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Carnation Etched Ring Virus Elimination Through Shoot Tip Culture

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ABSTRACT

***Carnation etched ring virus* (CERV) is the second most destructive virus which infects carnation and the only DNA virus among infecting viruses of carnation. In symptomatic leaves of carnation consist of mottling, necrotic and chlorotic flecks or blotches. Virus was detected by DAS-ELISA and PCR. Treatments consisted of different sizes of meristem and MS medium supplemented with different plant growth regulators (PGR_S) (0.5 mg/l benzyl adenine (BA), 0.5 mg/l gibberellic acid (GA_3) and medium without PGR_S.). The plantlets were analysed by PCR in order to evaluate virus eradication. Results of PCR *in vitro* culture revealed that explant size and type of PGR_S had a significant effect on elimination of CERV and the highest amount of it (100%) was observed on medium containing BA in meristem size of 0.4, 0.7 mm and the lowest amount of it (26%) was occurred on medium supplemented with GA_3 in meristem size of 1mm. So far, there is no reporting about influence of PGR_S on elimination of viruses.**

Key Words: *Carnation etched ring virus*, meristem culture, PCR, DAS-ELISA

INTRODUCTION

Carnation (*Dianthus caryophyllus* L.) is one of the most ornamental plants. The importance of this ornamental flower is due to its beauty, diversity of colors, excellent keeping quality and wide range of forms (Ali et al. 2008, Kanwar and Kumar 2009).

Eleven viruses, which in some cases can cause severe losses, affect carnation plants. Six of them are commercially important (Pallas et al. 1999). *Carnation etched ring virus* (*caulimoviridae*) is dsDNA virus and the second important virus that infect carnation. The utilization of healthy propagation materials has been considered as an important tool to prevent the productivity losses of carnation caused by viruses (Tan et al. 2010). Meristem tip culture has been used widely for production of virus-free plant materials in many species propagated mainly or exclusively by vegetative means (Vermeulen and Hean 1964). Meristem tips are generally free or have very low virus concentration and some viruses may be diminishing in the culture (Mori and Hosokawa 1977) and these regions have no connection with plant vascular system (Fayek et al. 2009). Although, in some of the viruses, such as CERV, meristem tip culture is very difficult to eliminate virus (Gosalvez-Bernal et al. 2006). On the other hand, CERV was not cure by thermotherapy (Hakkaart and Jordanova 1968). So the objective of this study was to evaluate the effects of different sizes of meristem tip and plant growth regulators (PGR_S) on elimination of CERV under *in vitro* condition.

MATERIALS AND METHODS

Index of infected mother plants

Fifty six carnations (Ivonne Orange bogr cultivar) in the length of 20 cm were collected from greenhouses (Chenaran- Khorasan Razavi province) of Iran. DAS-ELISA was employed using polyclonal antisera raised against CERV coat protein (AS-0146, DSMZ) and performed following a protocol described by Clark and Adams (1977). Each sample was tested twice and optical densities (OD_S) were recorded at 405 nm with a plate reader, 1 hour after adding the substrate. Infection was determined according to R=X+2SD where, X is average OD of negative controls and SD is standard deviation of this mean relative to its individual wells.

Total DNA extraction and PCR and sequencing

Total DNA was isolated from 0.3 g of a leaf tissue using CTAB method (Zhang et al. 1998). The primers used for PCR amplification were designed for PCR based on highly conserved regions of the CERV coat protein of Netherland isolate (Genbank accession number AJ619761.1, AJ619769.1) by Raikhy et al. (2007) (Table 1). The reaction was carried out in a 25 µl volume containing 1µl of each 10 pmoles/ul primers, 0.5 µl of 10 mmol/l dNTPs, 2.5 µl of 10X PCR buffer (Fermentas, USA), 1.5U of Taq DNA polymerase (Fermentas, USA) and 150ng of DNA. The reaction consisted of 37 cycles of 94 ^oC/30s, 53^oC/60s and 72 ^oC/100s and the final cycle of 72 ^oC/10mins carried out in termocycler (Biometra, USA). PCR products were electrophoresed in 1.5% agarose

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gel in 1X TAE buffer (0.04 mol/l, Tris-acetate and 0.001mol/l EDTA) stained with green viewer and observed under UV transilluminator and photographed using Gel documentation system.

Table 1. Primer pairs used for the detection of *carnation etched ring virus* (CERV) and amplicon produced by polymerase chain reaction.

Primer	Position based on NC-003498.1	Sequence	Amplicon size (bp)
CPU	1901-1872	TCCCCCGGGGGAATGAATAGAGAAGCTAT	1500
CPD	3356-3327	TCCCCCGGGGGATCATTCCGATTCTTCAG	

Purified PCR product (Bioneer, Seoul) and was used for sequencing. Both strands of DNA were sequenced by the dideoxy chain termination method, using an automated sequencer (ABI 3730xl DNA Analyzer, Applied Biosystems, USA) and the ABI BigDye® Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems). The nucleotide sequence of the Iranian isolate was deposited at the NCBI nucleotide dadabase under the Acc.No. JF957838.

Carnation Meristem Culture

Explants sterilization

Stem segments of carnation were selected in their active growth stage. After defoliation, shoot explants about 5-6 cm length were washed thoroughly with tap water for 30 minutes and surface sterilization was done by immersing them in 2% sodium hypochlorite solution containing Tween 20 (2drop/100ml solution) for 15 minutes. Then explants were rinsed 3 times with sterile distilled water.

Medium and plant establishment

Meristem tips in different sizes (0.4 mm: meristem without primordial; 0.7 mm: meristem with primordial; 1mm: meristem with primordia and small stem) (Fig. 1) were cultured on MS medium containing one of PGRs, benzyl adenine (0.5 mg/l), gibberelic acid (0.5 mg/l) and without any PGRs, 30 g/l sucrose, 8 g/l agar (Fig. 2, 3). The pH of the medium was adjusted to 5.7- 5.8 and autoclaved. The cultures were incubated in a growth chamber at 25±2^{o}C under 16 h light conditions. Subcultures were done every 4 weeks.

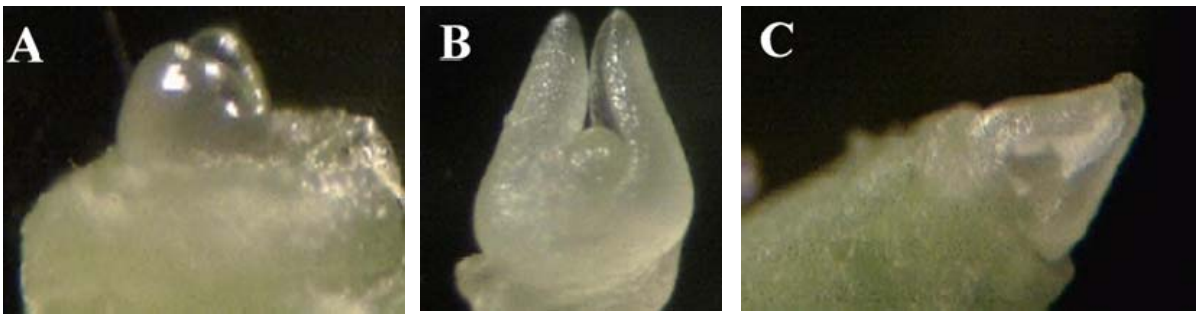

Figure 1. Different sizes of meristem. (A: meristem without primordial. B: meristem with primordia. C: meristem with primordia and small stem)

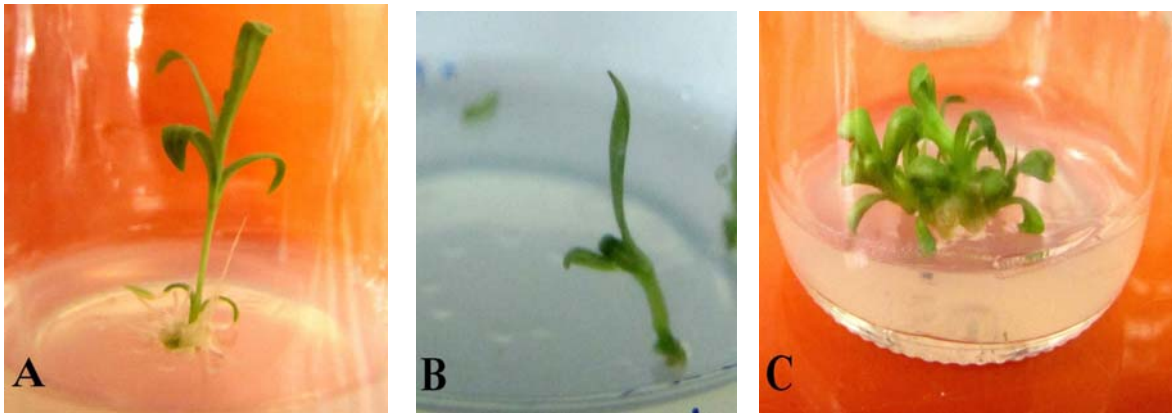

Figure 2. Effect of PGR_S on vegetative growth of carnation. (A: Medium with 0.5 mg/l gibberelic acid, B: Medium without any PGR_S, C: Medium with 0.5 mg/l benzyl adenine).

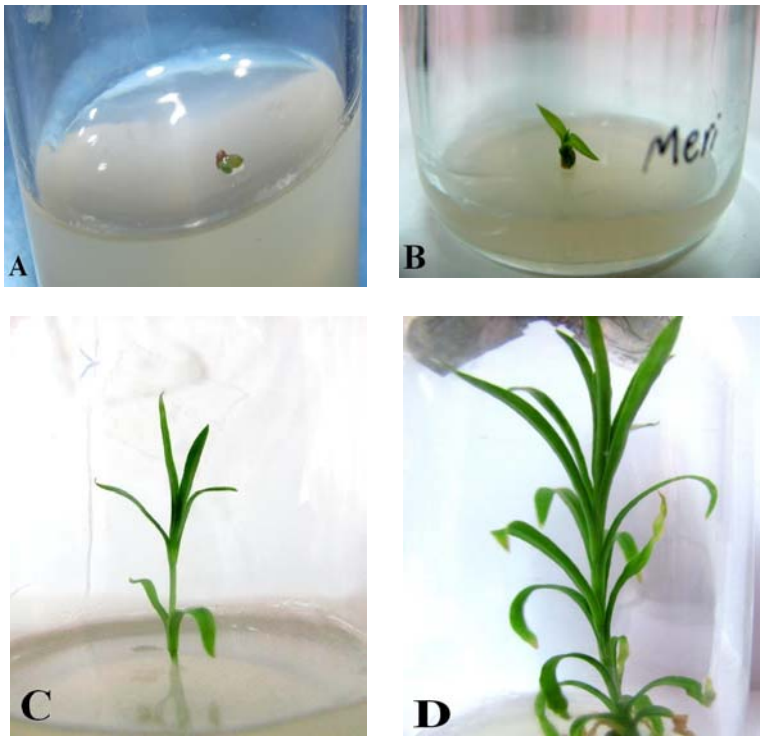

Figure 3. Different growth stage of carnation *in vitro* in medium supplemented with GA_3 (0.5 mg/l). A, B, C and D, respectively, 1, 2, 4 and 6 weeks after culturing.

Statistical analysis

A completely randomized design with ten replications was used. Treatment were verified based on ANOVA using MSTAT-C program and the means were compared using Duncan multiple range test (DMRT) at $P \leq 0.01$.

RESULTS AND DISCUSSION

Many factors influence the *in vitro* culture of carnation (Kharrazi et al. 2011). The factors considered in this study were the effect of different PGRs and explant size on shoot multiplication of carnation. Kind of PGRs in subculture stage affected on establishment, thus significant differences occurred among explants as shown in Table 2.

Table 2. The effect of the meristem tip size and kind of PGR_S on the production of carnation etched ring virus-free plantlets.

Size of explants (mm)			
1	0.7	0.4	Kind of PGR_S
26% (4-15)	9% (1-11)	9% (1-11)	GA_3 (0.5 mg/l)
4.76% (1-21)	0% (0-8)	0% (0-9)	BA (0.5 mg/l)

The effect of different kind of PGRs and explant size

The results of this experiment showed that the size of explants and kind of PGRs had a significant influence on rate of vitrification, rooting and height of explant. The highest amount of shoots number (5.1) was observed on medium containing BA in meristem with primordia and small stem explant. Significant differences were not occurred between different sizes of explants on medium containing BA, although in this medium, increasing size of explant led to increasing the rate of shoot regeneration. In addition, there was not shoot regeneration in the media supplemented with GA_3 (0.5 mg/l) and without PGRs and in GA3 containing medium, primary explants grew up normally, while in the medium without any PGRs there was little growth that considered as a failure to growth. Our results are in close agreement with Dagnino et al. (1991) that indicate GA_3 increase longitudinal growth of explant and did not have any influence on shoot regeneration (Table 3).

Table 3. Interaction effects of growth regulator type and explant type on some growth characteristic of carnation during *in vitro* condition.

Type of growth regulator	Explant size (mm)	Height of grown shoot (cm)	Rate of vitrification (%)	Rate of rooting (%)	Number of regenerated shoot
0.5 mg/l GA_3	1	2. 90 a*	0.00 e	100.00 a	0.00 b
	0.7	2.26 b	10.00 d	100.00 a	0.00 b
	0.4	2.20 b	20.30 c	81.80 b	0.00 b
0.5 mg/l BA	1	1.46 c	51.60 b	22.00 d	5.10 a
	0.7	1.64 c	50.00 b	0.00 e	4.80 a
	0.4	0.96 d	59.30 a	0.00 e	4.50 a
Without any PGRs	1	0.52 de	10.70 d	60.60 c	0.00 b
	0.7	0.36 e	10.90 d	59.40 c	0.00 b
	0.4	0.37 e	18.00 c	0.00 e	0.00 b

Means with similar letters in each column are not significantly different by Duncan's multiple range test ($p<0.05$).

In all explants, the maximum percentage of vitrification was observed in the media supplemented with BA. The highest amount of vitrification (59.30%) was observed on medium containing BA (0.5 mg/l) in meristem explants and the lowest amount of it (0%) was observed on medium containing GA_3 (0.5 mg/l) in meristem with primordial and small stem explant (Table 3). Our results are similar to Jain et al. (1997) and Jain et al. (2001) reports but in contrast to the finding of kim et al. (1998).

Mean comparison showed that size of explant and kind of PGR_S significantly influenced the height of plantlet. The highest height of plantlet (2.90 cm) was in medium containing GA_3 and meristem with primordia and small stem, while the lowest amount of it (0.36 cm) was observed in medium without any PGRs and meristem with primordia. In all sizes of explants, BA led to production of shorter plantlets in compare with GA_3 (Fig1-C). These results are in close agreement with finding of Jain et al. (1997).

The results showed smaller sizes of explant caused deduction in rooting rate. The highest root percentage for all sizes of explant were achieved on the medium supplemented with 0.5 mg/l GA_3, while no rooting was shown on the medium enriched with 0.5 mg/l BA and MS medium without any PGRs (Table3). These results are in according with the finding of Ullah et al. (2007) and Fayek et al. (2009). Our results and previous studies (Vu and Yelenosky 1988) have shown that application of BA in the medium stimulated micropropagation stage and the number of regenerated shoots, as BA stimulated the rate of mitotic process (Kiss et al. 2001), it is expected BA led to increase in shoot dry weight, number of leaf and leaf area index (data was not shown).

Virus detection

Primary index was done by ELISA, because our aim was selection of the most infected plants, although virus testing to confirm the infection carried out by PCR followed by sequencing. However, ELISA lacks the sensitivity required for the detection of virus, which occur in low concentration in these tissues. Furthermore, secondary index of virus elimination was performed by PCR. In PCR, the expected amplification of 1500bp was obtained in virus infected plants (naturally and *in vitro* grown) with CERV, while no amplification was obtained in CERV free plants (Fig. 4).

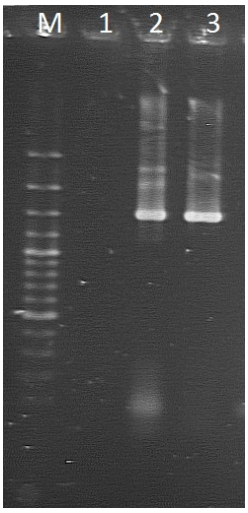

Figure 4. Agarose gel electrophoresis analysis of PCR product from total DNA extracted from carnation tissues individually infected with CERV. Lane 1, negative control. Lan 2, the virus specific amplified band 1500 bp. Lane 3, positive control. Lane M, 100 bp ladder.

Effect of meristem size on its establishment and virus elimination

Meristem tip of 0.4 mm size were found to be optimum for CERV elimination from infected carnations. Larger sizes of meristem (0.7 and 1 mm) in many cases were found to be virus infected (Table 2). This results and earlier study (Mori and Hosokawa 1977) show the elimination ratio of virus was higher when the size of meristem tip was smaller, although elimination limit of viruses differ considerably by the species of plants and viruses.

As it clear from the data in Table 2, there is a significant difference between two kinds of PGRs (BA and GA_3) in virus elimination. The most virus elimination was occurred on the culture medium enriched with BA. Although so far there is no report about influence of PGRs on virus elimination.

CONCLUSIONS

It become clear from the results, the relation between the size of explants to produce virus-free plantlets are generally in parallel, But effective size to produce virus- free plantlet independent on distribution, concentration or probably mixed infection virus in shoot apex of virus infected plants.

According to our results, meristem size 0.4 and 0.7 and medium containing enriched with BA is recommended to elimination of CERV using meristem tip culture under *in vitro* condition. Based on the results of this study, it can be concluded that simultaneously BA decreases infection of virus, and at the same time it increases the rate of vitrification. Consequently it is suggested that for obtaining normal plantlets with minimum

vitrification rate, lower concentration of BA should be used in the medium. More researches should be done for approaching to the method that led to increase the production of virus-free plantlet without any problem of vitrification at commercial levels.

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