APPLICATION OF THERMO- AND CHEMOTHERAPY *IN VITRO* FOR ELIMINATING SOME VIRUSES INFECTING *Prunus* sp. FRUIT TREES

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A B S T R A C T

Thermotherapy and chemotherapy *in vitro* were applied to eliminate *Apple chlorotic leaf spot virus* (ACLSV) and *Prunus necrotic ring spot virus* (PNRSV) from myrobalan (*Prunus cerasifera* var. *divaricata* Borgh.), PNRSV from 'Empress' plum (*Prunus domestica* L.) and *Prune dwarf virus* (PDV) from 'Early Rivers' sweet cherry (*Cerasus avium* (L.) Moench.) plants. ELISA assays conducted one year after therapy indicated that thermotherapy *in vitro* was highly effective for PNRSV and ACLSV elimination but it was not an efficient method for obtaining PDV-free 'Early Rivers' sweet cherry. Virazole® (Ribavirin) at concentration 25-100 mg l^{-1} was effective in eliminating ACLSV from myrobalan and PNRSV from 'Empress' plum shoots but this therapy was not successful in eliminating PNRSV from myrobalan and PDV from 'Early Rivers' sweet cherry shoots. Combination of thermotherapy with Virazole® treatment at concentration 50-100 mg l^{-1} enabled to eliminate PDV from 'Early Rivers' sweet cherry shoots and PNRSV from majority of treated shoots of myrobalan and 'Empress' plum. The higher concentrations of Virazole® (50-100 mg l^{-1}) were phytotoxic for treated shoots, especially in case of 'Empress' plum.

Key words: ACLSV, PNRSV, PDV, chemotherapy, thermotherapy

INTRODUCTION

Prunus necrotic ring spot virus (PNRSV), *Prune dwarf virus* (PDV) and *Apple chlorotic leaf spot virus* (ACLSV) are the most common viruses affecting *Prunus* species. They occur in many different strains and some of them cause serious diseases of fruit trees. The standard treatment for the elimination of fruit tree viruses is heat therapy followed by shoot tip

micropropagation (Mink et al., 1998). used to obtain virus-free plants from infected cultivars (Fridlund, 1989), a few scientists have explored the use of antiviral compounds for this purpose (Hansen, 1989).

The efficiency of virus elimination depends on the type of virus, plant host and virus combinations in mixed infection (Knapp et al. 1995). Spiegel et al. (1998) compared effectiveness of micropropagation of meristem and shoot tip cultures, thermotherapy for 28 days at 38°C/28°C, treatment with 20 mg l^{-1} of Virazole® and combination of thermo- and chemotherapy in elimination of PNRSV from plum shoots *in vitro.* The most efficient appeared to be combination of heat therapy and treatment with 20 mg l^{-1} of Virazole®.

Successful *in vitro* heat treatments have been reported for eliminating

PNRSV, PDV, ACLSV and *Plum pox virus* (PPV) from several stone fruit species (Snir and Stein, 1985; Deogratias et al., 1989; Stein et al., 1991; Gella and Errea, 1998; Manganaris et al., 2003). Howel et al. (2001) reduced levels of PNRS and PDV in shoots of *Prunus avium* grown in hydroponics using thermotherapy and chemotherapy.

This paper reports the study on elimination of ACLSV, PNRSV and PDV from myrobalan (cherry plum), 'Empress' plum and "Early Rivers' sweet cherry shoots *in vitro* using thermotherapy, chemotherapy and combination of the both methods.

Although heat therapy is generally

MATERIAL AND METHODS

Plant material and *in vitro* culture

The study as done on myrobalan (*Prunus cerasifera var. divaricata* Borgh) infected with complex of *Apple chlorotic leaf spot virus* (ACLSV) and *Prunus necrotic ring spot virus* (PNRSV), 'Empress' plum (*Prunus domestica* L.) infected with PNRSV and 'Early Rivers' sweet cherry (*Cerasus avium* (L.) Moench.) infected with *Prune dwarf virus* (PDV). The infection of the plants was confirmed by positive reaction in enzyme-linked immunosorbent assay (ELISA) using specific antisera.

The actively growing shoots were excised, rinsed in tap water for several hours, sterilized for one minute in 70% ethanol, 0.1% mercuric chloride ($HgCl_2$) for 90 s and then again rinsed three times in distilled water. The axillary buds were isolated and placed in the tubes containing 10 ml of Murashige and Skoog (MS) (1962) medium with the addition of 0.25 µM l^{-1} indole-3-butyric acid (IBA), 2.5 µM/l^{-1} 6-benzylaminopurine (BA), 30 g l^{-1} sucrose and solidified with 7 g l^{-1} of agar. For shoots multiplication the MS medium was enriched with 0.5 µM l^{-1} IBA and 5 µM l^{-1} BA. The pH of the medium was adjusted to 5.6 before being autoclaved. The cultures were maintained in a growth chamber at a day/night temperatures

24/21°C and 16 h photoperiod under a light intensity 2 Klux.

Thermotherapy *in vitro*

For heat treatment the cultured shoots were placed in a growth chamber where the temperature was gradually increased from 28°C to 36°C within a weak and kept at 36°C for following four weeks. Untreated shoots were kept at 22°C. At the end of the thermotherapy the survived shoots were removed, put on the fresh medium and maintained in a growth chamber at the day/night temperatures 24/21°C. After four weeks the individual shoots were transferred on the rooting medium with addition of 2 mg l^{-1} IBA. After rooting the shoots were potted and kept in a greenhouse.

Chemotherapy *in vitro*

The antiviral compound used in chemotherapy was Virazole® (Ribavarin) (ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA), a synthetic broad-spectrum antiviral nucleoside (1-ß-D-ribofuranesyl-1,2,4-triazole-3-carboxamide) at concentrations 10, 25, 50 and 100 mg l^{-1}. Four weeks after initiating the experiment shoot tips (1cm long) were excised and transferred to a Virazole®-free medium. Survived shoots were transferred to the jars containing the rooting medium and when roots were formed, potted and kept in greenhouse. Thermotherapy and chemotherapy treatment were also combined. The behaviour of shoots was observed during the heat treatment and chemotherapy to evaluate the survival rate.

ELISA

The virus content was assayed by DAS-ELISA (Clark et al., 1976) prior to the treatment and in surviving shoots two months and 1 year after the treatment. Treated plants were transferred to the cold room for 8 weeks to induce dormancy. Shoots of healthy and infected plants of the same species maintained *in vitro* were used as negative and positive controls.

RESULTS

Shoot survival

All shoots of 'Early Rivers' sweet cherry and the majority of myrobalan (80%) and 'Empress' plum (66.7%), which were kept for four weeks at 36°C, survived thermotherapy. 10-25 mg l^{-1} of Virazole® did not affect significantly the survival of the treated shoots. The higher concentration of Virazole® (50-100 mg l^{-1}) appeared to be phytotoxic for 'Empress' plum shoots, especially when combined with thermotherapy (Tab. 1). Shoots showed chlorosis and apex necrosis and most of them died during the therapy. In the course of the experiment only two out of 30 shoots treated at 36°C with 100 mg l^{-1} Virazole® survived. Myrobalan and 'Early Rivers' shoots showed better tolerance to the high concentration of this compound.

Virus elimination

ELISA method was applied to evaluate the effect of thermotherapy and chemotherapy *in vitro* on eliminating ACLSV, PNRSV and PDV from *Prunus* sp. fruit trees. The efficiency of the both therapy methods are shown in Table 2. Thermotherapy *in vitro* was not a sufficient method for obtaining PDV-free 'Early Rivers' sweet cherry but it was effective for PNRSV and ACLSV elimination. The effect of chemotherapy varied and depended on concentration of Virazole®, the virus and plant

Table 1. *Prunus* sp. shoot survival rate after *in vitro* treatment

Treatment	Number of survived shoots /number of treated shoots survival rate [%]		
	myrobalan	'Empress'	'Early Rivers'
Control	30/30 (100%)	30/30 (100%)	25/25 (100%)
Thermotherapy	24/30 (80%)	20/30 (66.7%)	25/25 (100%)
Virazole® [mg l^{-1}]			
10	27/30 (90%)	26/30 (86.6%)	25/25 (100%)
25	26/30 (86.6%)	24/30 (80%)	25/25 (100%)
50	26/30 (86.6%)	10/30 (33.3%)	22/25 (88%)
100	21/30 (70%)	8/30 (26.7%)	14/25 (56%)
Thermotherapy + Virazole® [mg l^{-1}]			
10	25/30 (83.3%)	22/30 (73.3%)	25/25 (100%)
25	22/30 (73.3%)	20/30 (66.7%)	25/25 (100%)
50	19/30 (63.3%)	10/30 (33.3%)	21/25 (84%)
100	18/30 (60%)	2/30 (6.7%)	15/25 (60%)

Table 2. Effect of *in vitro* treatment on the elimination of various viruses in *Prunus* sp.

Treatment	Number of ELISA negative plants/number of tested plants			
	myrobalan		'Empress' plum (PNRSV)	'Early Rivers' sweet cherry (PDV)
	PNRSV	ACLSV		
Control	0/30	0/30	0/30	0/25
Thermotherapy	14/24	16/24	15/20	0/25
Virazole® [mg l^{-1}]				
10	0/27	10/27	10/26	0/25
25	0/26	16/26	12/24	0/25
50	0/26	23/26	7/10	0/22
100	0/21	18/21	8/8	0/14
Thermotherapy and Virazole® [mg l^{-1}]				
10	16/25	14/25	22/22	0/25
25	17/22	20/22	20/20	3/25
50	19/19	19/19	10/10	8/21
100	18/18	15/18	2/2	14/15

species. Virazole® at concentration 25-100 mg l^{-1} was effective in eliminating ACLSV from myrobalan and PNRSV from 'Empress' plum shoots. However, independently of concentration used, it was impossible to eliminate PNRSV from myrobalan and PDV from 'Early Rivers' sweet cherry shoots using chemotherapy only. Combining of thermotherapy with 50-100 mg l^{-1} of Virazole® treatment was effective in eliminating PDV from shoots of sweet cherry cv. 'Early Rivers' Simultaneous use of the both methods enabled to eliminate PNRSV from most of the treated shoots of myrobalan and 'Empress' plum.

DISCUSSION

The temperature and time of exposure are limited by the heat tolerance of the host plant, depending on species and variety. It is known that pome fruit trees tolerate heat better than stone fruit trees (Gella and Errea, 1998; Stein et al., 1991). The use of alternating temperature 28°C/38°C in thermotherapy *in vitro* for 18-20 days was less harmful for shoots and in many cases allowed to eliminate PNRSV from shoots of peach (Spiegel et al., 1995).

In our studies, the survival rate of the shoots treated with thermotherapy was 66.7-100%, depending on the plant species. Deogratias et al. (1989) showed that all shoots of 'Noire de Meched' sweet cherry infected with ACLSV, treated with 32-34°C for 3 weeks, died at the end of the treatment period. However, in the same experiment 30% of 'Van 2D' sweet cherry shoots infected with PDV and PNRSV survived thermotherapy. Survival rate of shoots treated with 37°C for 21 days depended on species and cultivar of plants and a kind of virus. 90-100% of apricot shoot infected with ACLSV, 70% of sweet cherry shoots with PDV and PNRSV and 42-90% of peach shots with PNRSV and ACLSV survived thermotherapy (Gella and Errea, 1998). The differences between reported survival rates and results obtained in these studies could be due to the host species and cultivars and the method of high temperature application. Under the heat treatment sweet cherry tree growth and survival improved when trees were grown by hydroponics. This culture system allowed for prolonging heat treatment time that was sufficient to reduce levels of PNRSV and PDV in growing shoots of infected trees (Howell et al., 2001).

In our experiments heat treatment alone was successful in eliminating PNRSV and ACLSV from most of myrobalan and PNRSV from plum cv. 'Empress' shoots but was not sufficient for obtaining PDV-free sweet cherry cv. 'Early Rivers' shoots. This method was also successful in obtaining PNRSV- and PDV-free sweet and sour cherry (Deogratias et al., 1989) and PNRSV-free peach shoots (Barba et al., 1992; Stein et al., 1991). Depending on period of heat treatment, it was possible to eliminate PNRSV from 37-100%, ACLSV from 60-100% and PDV from 85-100% of *Prunus* sp. shoots (Gella and Errea, 1998). The differences between results obtained in our study and by other authors eliminating

PDV from sweet cherry could be explained by different cultivars and probably virus strains. Using thermotherapy for 28 days at 35°C, Snir and Stein (1985) successfully eliminated PNRSV from sweet and sour cherry shoots. Barba et al. (1992) showed that thermotherapy *in vitro* at 38°C for 40 days of plum and peach shoots infected with ACLSV resulted in 59% and 71% of healthy plantlets, respectively. It was difficult to eliminate ACLSV and PPV from apricot shoots using thermotherapy (Llácer, 1995).

In our experiments elimination of ACLSV from myrobalan and PNRSV from 'Empress' plum shoots was successful with Virazole® applied at concentration 10-100 mg l^{-1}. However, this compound alone was not sufficient to obtain PNRSV-free myrobalan and PDV-free 'Early Rivers' shoots. Deogratias et al. (1989) showed that Virazole® at concentration 25, 50 and 100 mg l^{-1} was effective in eliminating ACLSV from 50-100% of *Prunus mahaleb* shoots. However, in the same experiment it was necessary to apply 50 and 100 mg l^{-1} of this compound to eliminate PNRSV from 50-100% and PDV from 66-78% of shoots.

Combining thermotherapy and chemotherapy in this study was effective in eliminating PNRSV and ACLSV from *Prunus* sp., independently on Virazole® concentration used. The obtained results were better than in case of chemotherapy treatment only. Thermotherapy with Virazole® treatment at concentration 10 mg l^{-1} were not successful in obtaining PDV-free sweet cherry 'Early Rivers' shoots. When 25 mg l^{-1} of Virazole® was added to the media, only several shoots treated with 37°C were PDV-negative in ELISA test.

Virazole® at concentrations 50-100 mg l^{-1} was phytotoxic for 'Empress' plum shoots. Most of them showed chlorosis and necrosis and finally died during the therapy. The similar results obtained Deogratias et al. (1989) in eliminating PDV, PNRSV i ACLSV from sour cherry and Cieślińska and Zawadzka (1999) and Cieślińska (2002) in eliminating ACLSV from apple cv. 'Jonagold' and pear cv. 'Pierre Corneille' and *Raspberry vein chlorosis virus* (RVCV) from raspberry cv. 'Norna' To avoid the phytotoxic effect of chemotherapy it was better to apply 25 mg l^{-1} of Virazole® simultaneously with thermotherapy at 36-38°C for several weeks. Combining both methods allowed for elimination of ACLSV from 82% apple and of RVCV from 76% of raspberry shoots (Cieślińska and Zawadzka, 1999).

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ZASTOSOWANIE TERAPII *IN VITRO* W ELIMINACJI KILKU WIRUSÓW PORAŻAJĄCYCH DRZEWA OWOCOWE *Prunus* sp.

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S T R E S Z C Z E N I E

Celem prac było uwolnienie ałyczy (*Prunus cerasifera var. divaricata* Borgh.) od wirusa chlorotycznej plamistości liści jabłoni (*Apple chlorotic leaf spot virus*, ACLSV) i wirusa nekrotycznej plamistości pierścieniowej wiśni (*Prunus necrotic ring spot virus*, PNRSV), śliwy 'Empress' od PNRSV i czereśni 'Early Rivers' od wirusa karłowatości śliwy (*Prune dwarf virus*, PDV). W badaniach stosowano metodę termoterapii (utrzymywanie kultur *in vitro* porażonych pędów w temperaturze 36^{o}C przez 28 dni), chemioterapię z użyciem 10-100 mg l^{-1} Virazole® oraz kombinację obu metod. Pędy przed i po zabiegu testowane były na obecność wirusów testem DAS-ELISA. Termoterapia okazała się skuteczną metodą eliminacji PNRSV i ACLSV, lecz nie była przydatna w uwolnieniu pędów czereśni od PDV. Zastosowanie Virazole® w stężeniu 25-100 mg l^{-1} umożliwiło eliminację ACLSV z pędów ałyczy i PNRSV ze śliwy, lecz było nieskuteczne w przypadku PNRSV w ałyczy i PDV w czereśni. Eliminacja obu patogenów okazała się możliwa dopiero po zastosowaniu kombinacji termoterapii i chemioterapii przez dodanie do pożywki Virazole® w dawce 50-100 mg l^{-1}. Wysokie dawki tego związku, zwłaszcza w połączeniu z działaniem termoterapii miały fitotoksyczny wpływ prowadząc często do zamierania pędów, zwłaszcza w przypadku śliwy 'Empress'.

Słowa kluczowe: ACLSV, PNRSV, PDV, chemioterapia, termoterapia