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The Effect of Plant Growth Regulators on Callus Induction and Regeneration in Three Chrysanthemum (*Chrysanthemum grandiflorum* Ramat) Cultivars Under *In Vitro* Culture Condition

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Abstract

Chrysanthemums are known as one of the top three flowers in the floriculture industry. This ornamental plant is traditionally propagated by cuttings. The use of *in vitro* subculture is expanding for plants like chrysanthemums that are propagated by asexual methods. This research evaluated the effect of plant growth regulators on callus induction and regeneration in three chrysanthemum cultivars including 'Bonfire Yellow', 'Rambla', and 'Bella Rosa'. For callus generation, the leaf explants were cultivated in the Murashige and Skoog (MS) culture medium modified with with benzyl adenine (1, 2, or 3 mg/L BA) and naphthaleneacetic acid (0.5 or 1 mg/L NAA) in a factorial experiment based on a completely randomized design. The highest rate of regeneration of 'Bonfire Yellow'(31.25%), 'Rambla'(25%), and 'Bella Rosa' (31.25%) were obtained from the culture media containing 2 mg/L BA + 1 mg/L NAA, 1 mg/L BA + 1 mg/L NAA, and 3 mg/L BA + 0.5 mg/L NAA, respectively.

Keywords: Auxin, Benzyl adenine, Explant, Micropropagation, Tissue culture.

INTRODUCTION

Chrysanthemum (*Chrysanthemum grandiflorum* Ramat) is a famous and important ornamental plant in the horticulture industry whose cultivation dates back to 2000 years ago. This plant species is considered the second most important flower which has the highest production rate after roses (da Silva, 2003).

Chrysanthemums are industrially propagated by cuttings. However, this method seriously poses the mother plant to viral infection, which would be transferred to the next generations, on the one hand and increases the production cost on the other (Hahn *et al*., 1998). Propagation of chrysanthemum through tissue culture can produce superior plants in large and uniform quantities and sterile cultures (motherstock) can be obtained so that they can be used as material for further propagation (Firgiyanto *et al*., 2023). Popularity and demand have made *Chrysanthemum* one of the first commercial targets for micropropagation, allowing the use of tissue culture in the mass production of this flower. The primary method of *Chrysanthemum* propagation is generally done vegetatively with shoot cuttings and root suckers (Eman *et al*., 2022). Research on developing proper protocols for the *in vitro* culture of chrysanthemums has focused on various factors including the part of the mother plant where the explant is extracted, the physiological age of the explant, the culture medium used, the amount of different nutrients in the culture medium, and the type and concentration of the plant growth regulators (PGRs) used in the culture medium (Park *et al*., 2007).

The explant type is considered a crucial factor in the success of the *in vitro* culture of chrysanthemums (Lim *et al*., 2012). Ji *et al*. (2011) reported that different explants responded to PGRs in the culture medium differently. Various explants from the leaf (Naing *et al*., 2014), petals (Khalili *et al*., 2014), petioles (Lim *et al*., 2012), stems (Song *et al*., 2011), and thin cell layers (da Silva, 2003) have so far been experimented. In some research such as breeding by mutation, the use of callus to induce mutation, and then, the regeneration of the cell and/or tissue mutants are important steps in producing new cultivars. This research explored the effect of PGRs on callus induction and its regeneration in three chrysanthemum cultivars including ‘Bonfire yellow’, ‘Bella rosa’, and ‘Rambla’ .

MATERIALS AND METHODS

This research was conducted at the National Research Center of Flowers and Ornamental Plants in Mahallat, Iran from 2014 to 2018. Three cultivars of cut chrysanthemum stems that were available in Iran were used in the research. They included ‘Banfire Yellow’, ‘Rambla’, and ‘Bella Rosa’. First, 100 healthy terminal cuttings with a length of 10-15 cm were prepared from the cultivars in February. The cuttings that were viewed to be healthy with no symptoms of pests, diseases, or nutrient deficiency were used as the mother plants. After rooting in the culture tray containing cocopeat and perlite at a ratio of 40-60, they were transferred to the main pots. These plants, which were used as mother plants, were cultivated in long-day conditions. The explants used in the research were the leaf explants with an approximate length of 2 cm.

First, the explants were washed with water to remove dust and debris for which they were kept under tap water for 30 minutes in the laboratory and were, then, put in a hypochlorite 1% solution. After 10 minutes in this solution, they were immersed in ethanol 70% for 1 minute, were washed with sterile distilled water under a fume hood three times, and were dried on sterile tissue paper for 5 minutes. The parts damaged with the hypochlorite were removed and finally, the 1-cm-long explants were put in the culture medium taking care of the polarity. The research used the Murashige and Skoog (MS) culture medium containing 30 g/L of sucrose and

7 g/L of agar. To achieve *in vitro* seedlings, the explants were kept in the hormone-free culture medium for two months and were subcultured in a similar fresh culture medium every three weeks. A 1-cm^2 leaf sample was selected from every *in vitro* seedling. These explants were, then, transferred to a culture medium containing various combinations of 1, 2, or 3 mg/L of benzyl adenine (BA) and 0.5 or 1 mg/L of naphthaleneacetic acid (NAA) for callus induction (Table 1). The dense green calli were subcultured in fresh culture media every two weeks.

Table 1. Various treatments of plant growth regulators (PGR) used in the research.

	PGR treatments (mg/L)*
T1	0 BA + 0 NAA
T2	1 BA + 0 NAA
T3	2 BA + 0 NAA
T4	3 BA + 0 NAA
T5	0 BA + 0.5 NAA
T6	1 BA + 0.5 NAA
T7	2 BA + 0.5 NAA
T8	3 BA + 0.5 NAA
T9	0 BA + 1 NAA
T10	1 BA + 1 NAA
T11	2 BA + 1 NAA
T12	3 BA + 1 NAA

The calli were transferred to a regeneration culture medium containing 0.2 mg/L of indole butyric acid (IBA). About 30 calli were cultured in each treatment, and three replications were considered for each experiment. After one month and the emergence of the calli, their volume was evaluated by Hooker and Nabors's (1977) method for callus induction. The regeneration percentage and the viability rate of the induced calli were also recorded. The research was conducted as a factorial experiment based on a completely randomized design with three replications. Finally, the data were subjected to statistical analysis by the SAS (9.1) software package, and the means were compared by the LSD test at the 1% and 5% probability levels.

RESULTS AND DISCUSSION

Effect of the culture medium on chrysanthemum cv. 'Bonfire Yellow'

Two weeks after the establishment of the leaf explants in the MS culture medium containing different hormonal treatments, calli started to form from the wounds so that the explants completely swelled. In the treatments in which only BA was used, no calli formed. According to the mean comparison, the effect of BA and NAA was significant ($P < 0.05$) on callus induction in 'Bonfire Yellow'. It was revealed that the highest regeneration percentage (31.25%) was related to the MS culture medium containing 2 mg/L BA + 1 mg/L NAA. As well, this treatment recorded the highest growth rate and the highest viability rate of 81.25%. In appearance, the calli induced in this treatment were dense and compressed. The results revealed that the amount of PGRs applied to the explants was the most important factor for callus induction in different treatments. The exposure of explants to a suitable proportion of PGRs is a key factor in their morphogenesis under *in vitro* culture. Calli were obtained at acceptable amounts by the subculture of the calli in a similar culture medium. When the culture period of the explants was increased to about 35 days, callus induction increased significantly so that the whole surface of the explants was covered with calli after 45 days. Similar results have been reported for other plants (Ilahi *et al*., 2007; Borodulina *et al*., 2019). This finding agrees with

Thangmanee *et al*. (2012) who reported analogous results for the explants of chrysanthemum radial petals.

Table 2. The mean comparison for the effect of different PGR treatments on the regeneration, viability, and growth rate of chrysanthemum cv. 'Bonfire Yellow'.

Treatments	Regeneration percentage	Viability percentage	Growth rate
T1	-	-	-
T2	-	-	-
T3	-	-	-
T4	-	-	-
T5	11.5	45	7
T6	18	63.25	8
T7	22.25	77	13**
T8	11.25	66.50	9
T9	8.75	67	8
T10	29**	75.75**	9
T11	31.25**	81.75**	14**
T12	25.25	75**	12

**: Significant at $P < 0.01$ based on the LSD test.

Effect of the culture medium on the callus production by the leaf explants of chrysanthemum cv. 'Rambla'

In this cultivar too, in the treatments in which only BA was used, no calli formed. The comparison of means showed that the effect of BA and NAA was significant ($P < 0.05$) on callus production by cv. 'Rambla' based on the LSD test. It was found that the MS culture medium containing 1 mg/L BA + 1 mg/L NAA was related to the highest regeneration percentage of 25%. The same treatment recorded the highest viability rate of 50% too. Also, the produced calli were dense and compressed. According to the findings, the amount of endogenous PGRs in the explants plays a fundamental role in callus induction. Rambla' generally exhibited lower callus production and viability percentage than 'Bonfire Yellow'. It is clear that each cultivar shows different behavior when culture on media with PGRs levels and reported data by Lim *et al*. (2012) supported our claim.

Table 3. The mean comparison for the effect of different PGR treatments on the regeneration, viability, and growth rate of chrysanthemum cv. 'Rambla'.

Treatments	Regeneration percentage	Viability percentage	Growth rate
T1	-	-	-
T2	-	-	-
T3	-	-	-
T4	-	-	-
T5	9.5	43	9
T6	24	42.25	11
T7	22.25	57	13
T8	21.25	48.5**	9
T9	9.75	39	9
T10	25**	50**	18**
T11	23.25**	45.22**	16**
T12	24**	35	13

**: Significant at $P < 0.01$ based on the LSD test.

Effect of the culture medium on the callus production by the leaf explants of chrysanthemum cv. 'Bella Rosa'

In 'Bella Rosa', Two weeks after the cultured leaf pieces, the leaf explants started to form calli at the wound and they swelled. According to the comparison of means, the effect of BA and NAA was significant ($P < 0.05$) on the rate of callus induction in this cultivar. The results revealed that the MS medium containing 3 mg/L BA + 0.5 mg/L NAA was associated with the highest regeneration rate (31.25%). The same treatment had the highest growth rate (13%) and viability rate (62.25%). The calli produced in this treatment had a dense and compressed appearance. In this cultivar, when the explant culture duration was increased to about 35 days, the callus production increased significantly. Thangmanee *et al*. (2012) reported similar results for the explants of chrysanthemum radial petals for calli production and the features of calli which produced were like as we reported. Extensive research has addressed callus generation and induction in chrysanthemums and almost all have proven that MS is the ideal culture medium (Ji *et al*., 2011). In some studies, a combination of BA and 2-4,D have exhibited up to 100% callus generation (Borodulina *et al*., 2019). Like previous studies, the present research established that the most important factor in callus induction and generation in chrysanthemums was a suitable proportion of exogenous PGRs. The application of different auxins entailed different results for the callus generation of chrysanthemums (Naing *et al*., 2014). These compounds can be used separately or in combination with BA in which case they will be more effective in callus generation. Synthetic auxins, e.g., 2-4,D, at small concentrations increase cell division and the size of cells produced in the cultured explants (Ilahi *et al*., 2007). This increased cell size is accompanied by the increase in the rate of cell wall synthesis and, as such, the produced cells evolve rapidly. The combined application of BA and NAA has also been reported for callus induction and generation from the internode explants of chrysanthemums (Ilahi *et al*., 2007). These researchers reported that the best hormonal proportion was 0.5 mg/L BAP + 0.1 mg/L NAA. In this research, the explants cultured in hormone-free MS medium did not generate any calli, which corroborates the results of other studies (Deng *et al*., 2017; Jevremović and Subotić, 2018; Borodulina *et al*., 2019).

Table 4. The mean comparison for the effect of different PGR treatments on the regeneration, viability, and growth rate of chrysanthemum cv. 'Bella Rosa'.

Treatments	Regeneration percentage	Viability percentage	Growth rate
T1	-	-	-
T2	-	-	-
T3	-	-	-
T4	-	-	-
T5	8.5	33	8
T6	26	42	10
T7	21.25	55.22**	12**
T8	31.25**	62.25**	13**
T9	9.25	49	8
T10	25	42.25	11
T11	29.25	45.25	10
T12	31	48	12**

**: Significant at $P < 0.01$ based on the LSD test.

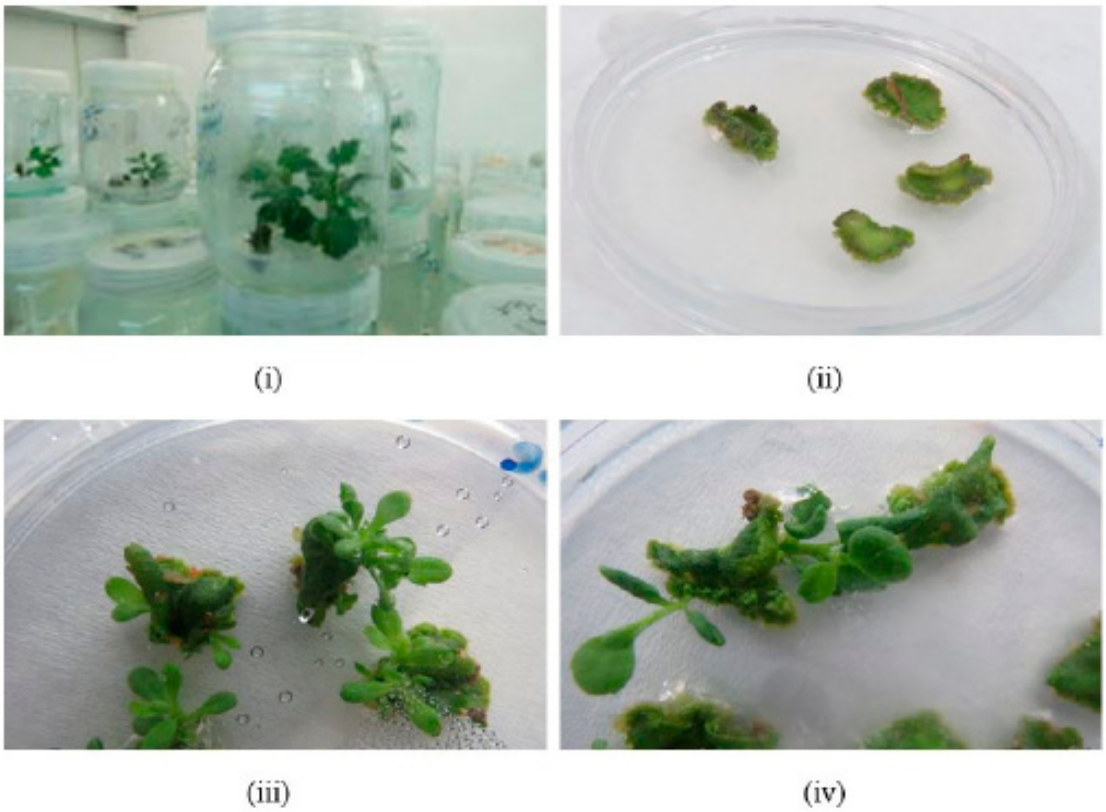

Fig. 1. The developmental stages of the experiment: (i) The production of *in vitro* plantlets to be used as explants, (ii) The production of calli from the leaf pieces, (iii) The regeneration of the calli and the shoot production of 'Bonfire Yellow', and (iv) The regeneration of the calli and the shoot production of 'Rambla'.

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