2012) reported maximum number of microshoots formed per petal explants of *Chrysanthemum morifolium* "Thai Chen Queen" on the MS medium supplemented with 4.0 mg l^{-1} BAP and 0.1 mg l^{-1} NAA. It has been reported that auxins and cytokinins are mutually dependent; hence, various physiological effects of these PGRs can be explained by their interaction. Exogenous manipulation of cytokinin levels results in an increase in the amount of endogenous auxins, thereby enhancing undifferentiated tissue production (Harberer and Kieber, 2000).

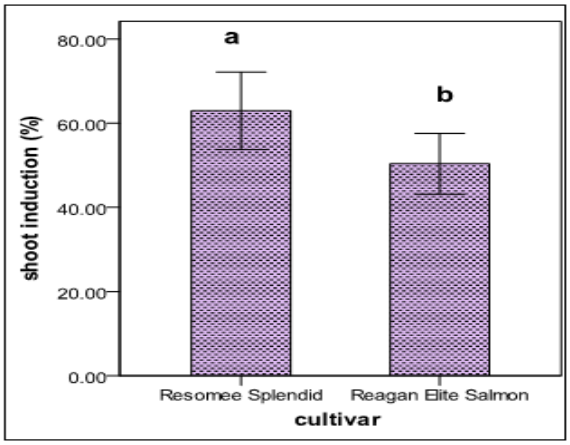

Fig. 1. Effect of cultivars on shoot induction percentage from petal explants of *Chrysanthemum morifolium* after six weeks of culture. Error bars represent standard errors.

As presented in Table 1, number of shoots per explant were totally higher on media supplemented with BAP than TDZ. The reason may lie behind that BAP accelerates bud initials development which results in higher number of buds primordial in chrysanthemum (Chagas *et al.*, 2004; Waseem *et al.*, 2011), while TDZ seems to be less responsive for organogenesis from chrysanthemum petal explants. Another possible explanation might be that the ratio between TDZ and NAA was not proper enough for shoot induction from petals in our study.

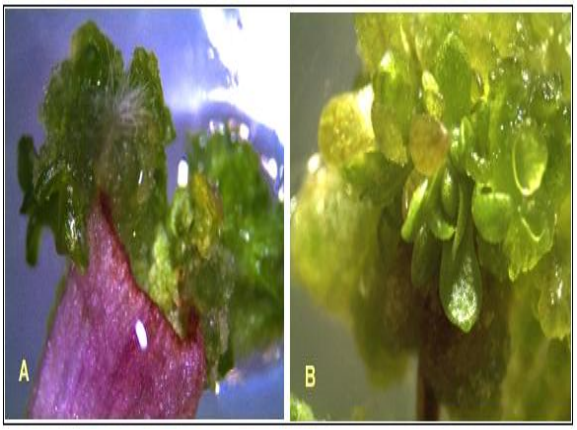

Fig. 2. Maximum number of shoots formed per petal explant in T12 (4.5 mg l^{-1} BAP + 1.0 mg l^{-1} NAA) through direct organogenesis in *Chrysanthemum morifolium*: (A) "Resomee Splendid" and (B) "Reagan Elite Salmon".

Callogenesis and regeneration type

Comparing two cultivars in Table 1, a greater callogenic response can be noticed in "Reagan Elite Salmon", which shows that appropriate levels of auxins and cytokinins are necessary for callus induction from each species or variety (Rout and Das, 1997). The efficiency of callus induction and callus growth rate has been reported to be partly genotype dependent (Barakat *et al.*, 2010; Kaul *et al.*, 1990). Moreover, as illustrated in table 1, range of callus differentiation into plantlets varied noticeably among different treatments, which indicates that the ability of fresh callus to differentiate into plantlets not only depends on the cultivar, but also relies on the hormone level of the induction medium (Barakat *et al.*, 2010). Interestingly, regeneration type also differed between the cultivars in T7 (3.0 mg l^{-1} BAP + 0.5 mg l^{-1} NAA) and T8 (4.5 mg l^{-1} BAP + 0.5 mg $^{-1}$l NAA) media. For more clarification, it can be mentioned that new shoots were formed indirectly on the "Resomee Splendid" petal explants, while, direct shoot formation occurred on "Reagan Elite Salmon" on the aforementioned media. Datta *et al.* (2005) reported direct organogenesis from ray florets of *Chrysanthemum morifolium* through application of 0.5 mg l^{-1} NAA in combination with 1,2 or 5 mg l^{-1} of either BAP or TDZ, while in our experiment, both

cultivars exhibited only indirect regeneration on the media supplemented with different concentrations of TDZ. It should be uttered that type of regeneration not only depends on the choice of cytokinin, but also attributes to the chrysanthemum genotype (Song *et al.*, 2011). Abilities to uptake and metabolize PGRs might also be different among various chrysanthemum genotypes (Nahid *et al.* 2007). Rademaker and de Jong (1990) reported that type of cultivar in comparison to the medium type had a greater effect on regeneration.

Finally, it can be recapitulated that an efficient protocol was developed for *in vitro* organogenesis of two chrysanthemum cultivars from petal explants in this survey through examining different combinations and concentrations of PGRs.

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