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Research Article

Elimination of *Bean yellow mosaic virus* through thermotherapy combined with meristem-tip culture in gladiolus corms

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Abstract: *Bean yellow mosaic virus* (BYMV, *Potyvirus*, *Potyviridae*) causes serious disease in *Gladiolus* spp. In this work, the possibility of obtaining BYMV free plant material from virus infected gladiolus corms was studied. Thermotherapy, meristem-tip culture and combination of both techniques on infected corms/meristem-tip explants (0.5–1 mm in length) resulted in BYMV elimination up to 15.38, 78.04 and 86.66%, respectively, as determined by double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) and reverse transcription-polymerase chain reaction (IC-RT-PCR). Individual virus-free shoots readily rooted in vitro and were transferred to corm formation medium. The results showed that thermotherapy promotes the survival rate of explants during meristem-tip culture steps (except regeneration step) and also plantlet acclimatization. Statistical analysis showed that the BYMV elimination in gladiolus corms was significantly ($P \leq 0.01$) affected by thermotherapy treatment of infected corms. Thermotherapy combined with meristem culture can greatly improve BYMV elimination efficiency from infected gladiolus corms, resulting in the production of BYMV free gladiolus plants.

Keywords: BYMV, Gladiolus, Meristem-tip culture, Thermotherapy, Virus free corm.

Introduction

The gladiolus (*Gladiolus* spp.) is an important component of the world floriculture industry and ranks among the top six flowers of export market (Anonymous, 1997). In the recent years, in spite of its high demand, decline in production has been observed in Iran. Viral diseases attain important status and many viruses have been reported to infect gladiolus cultivars. *Bean yellow mosaic virus* (BYMV) is the most prevalent one (Zaidi *et al.,* 1993), causing mosaic and flower color break symptoms (Raizada *et al.,* 1989) and sometimes it is symptomless (Nagel *et al.,* 1983). Occurrence of BYMV on *Gladiolus* sp. has been reported for the first time by Kamran and Izadpanah (1981) from Iran. Since gladiolus cultivars are vegetatively propagated, without maintaining their health standard, there is a great probability of virus transmission. So the utilization of healthy propagation materials has been considered as an important step to prevent the productivity losses of gladiolus plants caused by viruses. Meristem culture is a widely used method for virus eradication from horticultural plants, such as species propagated mainly by vegetative means (Faccioli and Marani, 1998; Ayabe and Sumi, 2001). Gene silencing is the main mechanism of virus eradication by meristem culture (Foster *et al.,* 2002). This technique has been used before to obtain virus-free ornamental plants (Allen, 1975; Fraga *et al.,* 2004; Ram *et al.,* 2005; Kumar *et al.,* 2009). However, production of virus-free plants by using meristem culture involves determination of optimal size range of shoot tips,

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supporting efficient virus elimination and also a high rate of plant regeneration (Wang *et al.,* 2006).

Heat treatment was originally applied by Kassanis in 1949 to eliminate viruses from plant tissue (Parmessur and Saumtally, 2001). Since high temperature can inhibit virus replication and movement, thermotherapy combined with meristem culture can greatly improve virus elimination efficiency by augmenting the virus-free region of treated shoot tips (Walkey, 1980). Thermotherapy combined with meristem culture was used to eliminate LSV form *Lilium elegans* (Nesi *et al.,* 2009). So far, virus elimination of ornamentals has been carried out only in a few cases in Iran. During two experiments, *Carnation etched ring caulimovirus* (CERV) and *Carnation mottle carmovirus* (CarMV) were eradicated from carnation explants by meristem culture alone (Ashnayi *et al.,* 2012) and combined with thermotherapy (Sepahpoor *et al.,* 2009), respectively. Virus testing of in vitro-derived planting material is vital for producing virus-free propagation material and requires more reliable and sensitive methods (Dovas *et al.,* 2001). The combination of an efficient in vitro protocol for virus elimination and a highly sensitive diagnostic technique can allow for the production of virus-free gladiolus propagation material for commercial use.

Owing to rapidly increasing demand of gladiolus in both local and global markets, its cultivation is increasing in different regions of Iran, such as Tehran and Markazi provinces. In this work, an attempt was made to obtain BYMV-free plant materials from gladiolus infected corms through meristem-tip culture and thermotherapy followed by meristem-tip culture.

Materials and Methods

Plant material

Distribution centers of *Gladiolus* spp. in Tehran and Markazi provinces of Iran were surveyed during 2010 and totally 341 corms with a mean weight of 12-16 g were randomly collected. Gladiolus corms were planted in pots under greenhouse conditions after breaking their dormancy by 100 ppm solution of Gibberllic acid for 24 h. Infection of corms with BYMV was checked by DAS-ELISA (Clark and Adams, 1977) using fresh leaf extract samples and BYMV polyclonal antibodies (AS-0471, DSMZ, Germany). The absorbance value of each well was measured at 405 nm by ELISA reader (Beckman AD 340S; Beckman Coulter, Fullerton, CA, USA). Samples were considered positive when OD values were more than twice the mean OD of the healthy control. After 170-190 days, the daughter corms, produced by BYMV infected maternal ones were used as the source of explants directly or after thermotherapy, following a period of dormancy at 4 ºC. Fresh corms were washed with tap water, surface disinfested with ethanol 70% (v/v) and NaOCl 10% (1.5% (v/v) commercial bleach) for 1.5 and 5 min, respectively, and finally rinsed three times in sterile distilled water, then some of them were used to prepare the explants directly (without thermotherapy). Thirty eight newly produced corms from BYMV infected maternal corms were subjected to thermotherapy.

Thermotherapy

Thirty eight newly produced corms from BYMV infected maternal corms were subjected to sequential thermotherapy treatment including 32 ºC for a week, 37 ºC for two weeks, and 39 ºC for three weeks in growth chamber. After heat treatment, 16 out of 38 were planted in pots to evaluate the efficiency of thermotherapy alone in the BYMV elimination from the obtained plants and the rest of treated corms were used as the source of excised explants.

Meristem-tip culture

Meristem-tip explants (0.5–1 mm apical dome plus 2–3 leaf primordia) were excised from apical and axillary buds of corms directly (without thermotherapy) or after application of sequential thermotherapy under aseptic conditions using a laminar flow cabinet. The experimental design was an unbalanced completely randomized design. For explants culture, the Murashige and Skoog (1962) medium, supplemented with 3% (w/v) sucrose and 0.75% (w/v) agar, was used as basal medium with pH adjusted to 5.8 before autoclaving. The meristem-tip explants, 0.5–1 mm in length, were cultured onto MS (MS with 3% (w/v) sucrose and

0.75% (w/v) agar) without plant growth regulators under a stereoscope furnished with a cold light source. After 5–6 weeks, all survivor explants were transferred to the same medium for shoot formation. Individual explants taken from cultures formed roots in a MS medium with 3% (w/v) sucrose and 0.45% (w/v) agar containing 0.002 mM NAA (α-naphthaleneacetic acid). The rooted plants were then transferred to the corm formation medium (MS medium containing 5% (w/v) sucrose, 0.45% (w/v) agar and 0.001 mM NAA). All cultures were maintained at daily temperature 28 ± 2 ºC and nightly temperature 24 ± 2 ºC with a photoperiod of 16 h and light irradiance of 40 µ E. m^{-2}. Sec^{-1} from cool white fluorescent lamps.

Plantlet acclimatization

Rooted shoots and produced corms from virus-free cultures were washed thoroughly to remove residues of the medium and planted in a peat/perlite and sandstorm mixture (1/2/2 v/v) in plastic trays, which then were transferred to a fog propagation system with 50% shading. In the fog, the relative humidity was adjusted to 96–99% during the first week and was gradually reduced to 85% over the next 2 weeks for acclimatization. During hardening, the young plantlets were sprayed with a commercial fungicide, twice a week, to avoid pathogen infections. The young plantlets were sprayed with a 0.1 dilution (10% concentration) MS medium during the first week.

Virus detection

Efficiency of meristem-tip culture and thermotherapy combined with meristem-tip culture in elimination of BYMV from gladiolus corms was evaluated by DAS-ELISA, IC-RT-PCR and back inoculation on *C. amaranticolor* using sap extract of leaves derived from in vitro meristem-tip explants and young acclimatized gladiolus plantlets. For IC-RT-PCR fresh leaf tissue was macerated in PBST buffer containing 2% PVP-40 and incubated in PCR vials which had been coated with BYMV IgG. cDNA was synthesized using M-MuLV reverse transcriptase (Fermentas GmbH, St. Leon-Rot, Germany) and BYMV-reverse primer (5′-CCAAAGTTCCAATCACCACC-3′) at 42 ºC for 60 min. Final PCR profile consisting of a single cycle of 2 min at 94 ºC; 38 cycles of 60 s at 94 ºC, 60 s at 53 ºC and 60 s at 72 ºC; and a final extension for 10 min at 72 ºC was performed using BYMV-forward (5′-CT(AC)CA(AG)ATGGAGAA(CT)CC(CT)GC-3′) and reverse primers. These primers were designed from conserved sequences in the helper component-proteinase (HC-Pro) protein region of BYMV genome by Nakazono-Nagaoka *et al.* (2004).

Statistical analysis

Data pertaining to the effect of thermotherapy on density of BYMV in gladiolus corms were subjected to one-way analysis of variance (ANOVA) test under completely randomized design. The data obtained were statistically analyzed using Stat graphics Centurion 16.1.11 (Stat Point, USA) and the differences among means were compared by Least Significant Difference (LSD).

Results

Detection of BYMV in plants obtained from 341 gladiolus corms by DAS-ELISA revealed infection of 274 of them, meaning 80.35% infection rate with no significant difference between Tehran and Markazi provinces. The choice of appropriate explant is critical in tissue culture (Ascough *et al.*, 2009). In gladiolus, the most popular tissue source has been corms (Nhut *et al.*, 2004) or inflorescence explants (Ziv and Lilien-Kipnis, 2000). In present investigation, gladiolus corms were used as the source of explants. Following thermotherapy step performed on 38 newly produced corms from maternal infected ones, the presence of BYMV in 16 corms (out of 38) was assayed by DAS-ELISA using fresh leaf extract. Although three out of 16 (18.75%) heat treated corms were unable to germinate in soil, serological assay results showed that only two out of 13 plants were free of the virus, explaining the low efficiency (15.38%) of thermotherapy on BYMV elimination from corms.

Meristem-tip explants (0.5-1mm in length) were derived from BYMV infected gladiolus corms with and without thermotherapy (Fig. 1A). For evaluation of meristem-tip culture and thermotherapy coupled with meristem-tip culture techniques efficiency in BYMV elimination, totally 121 and 73 meristem-tips were excised from non-heat-treated and heat-treated

gladiolus corms respectively (Table 1). In the establishment stage (Fig. 1B), the percentage survival of explants 1mm in length (38.46%, 45 out of 117) was higher than that for explants 0.5 mm (23.37%, 18 out of 77) in length and few of meristem-tip explants 0.5mm in length could grow and be established, and all died in rooting step (Table 1). The percentage survival of explants (0.5 and 1mm in lengths) excised from non-heat-treated corms (26.19% and 40.5% respectively) was more than heat treated corms (20% and 34.21% respectively). Shoots with 1-2 leaves and 10-15 cm length were produced in proliferation stage. All 41 and 30 proliferated explants from non-heat-treated and heat treated corms were tested for the presence of BYMV.

According to the DAS-ELISA results, the percentage of virus free shoots regenerated from meristems with 0.5 mm length (86.66%, 13 out of 15) was higher than larger meristems with 1mm length (80.35%, 45 out of 56). In our study, virus elimination occurred in most of the established cultures derived from heat-treated meristem-tips (Table 1), indicating thermotherapy followed by meristem-tip culture was more efficient in production of virus free explants than meristem-tip culture alone.

In root formation medium, 62.5% of shoots (35 out of 56) were rooted and produced 3-16 roots from 4-10 cm in length (Fig. 1C; Table 1). 60 % of all rooted plants (21 out of 35) were able to produce new corms (about10-15 mm in diameter) in corm formation medium (Fig. 1D and E; Table 1). The percentage of survivors during different steps of meristem-tip culture, including regeneration, corm production and acclimatization, explants derived from thermotherapy followed by meristem-tip culture was higher than that of meristem-tip culture alone. Finally six plantlets (28.57%) were acclimatized in acclimatization stage (Fig. 1F; Table 1), which were all free of BYMV based on DAS-ELISA. In IC-RT-PCR reactions which were performed using sap extract of leaves derived from in vitro meristem-tip explants and young acclimatized gladiolus plantlets and BYMV HC-Pro primer pair, no fragment was amplified while a DNA fragment of 1100 bp was amplified in positive control (Fig. 2). Back inoculation of meristem-tip explants and young acclimatized gladiolus plantlets leaf extract on *C. amaranticolor* did not induce any symptoms on leaves.

Statistical analysis showed that the BYMV elimination in gladiolus corms differed significantly by thermotherapy treatment of infected corms at $P \leq 0.01$ (Fig. 3). On the other hand, success in production of virus-free plants from infected gladiolus corms was not affected by the origin of corms as the source of change.

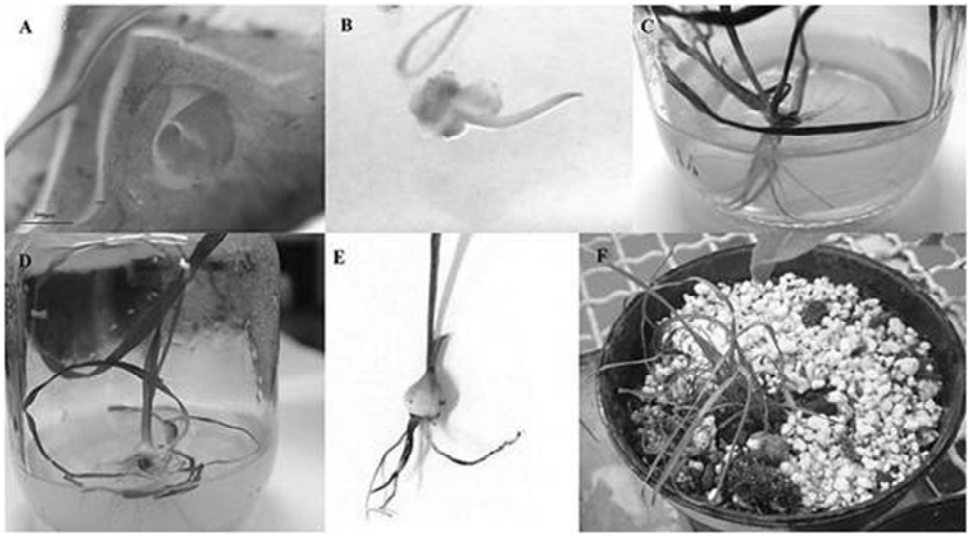

Figure 1 Different stages of in vitro regeneration of the explants derived from BYMV infected gladiolus corms. A, light microscopy of meristem excised from corm with an apical dome protected by several primordial leaves. B, establishment of meristem-tip explant. C, rooting of a virus-free gladiolus microcuttings on supplemented MS medium. D-E, corm production of a virus-free gladiolus plantlet on supplemented MS medium. F, virus-free gladiolus plantlet acclimatized in greenhouse.

Table 1 The percentage of survivor explants in different steps of BYMV-free gladiolus corm production by meristem-tip culture and thermotherapy coupled with meristem-tip culture techniques.

	Cultured explants No.			Proliferated explants No. (%)			BYMV free explants No. (%)			Rooted explants No. (%)		Corm produced explants No. (%)		Plants produced after acclimatization No. (%)	
	0.5 mm	1 mm	Total	0.5 mm	1 mm	Total	0.5 mm	1 mm	Total	0.5 mm	1 mm	0.5 mm	1 mm	0.5 mm	1 mm
Meristem- tip culture	42	79	121	6 (14.28)	35 (44.30)	41 (33.88)	5 (83.33)	27 (77.14)	32 (78.04)	0	24 (68.57)	0	13 (54.16)	0	3 (23.07)
Thermotherapy with meristem-tip culture	35	38	73	9 (25.71)	21 (55.26)	30 (41.09)	8 (88.88)	18 (85.71)	26 (86.66)	0	11 (52.38)	0	8 (72.72)	0	3 (37.50)
Total	77	117	194	15 (19.48)	56 (47.86)	71 (36.59)	13 (86.66)	45 (80.35)	58 (81.69)	0	35 (62.50)	0	21 (60.00)	0	6 (28.57)

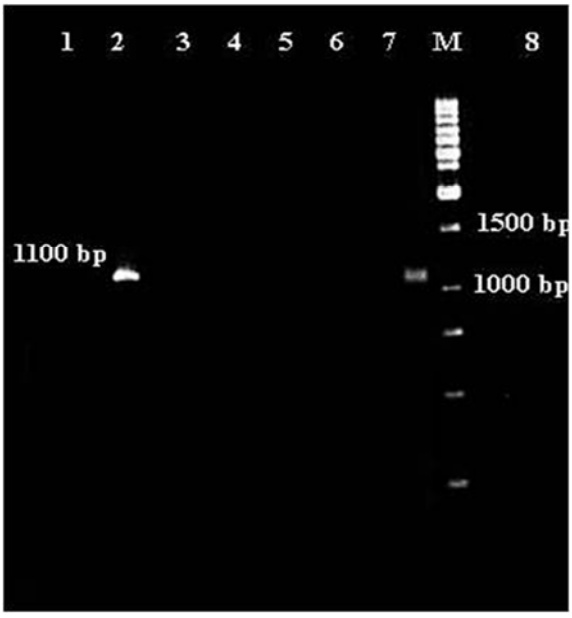

Figure 2 Electrophoresis pattern of IC-RT-PCR products on 0.8 agarose using BYMV HC-Pro primer pair and leaf sap extracted from BYMV-infected plants (lanes 2, 7); young acclimatized gladiolus plantlets derived from BYMV infected corm following meristem-tip culture only (lanes 1, 3, 4) and thermotherapy coupled with meristem-tip culture (lanes 5, 6, 8) techniques; M, 1Kb DNA ladder.

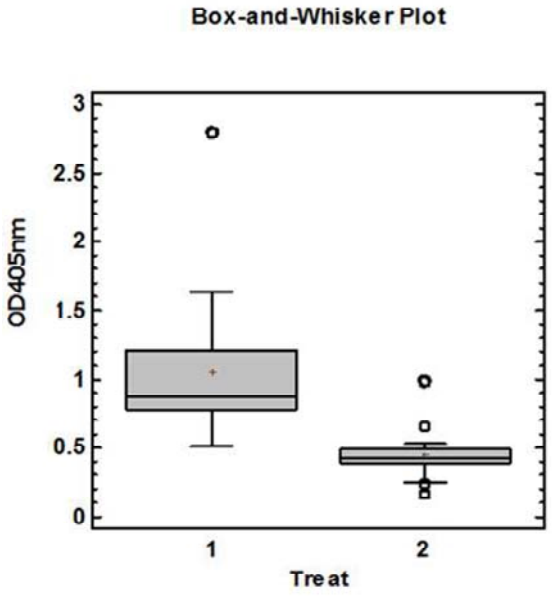

Figure 3 Analysis of thermotherapy effect on BYMV elimination in gladiolus infected corms using least significant difference (LSD) method based on OD_{405} values in ELISA, $p \leq 0.01$. treatment1 (with thermotherapy, *LS Mean* = 0.446) and treatment 2 (without thermotherapy, *LS Mean* = 1.051).

Discussion

The results presented in this study demonstrate partial success in the whole system of virus elimination by a combination of meristem-tip culture and thermotherapy. In our study, most of the established cultures which were derived

from heat-treated corms were free of BYMV, explaining the high efficiency (86.66%, 26 out of 30) of coupled thermotherapy/ meristem-tip culture in elimination of BYMV from corms. More than twice efficiency of thermotherapy combined with meristem-tip culture than meristem-tip culture alone has been reported for the elimination of CarMV from carnation plants (Sepahpoor *et al.,* 2009).

The specific differences in the regeneration potential of different explants of various sources and sizes were reported by a number of researchers (Nhut *et al.,* 2004; Memon *et al.,* 2010). In this study, the survival percentage of 1mm explants was more than those with 0.5 mm in length and the percentage of virus free shoots regenerated from 0.5 mm explants was more than explants 1mm in length. The capability of the meristem to develop into a full plant is directly related to the size of the explant. Although potential of big explants for establishment and growth was more than smaller ones, the efficiency of virus elimination in smaller explants was more than in bigger ones, as was expected from the previous studies (Faccioli and Marani, 1998; Ashnayi *et al.,* 2012). On the other hand, the application of thermotherapy with meristem-tip culture, allowed higher number of meristems to be excised, which is in accordance with previous research (Sepahpoor *et al.,* 2009;). In present investigation, the percentage of virus free shoots regenerated from heat treated corms was higher than explants excised from non-heat-treated corms. The application of meristem-tip culture to eradicate viral particles was initially based on the concept of meristem "immunity" towards viruses (Morel, 1948). Matthews (1991) suggested that in dividing cells, the plant mRNAs that are present in high concentrations in active cells might compete effectively with viral RNAs for the translation apparatus. Alternatively, cell-to-cell movement proteins are unable to function in meristematic cells due to their lack of selectivity for the sequence of nucleic acid they transport (Hake and Char, 1997). Moreover, the size and the number of plasmodesmata increase with respect to their location distally from the meristematic cell which would improve cell-to-cell virus translocation in the distal region. Different researchers have shown that the probability of obtaining virus-free plants is inversely related to the size of the meristem (Faccioli and Marani, 1998). However, the capability of the meristem to develop into a full plant is directly related to the size of the explants. In this study, we showed that thermotherapy in combination with meristem-tip culture could greatly promote the efficiency of virus elimination. Until now, we know little about the mechanism how thermotherapy enhances virus eradication from plants. Some early studies indicated that high temperature could augment the virus-free areas of treated plants by inhibiting virus replication or movement and improving the growth speed of treated plants (Cooper and Walkey, 1978) which resulted in the subsequent virus eradication from meristem-tips. Recently, it was found that viral RNA silencing could be significantly enhanced at the higher temperatures (Chellappan *et al.,* 2005). Northern blot analysis also revealed a drastic decrease in the amounts of *Raspberry bushy dwarf virus* (RBDV) RNA in raspberry shoot tips following 5 days of thermotherapy, and it was anticipated that the RNA silencing was enhanced and viral RNA was degraded in shoot tips during thermotherapy (Wang *et al.,* 2008).

In the present investigation, 50% of all shoots transferred to the root inducing MS medium containing 0.002 mM NAA, were rooted. Better rooting plays major role in acclimatization. The poor survival rate of transplanted plants is usually the result of poorly developed roots (Ziv *et al.,* 1970). Thus a major change in the pre-transplant stage is the change in culture environment to promote root initiation and shoot elongation. Investigations numerous groups of researchers have specified that various factors such as low concentrations or complete elimination of cytokinins and high concentrations of auxins (Logan and Zettler, 1985; Lilien-Kipnis and Kochba, 1987), low concentrations of inorganic salts (Sriskandarajal and Mullins, 1981), addition of sucrose (Kumar

et al., 1999), activated charcoal (Lilien-Kipnis and Kochba, 1987) and vermiculite (Logan and Zettler, 1985) instead of agar in the medium is required for better rooting response in in vitro cultures. Rooting in simple basal MS medium without plant growth regulators was also observed by Goo *et al.* (2003). The two most important plant growth regulators cytokinins and auxins have been reported as essential for in vitro regeneration and proliferation of shoots in gladiolus (Memon, 2012a). Regarding auxins, naphthalene acetic acid (NAA, 0.5-1 mg L) is the most commonly used plant growth regulator. In gladiolus, root length, number and its morphology was greatly affected by the increasing levels of NAA (Lilien-Kipnis and Kochba, 1987). Ahmad *et al.* (2000) also observed better rooting response on MS medium supplemented with NAA (0.5 mg L-1) or IBA (3.9 mg L-1).

In vitro corm formation by senescing shoots/plantlets of gladiolus has been reported by several workers (Sutter, 1986; De Bruyn and Ferreira, 1992). Sucrose concentration at a higher (4-10%) than normal level (3%) in tissue culture media favours the formation of storage organs in many species (Dantu and Bhojwani, 1987) such as tulip (Nishiuchi, 1980) and lilium (Takayama and Misawa, 1979). Increased levels of sucrose are also known to promote in vitro tuber formation in Dioscorea (Choy, 1988) and potato (Abbott and Belcher, 1986). Various explants such as nodal buds (Arora *et al.,* 1996), cormel tips (Arora *et al.,* 1996), inflorescence stalk (Ziv *et al.,* 1970), axillary buds of corm (Dantu and Bhojwani, 1987; Ahmad *et al.,* 2000) and slices of cormel sprouts (Sinha and Roy, 2002) have been utilized for in vitro cormel production in gladiolus on MS basal medium with different concentrations of growth hormones and sucrose. Sucrose plays an important role for in vitro cormel formation in gladiolus (Dantu and Bhojwani, 1987; Arora *et al.,* 1996; Sinha and Roy, 2002). In the present work, 60% of rooted plants produced corm in corm formation medium containing 5% (w/v) sucrose. The addition of sucrose had a positive effect on cormel weight and number in gladiolus, while the presence of 12% sucrose in MS basal medium exhibited elongated leaves but small cormels (Nagaraju *et al.,* 2002). This suggests that higher concentration of sucrose limit growth in general due to osmotic effect of sugars. Sinha and Roy, (2002) produced gladiolus corms in three sizes from rooted shoots cultured in half strength of MS supplemented with IBA (2 mg L) and sucrose (6%). Better corm production was also obtained on a medium supplemented with sucrose (6-9%) and cultured at 15°C (De Bruyn and Ferreira, 1992). Successful cormel formation of gladiolus varieties has been reported on medium containing IBA (1 mg L) and 7% sucrose (Memon *et al.,* 2010; 2012b).

In conclusion, on one hand the acquisition of virus-free plant material in gladiolus from meristem-tip explants derived from corms showed satisfactory results regarding explant survival and growth. On the other hand, the application of thermotherapy, followed by meristem-tip culture (allowing a larger meristem to be excised), gave encouraging results indicating that the acquisition of plant propagation material free from BYMV is possible even in the case of virus-infected gladiolus corms, which would otherwise be difficult to get rid of virus in meristem-tip culture. Gladiolus is vegetatively propagated and use of infected corm results in widespread occurrence of BYMV. A protocol has been developed to produce virus free plants through thermotherapy coupled with meristem-tip culture with the ultimate aim to release healthy corms for commercial propagation.

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حذف ویروس موزاییک زرد لوبیا (BYMV) از پداژه‌های گلایل با استفاده از گرمادرمانی و کشت مریستم انتهایی

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چکیده: ویروس موزاییک زرد لوبیا (*Bean yellow mosaic virus*, BYMV)، از جنس *Potyvirus* و خانواده *Potyviridae*، باعث بیماری گونه‌های مختلف گلایل (*Gladiolus* spp.) در دنیا می‌گردد. در این تحقیق امکان تولید پداژه‌های عاری از ویروس با استفاده از روش‌های گرمادرمانی، کشت مریستم انتهایی و کشت مریستم انتهایی به‌صورت توأم با گرمادرمانی مورد بررسی قرار گرفت. راندمان این روش‌ها در حذف BYMV از گیاهان حاصل از گرمادرمانی، کشت مریستم و کشت مریستم توأم با گرمادرمانی با استفاده از آزمون‌های DAS-ELISA، IC-RT-PCR به‌ترتیب ۱۵/۳۸٪، ۷۸/۰۴٪ و ۸۶/۶۶٪ برآورد گردید. گیاهچه‌های عاری از ویروس پس از ریشه‌دار شدن به محیط تشکیل پداژه انتقال داده شدند. براساس نتایج مشخص گردید که گرمادرمانی اثر فزاینده‌ای در میزان بقاء ریزنمونه‌ها در طول مراحل کشت مریستم (به‌جز مرحله باززایی) و سازگاری گیاهچه‌های حاصل دارد. تحلیل آماری داده‌ها نشان داد که گرمادرمانی تأثیر معنی‌داری در ویروس‌زدایی BYMV از پداژه‌های آلوده به این ویروس دارد. بنابراین کشت مریستم به‌صورت توأم با گرمادرمانی به‌عنوان روشی مؤثر و کارآمد در تولید پداژه‌های گلایل عاری از این ویروس می‌باشد.

واژگان کلیدی: BYMV، گلایل، کشت مریستم، گرمادرمانی، غده عاری از ویروس