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In Vitro Thermotherapy and Thermo-Chemotherapy Approaches to Eliminate Some Viruses in *Pyrus communis* L. cv. 'Natanz'

S. Karimpour[1], G. Davarynejad[1*], M. Zaki Aghl[2], and M. R. Safarnejad[3]

ABSTRACT

Production of virus-free stocks is crucial for efficient management of plant viruses in cultivation of pome fruits. Regarding the importance of producing the pre-basic stocks of valuable fruit trees, pear cultivar 'Natanz', an important local pear cultivar in Iran, was selected for virus eradication. In the present study, tissue culture combined with *in vitro* thermotherapy and thermo-chemotherapy techniques were used for elimination of *Apple Stem Pitting Virus* (ASPV) and *Apple Mosaic Virus* (ApMV). In thermotherapy approach, *in vitro* shoots were initially incubated for 55, 60, 65, and 70 days in alternating temperatures (32/38°C), then, meristems were cultivated on meristem medium. In thermo-chemotherapy approach, *in vitro* shoots were incubated for 50 days at 32/38°C, and then meristems were cultivated on a medium containing ribavirin. Virus detection by RT-PCR using specific primers was carried out after rooting and adaptation of the regenerated shoots. The percentage of survived shoots and meristem establishment were depended on thermo-duration. After 55 days, 83.33% of shoots survived, while it decreased to 33.33% after 70 days. Both ASPV and ApMV were eliminated after 60 days of thermotherapy. Ribavirin at 10 and 20 mg L^{-1} reduced the percentage of meristem establishment to 50 and 37%, respectively, compared to the control (88.88%). Thermo-chemothery was also effective for ASPV and ApMV eradication from pear shoots.

Keywords: Meristem culture, Pear, Ribavirin, RT-PCR, Virus elimination.

INTRODUCTION

Plant viruses are responsible for around 10% crop losses in agricultural crops. The best practice for combating against plant viruses in pome fruit is production of virus-free plants to diminish their harmful effect. There are several reports confirming presence of *Apple Stem Pitting Virus* (ASPV), *Apple Mosaic Virus* (ApMV), *Apple Chlorotic Leaf Spot Virus* (ACLSV), and *Apple Stem Grooving Virus* (ASGV) worldwide (Abtahi *et al*., 2017), which cause severe reduction in yield and fruit quality of pear (Plese *et al*., 1975; Yanase, 1983; Cembali *et al*., 2003; Shim *et al*., 2004). They induce different symptoms of chlorotic rings, line patterns, malformation in leaf (ACLSV), black necrotic leaf spot (ASGV; Shim *et al*., 2004) and xylem pits in the stem (ASPV; Stouffer, 1989). It is extremely important to produce virus-free mother plants because of vegetative propagation of fruit trees. Virus eradication is possible by *in vitro* isolation of the meristem in a very small size

[1]Department of Horticulture, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Islamic Republic of Iran.
*Corresponding author; e-mail: davarynej@um.ac.ir
[2]Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Islamic Republic of Iran.
[3]Department of Plant Viruses, Iranian Research Institute of Plant Protection, Agricultural Research Education and Extension Organization (AREEO), Tehran, Islamic Republic of Iran.

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(Bhardwaj *et al*., 1998; Wang *et al*., 2016). Since there is negative relationship between size and meristem establishment rate, the treatment of infected plant material before meristem culture can enhance the efficiency of virus elimination. Thermotherapy and chemotherapy or their combination followed by meristem culture has been used effectively to eliminate viruses in apples and pears. Thermotherapy using high temperature generates unfavorable conditions for virus multiplication in the cells (Panattoni *et al*., 2013) and reduces the virus titer in the shoot-tips (Liu et al., 2015). Temperature in the range of 35 to 42 ˚C was reported for virus elimination in pomes *in vitro* shoots (Knapp *et al*., 1995; Zilka *et al*., 2002; Cieślińska, 2002; Wang *et al*., 2006; Paprestein *et al*., 2008; Tan *et al*., 2010; Hu *et al*., 2012 and 2015; Lizarraga *et al*., 2017). Antiviral agents such as ribavirin with effective range (10-50 mgL^{-1}) have been successfully used for *in vitro* virus eradication in *Pyrus* and *Malus* spp. (Cieślińska and Zawadzka, 1999; Cieślińska, 2002; Nacheva and Milusheva, 2008; Sedlak *et al*., 2011; Hu *et al*., 2012; Nickel and Fajardo, 2012; Paprstein *et al*., 2013; 2014; Hu *et al*., 2015), while higher concentrations induced toxicity, which depends on species. Ribavirin affects the virus multiplication by inhibition of viral nucleic acid replication or generating catastrophic errors in the virus genome (Crotty *et al*., 2001). In all chemotherapy studies, meristems were cultivated after chemotherapy, normally 4-6 weeks, but James (2010) reported production of ASGV-free apple shoots after 9-12 weeks subculture of shoots on a medium containing 10 mg L^{-1} ribavirin.

The present study mainly aimed to optimize thermotherapy and thermo-chemotherapy protocols for generation of virus-free shoots of pear cultivar 'Natanz'.

MATERIALS AND METHODS

Plant Materials

In vitro shoots (3 cm, after 10 months subculture) of *Pyrus communis* L. cv. 'Natanz' derived from naturally infected pear trees from collection orchard (25.36 E, 58.54 N; altitude 1380 m) of Agricultural and Natural Resources Research and Education Centre of Shahrood, Semnan Province, Iran, were used for virus elimination treatments.

Virus Detection by RT-PCR

Presence of ACLSV, ASPV, ASGV and ApMV viruses in pear shoots (3 cm), were initially assessed using Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from plant materials by Qiagen RNA isolation kit (RNeasy Mini Kit). ACLSV, ASPV, ASGV, ApMV specific primer pairs and *nad 5* (NADH dehydrogenase subunit 5 as PCR internal control) were used for RT-PCR detection (Table 1). The uniplex two-step RT-PCR was optimized for virus detection. AccuPower CycleScript RT PreMix (Bioneer) and AccuPower PCR Premix (Bioneer) were used for RT and PCR,

Table 1. List of primers used in uniplex RT-PCR for four viruses.

Primer		Primer sequence in 5'–3' orientation	Product size (bp)	Reference
ACLSV	F	TTCATGGAAAGACAGGGGCAA	677	Menzel *et al*. (2002)
	R	AAGTCTACAGGCTATTTATTATAAGTCTAA		
ASPV	F	ATGTCTGGAACCTCATGCTGCAA	370	Menzel *et al*. (2002)
	R	TTGGGATCAACTTTACTAAAAAGCATAA		
ASGV	F	GAAGACGTGCTTCAACTAGC	579	Cho *et al*. (2015)
	R	TTTTAGACCAGTGGCAAAGT		
ApMV	F	AGGGTCCTGAGCAGTCGAGA	264	Cho *et al*. (2015)
	R	GTTTGGAGGGGCTTCCCACT		
Nad 5	F	GATGCTTCTTGGGGCTTCTTGTT	181	Menzel *et al.* (2002)
	R	CTCCAGTCACCAACATTGGCATAA		

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respectively. Cycling parameters were as follows: initial denaturation at 95°C for 4 minutes, followed by 40 cycles of 94°C for 30 seconds, 52°C for 1 minute, 72°C for 1.20 minutes, and a final extension step at 72°C for 10 minutes. PCR products were separated by electrophoresis in 1.5% agarose gels in TAE buffer, stained with GelRed™, and visualized under UV light.

Virus Eradication Methods

Naturally infected shoots (3 cm) in MS medium (Murashige and Skoog, 1962) supplemented with 1 mg L^{-1} BAP were treated using thermotherapy and thermo-chemotherapy methods. Then, meristems (0.5 mm) were extracted and incubated on modified MS medium enriched with 0.5 mg L^{-1} BAP, 0.05 mg L^{-1} IBA, 0.5 mg L^{-1} GA_3, 20 g L^{-1} sucrose, and 8 g L^{-1} agar.

A. Thermotherapy: After one day at 28°C and two days at 30°C, shoots were transferred to alternating temperatures of 32/38°C (4/4 hours) with 16/8 hours light/dark photoperiod. Meristem culture was performed after 55, 60, 65, and 70 days.

B. Thermo-Chemotherapy: After 50 days of alternating high temperatures of 32/38°C (4/4 hours) with 16/8 hours light/dark photoperiod (A. Thermotherapy), the meristems were extracted and incubated in meristem medium containing 0, 10, and 20 mg L^{-1} of ribavirin for 60 days, then transferred to ribavirin-free medium.

All of post-therapy plantlets as well as non-treated plantlets were induced to root and were gradually fed with water, ¼ and ½ MS solution three times a week up to five months.

Statistical Analysis

Survived shoots, established and browned meristems for thermotherapy, and established and browned meristems and callus production for thermo-chemotherapy were recorded and analyzed as a separate Completely Randomized Design (CRD) with 3 replications (4 plants for each replication: Totally 12 shoots) using SAS software (SAS Institute Inc., 1989; v. 9.4). LSD test at 5 % of probability level was used for mean comparison. The percentage of survived shoot, established, browned meristem and callus production were calculated as follow:

% Of survived shoot: % (No. survived shoots/No. incubated shoots)

% Of established meristem: % (No. established meristem/No. Survival shoots)

% Of browned meristem: % (No. Survival shoots-No. established meristems)

% Of callus production: % (No. generated callus/No. Survival shoots)

RESULTS

Virus Detection of *in Vitro* Untreated Pear Shoots

Virus detection results of untreated shoots indicated ‘Natanz’ pear cultivar was infected with ASPV and ApMV and infection to ACLSV and ASGV was not detected.

Effect of Thermotherapy on Survived Shoots, Established and Browned Meristems of *in Vitro* Pear Shoots

The effects of thermotherapy on the percentage of survived shoots, established and browned meristems are shown in Table 2. Increasing duration of the thermotherapy induced more shoot tip necrosis and, subsequently, led to decrease in the survived shoots from 91.67% at day 55 to 33.33% at day 70. Meanwhile, the percentage of meristem establishment was the highest, when they were extracted after 55 days (77.78%) and was the lowest after 70 days (44.44%) of thermotherapy. In addition, the percentage of meristem browning was affected by the duration of thermotherapy in contrary with meristem establishment percentage.

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Table 2. Survived shoots and established and browned meristems of *Pyrus communis* L. cv. 'Natanz' after 55 - 70 days of thermotherapy.

Period (Day)	Survived shoots (%)	Established meristem (%)	Browned meristem (%)
55	83.33 a (9/12) [a]	77.78 a (7/9)	22.22 a (2/9)
60	75.00 b (8/12)	66.67 a (5/8)	33.33 b (3/8)
65	66.67 b (8/12)	66.67 a (5/8)	33.33 b (3/8)
70	33.33 c (5/12)	44.44 b (2/5)	55.55 b (3/5)

[a] The number of samples in the identified trait/the total of samples. (a-c) Means followed by the same letter, within each group of means in each column are not significantly different at the 0.05 level based on LSD test.

Effect of Thermo-Chemotherapy on Established and Browned Meristems and Callus Production of *In Vitro* Pear Shoots

Regardless of the high temperature effects on the establishment of meristem, the concentration of ribavirin in the meristem medium had effects on the percentage of meristem establishment and meristem browning and the percentage of callus induction (Table 3). The highest establishment was obtained in ribavirin-free medium with 88.88% of incubated meristems. Adding ribavirin at 10 and 20 mg L^{-1}, 44.44 and 33.33% of meristems were established, respectively. Higher concentration of ribavirin was extremely toxic for the samples. When 10 and 20 mg L^{-1} were used in meristem medium, 33.33 and 55.55% of meristems were unable to grow and turned to brown, respectively. Also, 22.22% of the extracted meristems did not produce shoots and just produced callus when 10 mg L^{-1} of ribavirin was added to meristem medium. Twenty mg L^{-1} ribavirin induced the lower callus production (11.11%).

Efficiency of Virus Elimination in Pear Plants by Thermotherapy and Thermo-Chemotherapy

Shoots were derived from treated meristems were sub-cultivated each 3 weeks. Rooted plants after 5 months of adaptation were checked for virus infection using RT-PCR. The results indicated (Table 4) that at least 60 days of thermotherapy was needed for elimination of ASPV and ApMV from *Pyrus communis* L. cv. 'Natanz' shoots. Also, Chemo-thermotherapy of meristems using 10 or 20 mg L^{-1} of ribavirin were effective for ASPV and ApMV eradication from the 'Natanz' shoots. According to the results of these two procedures of virus elimination, chemo-thermotherapy in presence of ribavirin in the meristem medium reduced the time for production of virus-free 'Natanz' shoots (Table 4).

The effect of ApMV and ASPV elimination from pear cv. 'Natanz' was significant on plantlet growth. The virus-free post-therapy plants showed a 1.9-fold higher growth (19.75 cm) compared to untreated

Table 3. Established and browned meristems and callus production of *Pyrus communis* L. cv. 'Natanz' after thermotherapy on medium with different concentrations of ribavirin.[a]

Ribavirin (mg L^{-1})	Established meristem (%)	Browned meristem (%)	Callus production (%)
0	88.88 a (10/12) [a]	11.11 c (2/12)	00.00 c (0/12)
10	44.44 b (5/12)	33.33 b (4/12)	22.22 a (3/12)
20	33.33 c (3/12)	55.55 a (7/12)	11.11 b (2/12)

[a] The number of samples in the identified trait/ the total no. of samples. (a-c) Means followed by the same letter, within each group of means in each column are not significantly different at the 0.05 level based on LSD test.

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Table 4. Efficiency of *in vitro* thermotherapy and thermo-chemotherapy on virus elimination from *Pyrus communis* cv. 'Natanz' regenerated plants.

Thermotherapy (Day)	Ribavirin (mg L^{-1})	No of survived meristem/Total treated shoots	Number of virus-free/Tested Plants		Positive/Tested plants
			ASPV	ApMV	*nad 5*[a]
55	-	9/12	5/9	4/9	9/9
60	-	8/12	8/8	8/8	8/8
65	-	8/12	8/8	8/8	8/8
70	-	5/12	5/5	5/5	5/5
50	0	10/12	4/10	0/10	10/10
50	10	5/12	5/5	5/5	5/5
50	20	3/12	3/3	3/3	3/3

[a] PCR internal control.

plants infected with the ApMV and ASPV (10.5 cm) after 5 months of adaptation in the greenhouse (Figure 1).

DISCUSSION

Present study shows successful application of thermotherapy and thermo-chemotherapy approaches for producing virus-free pear plants. *In vitro* pear cv. 'Natanz' shoots had good survival in 32/38 °C thermos-cycles after 55 days, and after that, the survival rate were reduced. Also, the meristem establishment and the browning rates were affected. Thermotherapy with alternating temperatures helps shoots to survive more (Knapp *et al*., 1995; Zilka *et al*., 2002; Paprstein *et al*., 2008; Tan *et al*., 2010, Hu *et al*., 2012; Lizarraga *et al*., 2017). The effects of high temperature on meristem establishment were reported (Hu *et al*., 2015) although it also depends on cultivar (Tan *et al*., 2010). The concentration and duration of treatment by antiviral agents are two important factors in chemotherapy. According to previous studies, the effective concentration of ribavirin for virus elimination in apple and pear is 20-25 mg L^{-1} (Cieślinska and Zawadzka, 1999; Cieślińska, 2002; O'Herlihy *et al*., 2003; Nacheva and Milusheva, 2008; Sedlak *et al*., 2011; Hu *et al*., 2012; Paprstein *et al*., 2013; 2014; Hu *et al*., 2015). Higher concentrations of ribavirin inhibit growth of shoots and led to plant death (Cieślińska, 2002; Nacheva and Milusheva, 2008). We used ribavirin in the meristem medium instead of the shoot medium. The results indicated that ribavirin had an inhibitor effect on meristem establishment due to its toxicity.

Several factors including temperature, duration of thermotherapy regime, and size of explants may affect efficiency of virus eradication. The results indicated that

Figure 1. Virus-infected (untreated) and virus-free regenerated plantlets of *Pyrus communis* cv. 'Natanz' after five months of adaptation in greenhouse.

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increasing duration of thermotherapy regime increased chance of obtaining virus-free plants. Previously, temperatures between 37-39°C and cycles of 34/42°C were successfully used for generation of ASPV-free shoot-tips of *Pyrus* and *Malus* pp. (Paprstein *et al*., 2008; Tan *et al*., 2010; Guo *et al*., 2014; Hu *et al*., 2015). Also, ASPV-free 'Gala' (*Malus* × *domestica*) plants were generated by culturing of extra small explants (0.3 mm) (Wang *et al.*, 2016). Chemotherapy alone or combined with thermotherapy also have been reported for ASPV elimination in pomes. Effective concentration of ribavirin is depended on species. Application of 20 mg L^{-1} ribavirin for 28 days before meristem culture had variable efficiencies in generation of ASPV-free of 'Alexander Lucas' (79%), 'Bohemica' (80%), 'Elektra' (90%) and 'Rote Williams' (74%) pear cultivars (Sedlak *et al.*, 2011). Moreover, ASPV was eliminated from the 'Castel Gala' (100% at 5 mg L^{-1}) and 'Fuji' (25% at 5 mg L^{-1} and 33% at 7.5 and 10 mg L^{-1}) apple cultivars in different concentration of ribavirin after 12 weeks and when ribavirin at 20 mg L^{-1} for 28 days were used, 65% of 'Astra' and 'Erika' apple shoots were ASPV-free (Paprstein, 2014). Ribavirin at 15 mg L^{-1} also was completely effective for ASPV eradication from the *Malus* cv. 'Xinhongjiangjun' (Hu *et al*., 2015). Pretreatment of the *Malus* cv. 'Fragrance' shoots with 20 mg L^{-1} followed by application of 100 mg L^{-1} ribavirin improved efficiency of ASPV-free plants generation to 100% compared to 35% of 20 mg L^{-1} ribavirin (Paprstein, 2013). Chemo-thermotherapy of pear and apple plants using 15 or 25 mg L^{-1} ribavirin in shoot medium at 34-36°C resulted in 100% ASPV-free plants (Hu *et al*., 2015, 2018). In this study, ApMV and ASPV were eradicated by thermotherapy after 60 days at 32/38°C alternating temperatures or thermo-chemotherapy with 10 or 20 mg L^{-1} ribavirin in meristem medium. Limited studies are found for ApMV elimination in pomes. Bhardwaj *et al*. (1998) obtained ApMV-free plants of the apple cv. 'Tydeman's Early Worcester' with meristem culture in size 0.1 and 0.2 mm and also scion immersion in hot water (47°C for 30 minutes or 50°C for 15 miutes) or potted plant exposure to hot air (37°C for 4 weeks or 40°C for 2 weeks). Chemotherapy of the *Malus* cv. Remo *in vitro* shoots with 20 mg L^{-1} ribavirin produced ApMV-free plants (Nacheva and Milusheva, 2008). Similarly, James (2010) reported apple shoots treated with ribavirin in subculture medium (10 mg L^{-1}) for 3 months were virus free. Our results suggest that using ribavirin in meristem medium can increase the effectiveness of virus elimination in thermo-treated shoots and can be used for high temperature sensitive plants. These *in vitro* virus elimination procedures can work for other pome fruit species and their important viruses.

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دستآوردهای گرمادرمانی و گرما-شیمی‌درمانی درون شیشه‌ای برای حذف برخی از ویروس‌ها در گلابی رقم 'نطنز' (*Pyrus communis* L. cv. 'Natanz')

س. کریم پور، غ. داوری‌نژاد، م. زکی‌عقل، و م. ر. صفرنژاد

چکیده

تولید گیاهان عاری از ویروس برای مدیریت موثر ویروس‌های گیاهی باغات درختان میوه دانه‌دار حیاتی است. بدلیل اهمیت تولید هسته‌های پیش تکثیری نهال درختان میوه با ارزش، گلابی رقم 'نطنز' که یکی از ارقام مهم بومی ایران است برای ویروس‌زدایی گزینش گردید. در این مطالعه، تکنیک‌های کشت بافت به همراه گرمادرمانی و گرما-شیمی‌درمانی درون شیشه‌ای برای حذف ویروس ساقه گودکی سیب (ASPV) و ویروس موزائیک سیب (ApMV) مورد ارزیابی قرار گرفت. در روش گرمادرمانی، مریستم‌ها بعد از ۵۵، ۶۰، ۶۵ و ۷۰ روز قرار گرفتن شاخه‌ها در تناوب گرمایی (۳۸/۳۲ درجه سانتیگراد) کشت شدند و در روش گرما-شیمی درمانی، مریستم‌ها بعد از ۵۰ روز قرار گرفتن شاخه‌ها در تناوب گرمایی (۳۸/۳۲ درجه سانتیگراد) در محیط کشت حاوی غلظت‌های مختلف ریباویرین کشت شدند. شاخه‌های حاصل از مریستم‌های تیمار شده ریشه‌دار شده و بعد از سازگاری ردیابی ویروس با آغازگرهای اختصاصی انجام شد. شاخه‌های زنده مانده بعد از تیمار گرمادرمانی و همچنین استقرار مریستم بستگی به طول دوره گرمادرمانی داشت. بعد از ۵۵ روز ۸۳/۳۳ درصد از شاخه ها زنده ماندند درحالیکه این میزان در روز ۷۰ به ۳۳/۳۳ درصد کاهش یافت. هر دو ویروس ASPV و

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ApMV بعد از ۶۰ روز از گرمادرمانی حذف شده بودند. ریباویرین در ۱۰ و ۲۰ میلیگرم بر لیتر درصد استقرار مریستم را به ترتیب به ۵۰ و ۳۷ درصد نسبت به شاهد (۸۸/۸۸ درصد) کاهش داد. تیمارگرما-شیمی‌درمانی با استفاده از ریباویرین نیز موجب حذف ASPV و ApMV از شاخه‌های گلابی شده بود.

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