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RESEARCH ARTICLE

Optimizing the callogenesis and determining the gamma-ray intensity in leaf explant of cut carnation standard cultivars

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ABSTRACT: The present study has been designed and executed to determine the best growth-regulating compound for callus induction as well as to specify the optimum dose of gamma irradiation in carnation cultivars (Tabasco, Nobless, Cameron, Tabor, Eskimo, and Mariposa). In this experiment, an MS culture medium was used to evaluate the various levels of growth regulator concentrations including NAA in four levels (0, 0.5, 1, and 2 mgl^{-1}), 2,4-D in five levels (0, 0.5, 1, 2, and 3 mgl^{-1}), and BA in two levels (0.5 and 1 mgl^{-1}). Irradiating the callus of leaf explants was carried out three weeks after cultivation at 0, 15, 25, 35, 45, and 55-gray doses-to determine the optimum dose of gamma radiation. The analysis of data and illustration of graphs were carried out via Excel software and according to the obtained results, the radiation level that killed 50% of the calluses was selected as the optimum dose for further experiments. The results have indicated that all main effects and the interaction effects regarding the characteristics of callogenesis percentage and callus volume were significant at a probability level of 1%. Means were grouped using Duncan's multiple range test, revealing that the highest level of callus induction was in Eskimo cultivar with a 73% overall mean. Overall, the results indicate that 2 mgl^{-1} 2,4-D, 0.5 mgl^{-1} BA is the best regulatory compound for callogenesis in carnation cultivars. Moreover, it was found that on average, the 25-gray dose leads to suitable results in the callus explants of all cultivars.

KEYWORDS: callus induction, carnation, PGRs, *in vitro* mutagenesis, mutation breeding.

INTRODUCTION

The carnation, *Dianthus caryophyllus* L., is a dicotyledonous plant from the *Caryophyllaceae* family. Carnations are one of the top ten standout cut flowers across the world and thus the plant breeding of this valuable plant, with the goal of improving its qualitative and quantitative traits, is of particular importance. Implementing the techniques of plant tissue culture generally decreases many issues and drastically increases the efficiency of the *in-vitro* breeding techniques [3]. A major application of tissue culture is the production of callus from various plant organs for subsequent plant breeding practices. In this respect, Karimi *et al* [8] tested various sucrose and mannitol concentrations with the aim of producing calluses in carnation petals explants. The result of this research was indicating of the fact that increasing the level of mannitol significantly increases the rate of somatic embryogenesis. The optimum treatment in this research was using MS medium with 3% sucrose and 12%

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mannitol. In another research, Karimi *et al.* [7] investigated the effect of various concentrations of plant PGRs in petal explants for secondary somatic embryogenesis. In this study, it was found that the treatment involving 2 mg/L of 2,4-D and 0.2 mg/L of BA with 9% sucrose in an MS basal medium produces the best results regarding the callus induction. In order to induce callus in the leaf explants of carnation, Sharma *et al.* [15] designed an experiment using different concentrations of plant PGRs and discovered that the best treatment was the MS medium contained with 2 mgl^{-1} of NAA and 0.5 mgl^{-1} of 2,4-D. Another research study, conducted by Esmaiel *et al.* [4] on leaf explants of carnation, used different concentrations of plant PGRs to optimize callus production in carnation and found that the 0.5 mgl^{-1} NAA + 0.5 mgl^{-1} BA led to the highest level of callus production. Response to PGRs in different carnation cultivars for callus induction may be different. In this regards, Teixeira Da Silva [14] conducted a study using various PGR compounds on fifteen carnation cultivars and using internode explants with the goal of producing the highest number of calluses. The results showed that the best treatment for callus production in carnation is the solid MS basal medium along with the 0.5 mgl^{-1} NAA, 1.0 mgl^{-1} BA, and 1.0 mgl^{-1} 2,4-D compound. Considering the fact that the level of reaction to growth regulators differs among cultivars, it is essential to find the most optimal treatment for each cultivar.

Breeding in ornamentals is dependent on genetic variation, and new variation in breeding programs is important for produce of new traits. Mutation breeding is one of the most important method for increasing genetic variation in different ornamental plant. Nowadays, it is notable that due to the characteristics evaluated in ornamental plants, utilizing induced mutation techniques accompany with plant tissue culture techniques is of great efficiency [3-12].

In fact, using the gamma-ray to create mutations is highly efficient such that over 61% of mutants have been produced via the gamma-ray [3]. In this regard, Sabaghi *et al.* [14] Used gamma radiation to develop a mutation in the seed of cut stock (*Matthiola incana*) flowers and, investigating the second generation of the mutant lines, suggested that this method is highly efficient in creating diversity in characteristics. Different research studies have used various tissues for irradiation. In this regard, Bala and Singh [2] investigated the effect of gamma radiation (5, 10, 15, 25, 40, 55, 65, 70, and 80 gray) on single-bud cuttings of rose flowers in an MS medium and tissue culture conditions. The evaluation of morphological traits to investigate the effect of mutation breeding on rose flowers found that using gamma-ray at a 40-gray dose has favorable effects on increasing the degree of diversity.

Kumar *et al.* [9] used gamma radiation to induce mutations in *Chrysanthemum* flowers. They were used 0, 10, 20, and 30 gray doses were used on tissue culture explants of young leaves. The results showed that increasing the dose, enhanced the diversity of vegetative characteristics and the flower-related characteristics, and the 20-gray selected as optimum irradiation dose.

The present research was conducted to determine the optimal PGRs treatment for the callus induction, as well as to determine the optimal dose of gamma irradiation on callus explants of cut carnation standard cultivars.

MATERIALS AND METHODS

Location and plant material

The present study was conducted in the plant tissue culture laboratory of the National Ornamental Plants Research Center in Mahallat, Iran, in the winter of 2015 and spring of 2016. The plant material used in this research included leaf explants from *in-vitro* mother plants of carnation cultivars (Tabasco, Nobless, Cameron, Tabor, Eskimo, and Mariposa). To do so, one-third of the leaf tips (the part attached to the stem), at an approximate length of 5 to 7 mm, were used as explants in this experiment.

Callogenesis

Sterilized plant leaves were used for callus induction. To determine the optimum level of growth regulators for callogenesis, growth regulators including NAA in four levels (0, 0.5, 1, and 2 mgl^{-1}), 2,4-D in five levels (0, 0.5, 1, 2, and 3 mgl^{-1}), and BA in two levels (0.5 and 1 mgl^{-1}) were used to design and execute a factorial experiment based on a completely randomized design. The explants were cultured in an MS medium with various hormone concentrations and a 5.8 pH level and kept under the conditions of 24°C temperature and 16-hours light, 8-hours dark lighting. After six weeks, the produced calluses were examined in terms of callogenesis volume and percentage, and the obtained data were recorded.

Determining the optimum irradiation dose

To determine the optimum irradiation dose, 20 calluses were mass-cultured in an 8-cm petri dish one day before

irradiation and in five repetitions, kept under the conditions of 24°C temperature and 16-hour light, 8-hour dark lighting. To determine the optimum dose of gamma-ray, irradiation was performed with doses of 0, 15, 25, 35, 45, and 55 gray. After irradiation, the calluses were transferred to an MS culture medium containing 2 mg/L TDZ + 0.5 mg/L NAA for regeneration. At this stage, seven calluses were cultured in each 8-cm petri dish. The explants were sub-cultured every three weeks and maintained under controlled conditions until the time of regeneration.

Characteristics Collection and data analysis

The calluses were examined after 21 days in terms of survival, and after 12 weeks in terms of regeneration rate. To determine the volume of calluses, they were scored from one to five for small to large-size, respectively, and the callogenesis rate was stated in percentage. The data obtained from the experiment were analyzed in SAS software. Furthermore, the data obtained from irradiating the calluses were analyzed in Excel software and the charts related to survival rate and regeneration rate were designed using this software.

RESULTS AND DISCUSSION

Callogenesis

The analysis of variance results of the data obtained from the callogenesis treatments of carnation leaf explants showed that the model used in this research study was significant at a 1% level with R^2= 86% and CV=37 regarding the callogenesis percentage trait, and at a 1% level with R^2= 88% and CV=34 regarding the callus volume trait. Also, the analysis of variance results for the main effects and interaction effects of the factors were all significant at a 1% level (Table 1). The comparison of means, based on Duncan's multiple range test, showed that the Eskimo cultivar treated with 2 mgl^{-1} 2,4-D along with 0.5 mgl^{-1} NAA and 0.5 mgl^{-1} BA had the highest mean, the difference of which was significant at a probability level of 5%, placing it in the first group according to Duncan grouping. On average, 2 mgl^{-1} of 2,4-D, 1 mgl^{-1} of NAA, and 0.5 mg/L of BA resulted in optimum callogenesis among the cultivars. The general deduction is that the ternary compound of 2,4-D, NAA, and BA in various concentrations can be used for optimal callogenesis in carnation cultivars. The results of the present study imply that growth regulators have different effects on various carnation cultivars, which is consistent with the results of Arif *et al.* [11]. Moreover, it is evident that using a combination of growth regulators is much more effective on callus induction in carnation leaf explants than their individual application, which is in line with the results of DaSilva [17].

Table 1. Analysis of variance for callus induction (%) and callus volume (%) of Dianthus caryophyllus including individual and Compound for each studied independent variable.

S. O. V.	D.F.	M.S.	
		callus induction (%)	callus Vol (%)
Var	5	54934.44**	46.19**
2,4-D	4	50015**	55.26**
NAA	3	2213.33**	5.5**
BA	1	4000**	5.6**
Var × 2,4-D	20	4740**	4.34**
Var × NAA	15	1752**	0.88**
Var × BA	5	4243**	2.71**
2,4-D × NAA	12	3595**	2.79**
2,4-D × BA	4	2430**	0.78**
NAA × BA	3	974.81**	4.75**
Var × 2,4-D × NAA	60	2170.74**	2.4**
Var × 2,4-D × BA	20	1357.22**	1.62**
Var × NAA × BA	15	1190.55**	0.81**
2,4-D × NAA × BA	12	1190.55	2.95**
Var × 2,4-D × NAA × BA	35	1313.8**	0.71**

Table 2. LD_{50} rate of gamma radiation in callus explants. The optimum doses for different cultivars can be seen in the table.

Cultivars	LD_{50}
Nobless	31.4
Cameron	22
Tabor	23.8
Mariposa	22
Tabasco	28.5
Eskimo	24

Determining the optimum dose

The results of irradiating the explants of carnation calluses showed that by increasing the radiation dose, the survival percentage and the regeneration rate declined in all cultivars (Fig. 1 and Fig 2), which is consistent with the results of Roychoudhury *et al* [13]. Experiment on carnation flowers using chemical mutagens. Also, the LD_{50} was calculated via linear regression, the details of which are depicted in Table 2. According to the results and in terms of callus survival, even though the Eskimo cultivar revealed favorable callogenesis, its reaction to

gamma radiation was extreme, such that survival in doses higher than 15 grays declined to a high degree and callus regeneration was observed only in 15 gray and no regeneration was observed in higher radiation doses in this cultivar (Table 2). According to the results, the Eskimo cultivar was identified as most sensitive to radiation versus the Nobless cultivar which can be introduced as the most resistant to gamma radiation. The results indicate that doses higher than 35 grays drastically reduced the survival and regeneration rate in all cultivars,

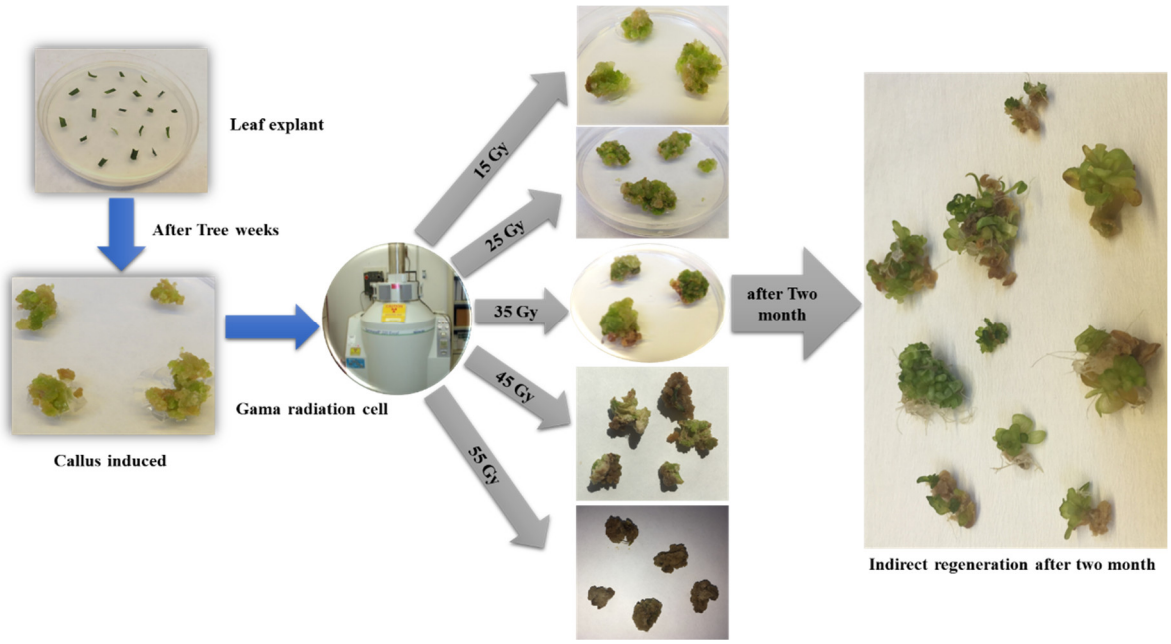

Figure 1. The process of callus induction and in vitro gamma ray irradiations on carnation leaf explants. Left: Plant growth regulators have optimally led to the production of callus from the leaf explant in different carnation cultivars. Middle: Responses of callus explants to gamma radiation are visible.

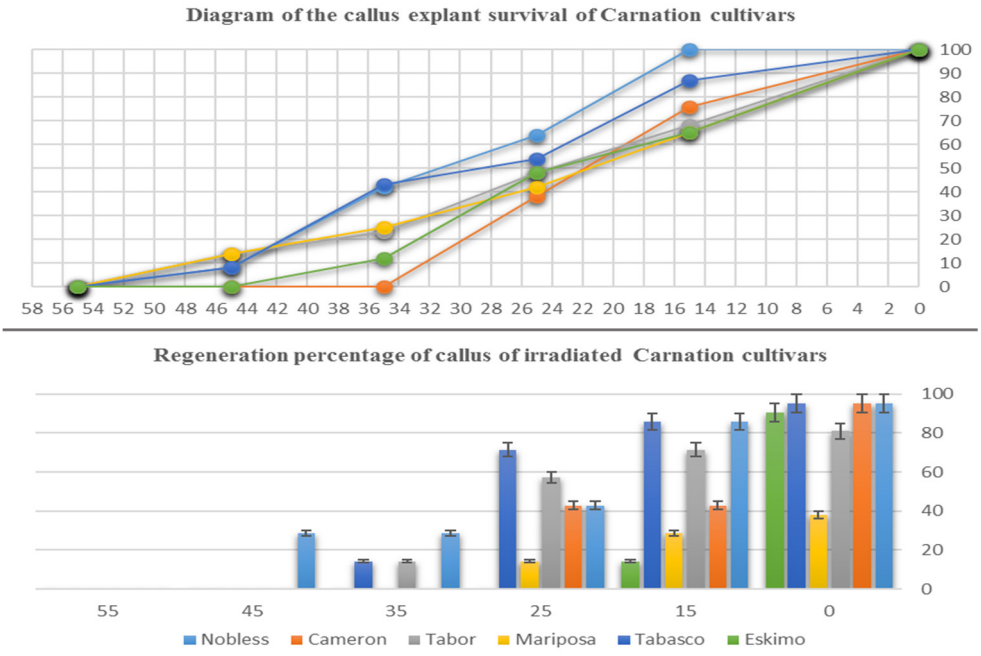

Figure 2. The irradiation results for carnation callus explants showed that increasing the amount of radiation decreased survival percentage. Top: Line diagram of survival rate for callus explants of standard carnation cultivars at different gamma ray intensities. According to the bottom graph, there is a sharp decrease in regeneration at doses higher than 25 Gy. As shown in the figure, by increasing the dose, the rate of survival decreased, and no live callus was observed at 55 Gy.

and only a few cultivars survived with a very low percentage in the 45-gray dose. The regeneration results revealed that only the Nobless cultivar regenerated by 28.57% in the 45-gray dose. It is noteworthy that all calluses were destroyed in the 55-gray dose in all cultivars. According to the results, it can generally be stated that various doses of mutagens have significant effects on the survival and regeneration of carnation flowers, which has also been pointed out by Ibrahim et al [6].

CONCLUSION

In this study, we have identified the best combination of callus induction and effective gamma radiation on callus explants in different carnation cultivars. We found that *in-vitro* leaf explants are highly efficient for *in* vitro breeding in carnation that is confirmed Lata *et al* [10] experiment. Moreover, the combination of mutation breeding methods and *in-vitro* techniques has solved the majority of mutagenesis problems. *In-vitro* mutagenesis methods provide a useful way to rapidly determine LD_{50} and lethal doses in mutation breeding projects (Fig. 2). This method proved to be a useful tool both in callus induction and in determining the efficient gamma radiation dose in carnation cultivars. Compared to the other methods employed for carnation, one of the advantages of *in vitro* mutagenesis is that it can produce a large M_2 population; it has also proved useful in identifying high-rate mutants [18]. Therefore, using the resources and methods outlined in this paper can help researchers in breeding ornamental plants using *in-vitro* mutation breeding methods.

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بهینه سازی کالوس زایی و تعیین شدت پرتو گاما در ریزنمونه های برگی ارقام استاندارد میخک شاخه بریده

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چکیده

میخک به عنوان یکی از مهمترین گل‌های شاخه بریده جهان شناخته شده و تولید ارقام جدید در این گیاه صرفه اقتصادی بالایی دارد. امروزه با استفاده از تکنیک‌های القای جهش در بسیاری از گونه‌ها ارقام جدید تولید شده است. در پژوهش حاضر به منظور تعیین بهترین ترکیب تنظیم کننده رشد جهت تولید کالوس و همچنین تعیین دوز بهینه پرتوتابی با اشعه گاما در ارقام میخک (Tabasco، Mariposa، Eskimo، Tabor، Cameron و Nobless) طراحی و اجراء شد. در این پژوهش از محیط کشت MS و سطوح غلظت‌های مختلف تنظیم کننده های رشد شامل NAA درچهار سطح (0,0.5,1,2 mg/l)، 2,4-D در ۵ سطح (0,0.5,1,2,3 mg/l) و BA در دو سطح (0.5,1mg/l) استفاده شد. پرتوتابی کالوس ریز نمونه برگی در هفته سوم پس از کشت، جهت تعیین دوز بهینه پرتو گاما در دوزهای ۰، ۱۵، ۲۵، ۳۵، ۴۵، ۵۵ گری انجام شد. پس از پرتوتابی کالوس‌ها به محیط باززایی منتقل شدند و پس از ۲۱ روز تعداد کالوس های زنده در ارقام مختلف یادداشت شد. آنالیز داده ها و رسم نمودار توسط نرم افزار اکسل انجام و با توجه به نتایج، میزان پرتویی که سبب مرگ ۵۰ درصد از کالوس‌ها شد، به عنوان دوز بهینه جهت آزمایشات بعدی انتخاب شد. نتایج نشان داد تمامی اثرات اصلی و اثرات متقابل در مورد هر دو صفت درصد کالوس‌زایی و حجم کالوس در سطح احتمال ۱ درصد معنی‌دار بود. گروه‌بندی میانگین‌ها با استفاده از آزمون چند دامنه‌ای دانکن انجام شد و بیشترین میزان تولید کالوس در رقم اسکیمو با میانگین کلی ۷۳ درصدمشاهده شد. به طور کلی نتایج نشان داد که بهترین ترکیب تنظیم‌کننده جهت کالوس‌زایی در ارقام میخک 2,4-D 2mg/l, BA 0.5 mg/l می باشد. همچنین در ریزنمونه‌های کالوس ارقام میخک به طور میانگین دوز ۲۵ گری در تمامی ارقام نتیجه مطلوبی به همراه داشت.

کلمات کلیدی: القای کالوس، میخک، تنظیم‌کننده‌های رشد گیاه، جهش‌زایی این ویترو، اصلاح موتاسیونی