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Full Length Article

In Vitro Elimination of PPV from Infected Apricot Shoot Tips via Chemotherapy and Cryotherapy

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Abstract

Plum pox potyvirus (PPV) is the most serious viral disease of stone fruits of *Prunus* sp. and elimination of the virus could be achieved by using *in vitro*-applied techniques. Thus, the potential of usage of thermotherapy, chemotherapy and cryotherapy techniques was assessed by *in vitro* treatment of infected apricot shoot tips. Survival was declined with prolonged application of thermotherapy whereas promising results were scored after chemotherapy and cryotherapy. The highest survival (100%) of the shoot tips was achieved with quercetin containing medium while relatively lower (80%) survival was scored with ribavirin. In addition, inclusion of the 8-azaguanine had found to be phytotoxic as no survival was achieved after treatment. In the case of cryotherapy, two-step freezing technique was applied and 10% of survival was recorded in cryopreserved apricot shoot tips. RT-PCR analysis showed that PPV was eliminated in the survived ones both after chemotherapy and cryotherapy, which revealed that those techniques could be used for *in vitro* elimination of PPV from infected apricot as well as other stone fruits.

Keywords: *Prunus armeniaca* L.; Ribavirin; Quercetin; 8-azaguanin; Two-step freezing

Introduction

Plum pox potyvirus (PPV), belongs to *Potyvirus* genus in Potyviridae family, has long, flexuous rod particles and positive sense single stranded (ss) RNA with a molecular weight of 3.5 x 10^6 Da (Shukla *et al*., 1994). The virus infects nearly all *Prunus* species including the ornamental ones [i.e., *P. cerasifera* (Elibuyuk, 2006), *P. spinoza* (Polák, 2006)]. However, it gives most destructive harm to apricots, plums, and peaches (Ozden *et al*., 2011). The symptoms of the disease (i.e., chlorotic spots, bands, rings, deformations and a bitter taste) are observed in leaves, fruits, pits, and flowers. Although it is not dangerous to consumer's health like other plant virus diseases, the disease causes both direct (loss of the fruits and marketing problems) and indirect losses (controlling costs including quarantine efforts, costs of research, nursery control and diagnosis).

PPV distributes with long distance movement by infected propagation material and non-persistent transmission by several aphids (Németh, 1994). Thus, the disease can be controlled by spraying aphid vectors, using clean propagation material, planting naturally resistant or tolerant varieties (if available) (Németh, 1986). As *in vivo* treatment of the virus could not be achieved, infected trees are eliminated and this solution not only causes the loss of the productive tree but also increases the economic loss. Thus, *in vitro*-applied biotechnological techniques that have potential to cure viral diseases as well as PPV gain importance both to conserve the productive healthy tree and to decrease economic loss.

Today, several biotechnological methods including meristem culture, micrografting (such as transmicrografting), thermotherapy (application of increased temperature to infected plants for the inhibition of viral propagation and diffusion), chemotherapy (treatment of infected plants by broad-spectrum synthetic antiviral nucleoside for inhibiton of viral replication) and recently developed electrotherapy (treatment of infected plants with electric current), cryotherapy (infected tissues are maintained in ultra-low temperatures) or combination of these methods was applied for the elimination of different virus as well as PPV from various *Prunus* species. In the case of apricot, thermotherapy was applied by several researchers (i.e., Koubouris *et al*., 2007; Křižan and Ondrušiková, 2009), however the potential of not only usage of different chemotherapeutic agents for chemotherapy but also application of various cryogenic methods for cryotherapy still needs to be assessed.

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Thus, the aim of the study concerned; (i) the incubation of microshoots at 37°C for several weeks for thermotherapeutic response, (ii) the usage of ribavirin, quercetin, and 8-azaguanine for chemotherapeutic response, (iii) the application of two-step freezing technique for cryotherapeutic-elimination of PPV from apricot in *in vitro* conditions.

Materials and Methods

In Vitro Proliferation

Shoot tips were excised from infected mature apricot (*Prunus armeniaca* L.) tree in the campus of Faculty of Agriculture of Ankara University and sterilized by dipping 70% ethanol for 30 sec followed by 1.5% NaOCl for 15 min. Shoot tips then washed thrice in dH_2O and transferred to MS (Murashige and Skoog, 1962) medium containing 2 mg/L BA and 0.5 mg/L GA_3 for proliferation (proliferation medium). Cultures were incubated at 25 ± 2°C under 16 h photoperiod of 36 µmol m^{-2} s^{-1} irradiance provided by cool daylight fluorescent lamps (proliferation conditions). Upon establishment of aseptic cultures, regenerated shoots were multiplied in order to obtain sufficient number of shoots for setting up the experiment with thermotherapy, chemotherapy and cryotherapy. Following at least 3 subculture (each subculture was 30 days), shoot tips were excised from *in vitro* proliferated microshoots and used for chemotherapy and cryotherapy.

Thermotherapy

In vitro-propagated microshoots were incubated at 37°C for 3, 4, 5, 6 and 7 weeks and then shoot tips were excised from each treatment and transferred to proliferation medium and conditions. Survival was scored not only immediately after incubation at 37°C but also after first and second subculture of shoot tips excised from treated microshoots.

Chemotherapy

Shoot tips excised from *in vitro*-propagated microshoots were transferred to proliferation MS medium containing 1 mg/L different chemotherapeutic agents such as 8-azaguanine, ribavirin and quercetin for chemotherapy. Due to their thermolability, antiviral agents were added through filter sterilization by Millipore filter of 0.22 µm upon media autoclaving. Shoot tips were cultured for 30 days in proliferation conditions. Survival of the explants was scored and then leaves were excised and used for RT-PCR reactions.

Cryotherapy

Cryopreservation protocol was applied according to Brison *et al*. (1997). In brief, explants were pre-cultured in 1 mg/L BA, 5% dimethylsulfoxide (DMSO) and 2% mg/L proline containing MS medium at 4°C for 1d and then treated with 40 min in PVS2 solution. Slow cooling of shoot tips was achieved by transfer of the cryovials to Mr Frosty (Nalgene®) device, which was kept in a -80°C freezer until the temperature was reduced to -40°C. Afterwards, cryovials were rapidly plunged to liquid nitrogen (LN). After storage for at least 1 h in LN, explants were thawed rapidly in water bath (1 min at 40°C) and transferred to washing solution (1.2 M sucrose for 30 min) and cultured in proliferation medium for recovery.

RNA Isolation and RT-PCR

RNA was isolated from leaves excised from PPV-infected tree in which *in vitro* cultures were initiated (positive control) and chemo- (both quercetin and ribavirin) and cryo-treated samples according to Spiegel *et al.* (1996). One-step RT-PCR was carried out according to Wetzel *et al*. (1991) with using P1 (5′ ACCGAGACCACTACACTCCC 3′) and PM (species-specific, 5′ CTTCAACAA CGCCTGTGCGT 3′) for chemo-treated; P1 and P2 (5′ CAGACTACAGCCTCGCCAGA 3′) primers amplifying C-end of the coat protein (CP) gene for cryo-treated samples. PM primer was used with P1 primer to understand the strain of the virus. PCR protocol performed in a volume of 25 µL containing 0.5 mg template RNA, 1 µM of each of the primers, 20 u/µL reverse transcriptase, 12 u/µL RNase inhibitor, 1×PCR reaction buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl], 1.5 mM $MgCl_2$, 0.4 mM dNTP mix and 1 unit Taq DNA polymerase. Reaction mixture was incubated in 37°C for 60 min for cDNA synthesis and then the amplification reaction was performed as follows, 94°C for 4 min, 54°C for 2 min, 72°C for 3 min, 94°C for 2 min, 54°C for 2 min, 72°C for 3 min followed by 40 cycles of 94°C for 30 s, 54°C for 1 min and 72°C for 1 min with a final extension at 72°C for 10 min. PCR products were analyzed by 1.5% agarose gel electrophoresis in TAE buffer.

Data Collection and Statistical Analysis

Ten Petri dishes, each one with at least four shoot tips, were used for each proliferation treatment and each experiment was repeated at least twice. For determination of survival, the percentage of green microshoots was determined immediately after incubation at 37°C for thermotherapy together with the percentage of green and proliferated shoot tips were scored for all tested therapies. Proliferation was defined as the shoot tips that produced normal shoots (minimum 5 mm) after 30 days of *in vitro* culture on proliferation medium. Statistical analysis of the non-parametric data (frequencies) was carried out by the test for homogeneity of proportions and significant treatment differences selected by a non-parametric statistical test, the Post Hoc Multiple Comparisons Test.

Results

Thermotherapy

The maximum survival of microshoots (100%) was obtained with incubation at 37°C for 3 weeks (Table 1). However, a decline in survival was obtained with prolongation of incubation time and totally inhibited when incubation was carried out for over 4 weeks. Moreover, survival was also reduced when shoot tips were excised from treated microshoots and cultured at 25°C for 4 weeks (1st subculture). Moreover, total mortality was occurred following 2nd subculture of shoot tips.

Chemotherapy

Usage of antiviral agents has been reported to be effective in the elimination of various viruses (James *et al.*, 1997; Nascimento *et al*., 2003; James, 2010). Thus, in the present study 3 chemotherapeutic compounds were used for elimination of PPV from apricot shoot tips. The highest survival (100%) was scored with incorporation of 1 mg/L quercetin to proliferation medium, while 80% of shoot tips were survived in ribavirin containing medium (Fig. 1 and Fig. 2). RT-PCR analysis also showed that PPV was eliminated in the apricot tissues after treatment with quercetin and ribavirin (Fig. 3A). On the contrary, inclusion of 8-azaguanine to the proliferation medium was found to be phytotoxic as no survival was obtained.

Cryotherapy

In the case of cryotherapy, 10% of survival was recorded in cryopreserved apricot shoot tips and RT-PCR analysis showed that PPV was eliminated in the survived ones after cryotherapy (Fig. 3B).

Discussion

Different *in vitro* therapies including thermotherapy, chemotherapy and cryotherapy were applied in this study for the elimination of PPV from apricot. Although *in vitro* thermotherapy in combination with shoot tip excision proved to eliminate PPV effectively in apricot cv 'Bebecou' (Koubouris *et al*., 2007), the survival rate of the explants subjected to *in vitro* thermotherapy in that study barely reached to 28%. This result is inconsistent with our findings as a decline in survival was observed after prolonged incubation of microshoots to 37°C, implying that woody plants were sensitive to prolonged exposure to extreme high temperatures (Spiegel *et al*., 1995).

In the case of the chemotherapy, quercetin, ribavirin and 8-azaguanin were applied to PPV-infected apricot tissues. Quercetin (3,3',4',5,7-pentahydroxyflavone) is a natural product flavonoid. Flavonoids have been shown to be effective antivirals against several plant viruses

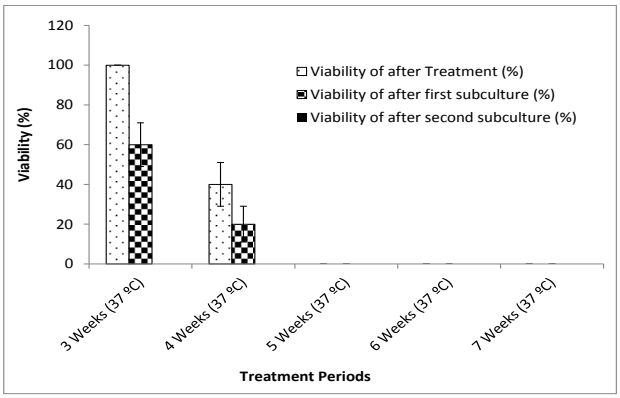

Fig. 1: Influences of different chemotherapeutic agents on *in vitro* survival of apricot shoot tips

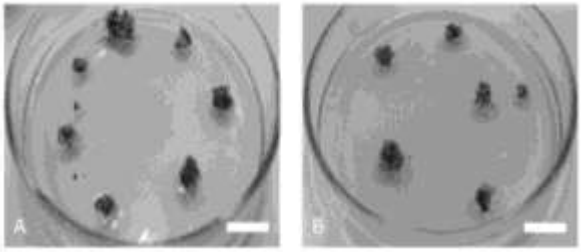

Fig. 2: Influences of quercetin (A) and ribavirin (B) on *in vitro* survival and bud formation of apricot shoot tips. Bars were 1.75 cm and 1.70 cm in A and B, respectively

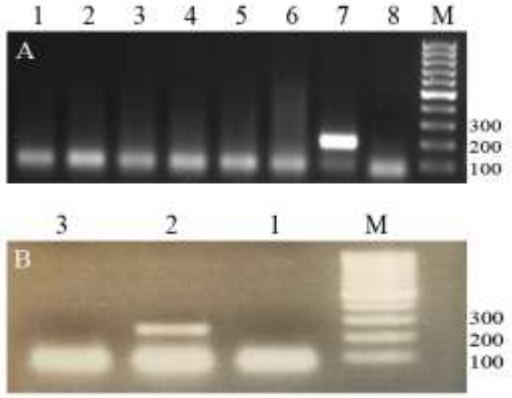

Fig. 3: RT-PCR of samples after chemotherapy (A) and cryotherapy (B). A. Position 1-3 ribarivin-treated samples; 4-6 quercetin-treated samples; 7 positive control (PPV-infected tree used for initiation of *in vitro* culture; 8 negative control; M Marker 100 bp DNA Ladder (Fermentas). B. Position 1 negative control; 2 positive control; 3 cryotherapy applied sample; M Marker 100 bp DNA Ladder (Fermentas). Arrows indicated the presence of 198 bp band for chemo-treated samples with P1 and PM primers, while 243 bp for cryo-treated samples with P1and P2 primers in A and B, respectively

Table 1: The survival of microshoots/shoot tips after incubation of PPV-infected apricot microshoots at 37°C for several weeks for *in vitro* thermotherapy

Incubation time (weeks at 37°C)	Survival after incubation[a] (%)	Survival after 1st subculture[b]	Survival after 2nd subculture[b]
3	100a[c]	60a[c]	0a[c]
4	40b	20b	0a
5	0c	0c	0a
6	0c	0c	0a
7	0c	0c	0a

[a]Microshoots
[b]Shoot tips
[c]Percentages followed by the same letter are not significantly different at $P \leq 0.05$ by the Post Hoc Multiple Comparisons Test (i.e., percentage significativity is per vertical lines)

(French *et al*., 1991; French and Towers, 1992; Malhotra *et al*., 1996). Flavonoids enhance cAMP levels by inhibiting cAMP phosphodiesterase (Mucsi and Pragai, 1985) and this may affect virus replication. Although, quercetin was found to be effective in elimination of tomato ringspot virus (TomRSV) (Malhotra *et al*., 1996) and apple stem grooving virus (James, 2010), its influence was assessed for the first time in our study for PPV elimination from apricot. Our results showed that 1 mg/L quercetin could also be used for chemotherapeutic treatment of PPV as the shoot tips treated with that agent survived (Fig. 1) and caused proliferation of buds (Fig. 2A). Moreover, usage of this agent also produced PPV-free apricot plantlets (Fig. 3A).

Ribavirin, which is proposed to inhibit 5' capping of viral RNA (Lerch, 1987) and/or inhibit systemic virus movement (Cassells and Long, 1982), was applied to different plant species [i.e., potato (Nascimento *et al*., 2003); Begonia (Verma *et al*., 2005); apple (Hansen and Lane, 1985) for elimination of various viruses [PVY, PNRSV and Apple chlorotic leaf spot virus (ACLSV), respectively]. It was also one of the most used chemotherapeutic agents for PPV elimination in apricot. The positive influence of ribavirin on elimination of PPV was also detected in our study as relatively higher survival rate (Fig. 1 and 2B) together with achievement of PPV-free material was obtained (Fig. 3B).

The optimal concentration of ribavirin was found to be 10 mg/L and time period of the treatment was reported to be between 12 and 20 weeks in PPV-infected apricot cv. Hanita (Hauptmanová and Polák, 2011) and 4 or 6 weeks of application was found to be ineffective for PPV elimination (Gabova, 1995). However, our results showed that a relatively lower concentration (1 mg/L) and shorter period (4 weeks) of treatment could also be adequate for *in vitro* elimination of PPV from apricot. This result could be due to the fact that the efficiency of ribavirin in elimination of plant viruses depends on its concentration and the genotype of the both host plant and virus (Knapp *et al*., 1995).

Various structural analogues of the natural purines and pyrimidines are known to be capable of replacing the corresponding bases in nucleic acids (Smith and Matthews, 1957) and thus could be used for virus elimination. Among them, 8-azaguanin that replaces guanine in several ribonucleic acids and interferes in some way with viral RNA synthesis and function (Matthews, 1981) was assessed in this study in order to reveal its influence on PPV. Although it was previously reported that it delayed or prevented systemic development of BMV in wheat without toxicity, it was found to be phytotoxic in apricot as no survival was obtained after treatment (Fig. 1). This result was inconsistent with Balamuralikrishan and co-workers (2002) as this chemotherapeutant exhibited phytotoxicity and reduced regeneration of plantlets in sugarcane. Due to this narrow margin in dosage between plant toxicity and antiviral effectiveness (Matthews, 1981), the usage of this chemical was limited in plants.

In cryotherapy, lethal injury of cells that are located more distant from the apical dome and are more likely to be infected by viruses during cryo-treatment is utilized to kill infected tissues. This therapy was successfully utilized initially to eliminate PPV from an interspecific *Prunus* rootstock (Brison *et al*., 1997) and, now, it was also found to be effective in elimination of this virus from apricot. Usage of cryotherapy is promising as successful results were obtained in different plant species [i.e., banana (Helliot *et al*., 2002), grapevine (Wang *et al*., 2003), potato (Wang *et al*., 2006), sweet potato (Wang and Valkonen, 2008)] for elimination of several viruses (Wang *et al*., 2009).

Conclusion

Cryotherapy and chemotherapy can be used to eliminate PPV from infected apricot shoot tips. It is expected that the elimination technique applied and results obtained in apricot will be a model study for the elimination of various viruses from different plant species especially *Prunus* spp. Application of cryopreservation methods, which enables the storage of plant tissue at ultra-low temperatures, is very important as it will be used as a combined method for virus elimination and conservation of germplasm. It should also be noted that the relatively lower survival rate obtained by application of two-step freezing method should be increased by using other cryogenic techniques such as one-step freezing techniques.

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