ELIMINATION OF *PRUNUS NECROTIC RING SPOT VIRUS* (PNRSV) FROM PLUM 'EARLIBLUE' SHOOTS THROUGH THERMOTHERAPY *IN VITRO*

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A B S T R A C T

The study aimed to eliminate *Prunus necrotic ring spot virus* (PNRSV) from 'Earliblue' plum shoots by thermotherapy *in vitro* using several temperature regimes (38°C/36°C, 38°C/36°C + 35°C/35°C, 37°C/36°C) and treatment duration (10 to 29 days). The media used for therapy based on modified Murashige and Skoog (1962) and Lloyd and McCown (1980) media. Presence of virus in plant tissue before and lack of virus after heat treatment was proved by enzyme-linked immunosorbent assay (ELISA). The higher percentage of 'Earliblue' shoots survival was obtained on media with decreased minerals content, at lower temperature and shorter length of therapy, and at higher light intensity. Except from one combinations, 100% of shoots tested were freed of the virus.

Key words: PNRSV, thermotherapy *in vitro,* ELISA

INTRODUCTION

Prunus species are often infected by viruses (PDV, PPV, PNRSV, ACLSV) which cause serious diseases of trees resulting in considerable decrease of crop and fruit and tree quality. Thermotherapy conducted *in vivo* is time consuming and results in low percentage of survived trees. *In vitro* thermotherapy combined with shoot tip culture is 5.8 times more effective than conventional thermotherapy (potted plants kept at 30 °C-35°C for 8 weeks) while taking half the time (Koubouris et al., 2007). Thus, the sterile cultures gives opportunity for elimination of viruses from plant tissue with great commercial importance.

The most effective methods used for virus elimination from plant material are thermotherapy *in vitro* (Spiegel et al., 1995), chemotherapy or a combination of both procedures

(Cieślińska, 2007). Sometimes recovery of virus-free plants can by obtained by *in vitro* shoot-tip grafting (Rizqi et al., 2001). The efficiency of virus elimination depends on type of virus, plant host and possible virus combinations (Knapp et al., 1995). Stein et al. (1991) proved that alternating high and low temperature thermotherapy regimes were more effective in decreasing virus titre than constant high temperature. Howell et al. (2001) using combined method of chemotherapy and hydroponics reduced levels of PNRSV and PDV in shoots of *Prunus avium*. Most often ELISA assays is used for screening the infected tissue, but sometimes the pathogen titre is below the threshold of detection. In this case serodiagnostics currently in use are of little value for early screening and the new, sensitive detection techniques developed for *in vivo* application could give more reliable results. Broad and specific assays using molecular techniques for virus and phytoplasma detection were applied (Laimer da Mâchado et al., 2001).

The aim of the presented study was to eliminate *Prunus necrotic ring spot virus* (PNRSV) from the shoots of 'Earliblue' plum by thermotherapy *in vitro*.

MATERIAL AND METHODS

The study was carried out on 'Earliblue' plum (*Prunus domestica* L.) shoots infected with PNRSV. The infection of shoots was confirmed by positive reaction in enzyme-linked immunosorbent assay (ELISA) using specific antiserum (Clark et al., 1976). For heat treatment cultured shoots were placed in Erlenmayer flasks containing 25 ml of Murashige and Skoog (1962) (MS) or Lloyd and McCown (1980) (WPM) media. The media were modified as presented in Table 1.

All media, with exception of T5, were supplemented with glycine (2.0 mg l^{-1}), nicotinic acid (0.5 mg l^{-1}), pyridoxine hydrochloride (0.5 mg l^{-1}), thiamine hydrochloride (0.5 mg l^{-1}), myo-inositol (100 mg l^{-1}), Na_2EDTA + $FeSO_4$ – (40 mg l^{-1}), sucrose (20 g l^{-1}) and solidified with 6% agar (Biocorp). Medium T5 was supplemented with 6% agar only. The pH of the media was adjusted to 5.5 before being autoclaving. 'Earliblue' plum shoots 1 cm long were inserted in the media. The cultures were maintained in therapy chamber under two levels of photon flux density (PFD): 54.3 µmol s^{-1} m^{-2} and 17.3 µmol s^{-1} m^{-2} at 16h photoperiod. Several temperature regimes (38°C-/36°C, 38°C-/36°C + 35°C/35°C and 37°C/36°C), duration of heat therapy (10-29 days) were applied. As a control conditions a 16h photoperiod at PFD 92.8 µmol $s^{-1}m^{-2}$ and temperature 23±1°C was applied.

Tips (0.5 cm) from the shoots which survived the therapy were cut off and transferred onto fresh MS or WPM medium enriched with 3% sucrose and 0.5 mg l^{-1} BA for multiplication. The propagated plant material was tested for the virus content by DAS-ELISA about two month after treatment.

Table 1. Media used for heat treatment of 'Earliblue' plum shoots

Medium	Composition
T1	MS salt medium
T2	Modified MS medium: NH_4NO_3, 825.0 mg l^{-1} KNO_3, 950.0 mg l^{-1}
T3	WPM salt medium
T4	Modified WPM medium: NH_4NO_3, 200.0 mg mg l^{-1} $Ca(NO_3)_2$, 193.0 mg mg l^{-1} $CaCl_2$, 36.2 mg mg l^{-1}
T5	Distilled water

RESULTS

Survival of 'Earliblue' plum shoots

The shoots survived in different rate depending on the medium used, duration and temperature of thermo-therapy, and light intensity. Some of treatment combinations resulted in 100% shoot death (Tab. 2 and 3).

Each of investigated factors affected the survival rate of shoots separately and in combination with other factors. Thus, the obtained results can be analyzed in different way.

The light intensity during heat therapy affected significantly the shoot survival rate. In general, under a stronger photon flux density (54.3µmols^{-1}m^{-2}) (Tab. 2.) the rate of shoots survival was higher than under a lower PFD (17.3 µmols^{-1}m^{-2}) (Tab. 3). Under lower light intensity the higher temperature range (38°C/36°C) resulted in 100% of dead shoots (Tab. 3). In temperature range of 38°C/36°C the percentage of survived shoot decreased with the length of heat treatment (Tab. 2). Decreasing the temperature by one degree during the day-time resulted in increased survival rate of shoots maintained on T1, T2 and T3 media, even at a longer time of thermo-therapy. Treatment with combination of temperature ranges (38°C/36°C + 35°C/35°C) resulted in a better survival of shoots on media T3 and T4 if they were kept at a higher temperature for a shorter time (Tab. 2). Obtained results show certain regularity – the higher were temperatures during thermotherapy the lower were the rates of shoot survival at a given length of treatment duration. Conducting the therapy at a lower temperature for a longer time enabled to obtain higher percentage of alive shoots (Tab. 2).

Type of media applied is one of the factors influencing the survival of the shoots. In the study presented the mineral content of some media was decreased and all were hormone-free. The relationship between type of a medium tested and percentage of survived shoots was noticeable (Tab. 2 and 3). Distinctly higher shoot survival rate, even after longer time of therapy, was obtained on T3, T4 and T5 media which contained less mineral components than T1 or T2.

Table 2. Effect of exposure to alternating temperature on survival of 'Earliblue' plum shoots after thermotherapy *in vitro* at PFD 54.3 µmol s^{-1} m^{-2}

Duration of thermotherapy (days)	Survival rate [%] of shoots grown on various media				
	T1	T2	T3	T4	T5
24°C/23°C					
Control	100	100	100	100	100
38°C/36°C					
10	28.6	28.6	14.2	57.1	-
14	5.4	8.1	8.1	-	-
19	0	0	0	-	-
38°C/36°C + 35°C/35°C					
0+18	-	-	52.0	-	-
6+4	-	-	-	71.4	42.9
6+6	-	-	-	14.2	42.9
12+1	0	0	28.6	-	-
12+4	0	0	14.2	14.2	-
37°C/36°C					
21	3.6	21.4	48.6	51.4	13.6
29	-	-	-	28.6	-

(-) not investigated

Table 3. Survival rate of 'Earliblue' plum shoots after thermotherapy *in vitro* at PFD 17.3 µmol s^{-1} m^{-2}

Duration of thermotherapy (days)	Survival rate [%] of shoots grown on various media				
	T1	T2	T3	T4	T5
38°C/36°C					
14	0	0	0	-	-
19	0	0	0	-	-
37°C/36°C					
21	0	0	2.9	28.6	13.6
29	-	-	-	4.8	4.8

(-) not investigated

Elimination virus from 'Earliblue' plum shoots

PNRSV was eliminated from 'Earliblue' plum shoots in all treatment combinations tested except one conducted at 38°C/36°C for 10 days on T4 media, where 100% of survived shoots were still infected with the virus (Tab. 4 and 5).

Table 4. Elimination virus from of 'Earliblue' plum shoots after thermotherapy *in vitro* at PFD 54.3 µmol s^{-1} m^{-2}

Duration of thermotherapy (days)	Virus free shoots [%]				
	T1	T2	T3	T4	T5
24°C/23°C					
Control	0	0	0	0	0
38°C /36°C					
10	100	100	100	0	-
14	100	100	100	-	-
19	*	*	*	-	-
38°C /36°C + 35°C /35°C					
0+18	-	-	100	-	-
6+4	-	-	-	100	100
6+6	-	-	-	100	100
12+1	*	*	100	-	-
12+4	*	*	100	100	-
37°C /36°C					
21	100	100	100	100	100
29	-	-	-	100	-

(-) not investigated
*all shoots died after thermotherapy

Table 5. Elimination virus from of 'Earliblue' plum shoots after thermotherapy *in vitro* at PFD 17.3 µmol s^{-1} m^{-2}

Duration of thermotherapy (days)	Virus free shoots [%]				
	T1	T2	T3	T4	T5
38°C/36°C					
14	*	*	*	-	-
19	*	*	*	-	-
37°C/36°C					
21	*	*	100	100	100
29	-	-	-	100	100

(-) not investigated
*all shoots died after thermotherapy

DISCUSSION

Obtained results showed a great influence of light intensity on survival of 'Earliblue' plum shoots during thermotherapy. In general, under a stronger photon flux density (54.3 µmols^{-1}m^{-2}) the survival of plum shoots was better. Other authors applied defined light conditions in experimental chamber but they did not compare different light intensities

and their effect on shoot survival rate. Deogratias et al. (1989) carried out the thermotherapy of sweet cherry shoots at temperature raised gradually within three weeks, at much higher photon flux density (200 µmol $s^{-1}m^{-2}$). Cieślińska (2007) maintained the plum and sweet cherry shoot cultures under a light intensity of 2000 lux (app. 24-40 µmol $s^{-1}m^{-2}$), Gella and Errea (1998) kept *Prunus* culture under a light intensity 5000 lux (app. 60-100 µmol $s^{-1}m^{-2}$), Knapp et al. (1995) maintained *Malus* and *Prunus* culture under light intensity 4400 lux (app. 53-88 µmol $s^{-1}m^{-2}$), Wang et al. (2006) placed *Pyrus pyrifolia* 'Huang-hua' culture under light intensity 1500 lux (app. 18-30 µmol $s^{-1}m^{-2}$).

The more important factor influencing shoot survival is temperature range. The most often temperature is measured in a growth chamber. Some authors proved that air temperature measured in a chamber was 1°C Stein et al. (1991) or 2°C Deogratias et al. (1989) lower than air temperature measured inside the test tubes. The temperature regime and the length of heat treatments should be applied individually to cultivars tested. The choice of temperature regime is a balance between the relatively low shoot mortality and high percentage of virus free shoots. In the study presented the applied temperature range enabled to achieve that goal. The survival rate of shoots was 3.6-71.4% and 2.9-28.6% under higher and lower light intensity, respectively, depending on the temperature range. Applied day/night temperatures differed within 1-2°C (38°C/36°C or 37°C/36°C) or temperature was constant (35°C/35°C). Cieślińska (2007) obtained the survival rates of 'Empress' plum shoots and 'Early Rivers' sweet cherry shoots of 66.7% and 100%, respectively. Spiegel et al. (1995) achieved the survival of 'Summerset' and 'Hermosa' peach shoots at the level of 48% and 88%, respectively. In another study the survival rates of 'Summerset' and 'Hermosa' peach shoots were 51% and 81%, respectively (Stein et al., 1991). Sometimes heat treatment causes high mortality of shoots, even up to 90% (Snir and Stein, 1985).

In the study presented among the tested temperature ranges the most effective regarding the percentage of survived and virus free shoots was combination 38°C/36°C + 35°C/35°C. Spiegel et al. (1995) showed that thermotherapy at night/day temperature range 38°C/28°C during 18-20 days was efficient in eliminating PNRSV from peach shoots. Snir and Stein (1985) applied constant temperature 35°C for 4 weeks. However, Stein et al. (1991) proved that constant temperature regimes were either ineffective in reducing virus titre or lethal to the shoots. Howell et al. (2001) suggest usefulness of alternating temperature even every four hours, between 40°C and 32°C. Cieślińska (2007) recommended to increase temperature gradually from 28°C to 36°C within a week and maintain at 36°C for following four weeks. Other authors (Stein et al., 1991; Deogratias et al., 1989; Spiegel et al.,

1995) suggest the necessity of individual treatment of each cultivar in defined conditions.

In the study presented the effectiveness of thermotherapy, measured as percentage of virus free shoots, was very high. Only in one combination (38°C/36°C) virus was not eliminated from plum shoots. Possible reason was too short duration of thermotherapy – only ten days. In the other studies the percentage of shoots from which the virus was eliminated varied in high degree. Cieślińska (2007) succeeded in eliminating PNRSV from 75% of 'Empress' plum shoots. Stein et al. (1991) obtained 40% of 'Summerset' and 90% of 'Hermosa' peach shoots virus free. Snir and Stein (1985) eliminated this virus from 40% of 'Early Ruby' sweet cherry shoots. Similarly, Deogratias et al. (1989) evidenced that 30% of 'Van' sweet cherry shoots was released from virus.

The results of the experiment presented proved that mineral composition of a media plays an important role in the efficiency of thermotherapy of 'Earliblue' plum. The best survival of shoots was achieved on the media with reduced mineral content. Postman and Hadidi (1995) applied medium without hormone for eliminating apple scar skin viroid from pears shoots. In other reports, proliferation (Snir and Stein, 1985; Deogratias et al., 1989) or elongation (Stein et al., 1991) medium is recommended for heat therapy. Snir and Stein (1985) enriched media for heat therapy with IBA and BA, both at concentration of 1.0 mg l^{-1}.

In conclusion, the applied treatments enabled the shoots to survive in different degree depending on the combination of tested factors and enabled to eliminate PNRSV from 'Earliblue' plum shoots.

REFERENCES

Cieślińska M. 2007. Application of thermo-and chemotherapy *in vitro* for eliminating some viruses infecting *Prunus* sp. fruit trees. J. FRUIT ORNAM. PLANT RES. 15: 117-124.

Clark M.F., Adams A.N., Barbara D.J. 1976. The detection of plant viruses by enzyme-linked immunosorbent assay (ELISA). ACTA HORTIC. 67: 43-49.

Deogratias J.M., Dosba F., Lutz A. 1989. Eradication of *prune dwarf virus*, *prunus necrotic ringspot virus* and *apple chlorotic leaf spot virus* in sweet cherries by a combination of chemotherapy, thermotherapy and *in vitro* culture. CAN. J. PLANT PATHOL. 11: 337-342.

Gella R., Errea P. 1998. Application of *in vitro* therapy for *Ilavirus* elimination in three *Prunus* species. J. PHYTOPHATHOL. 146: 445-449.

Howell W.E., Eastwell K.C., Li T.S.C. 2001. Heat treatment, chemotherapy and hydroponic culture for obtaining virus-free trees of sweet cherry. ACTA HORTIC. 550: 455-458.

Knapp E., Hanzer V., Weiss H., da Câmara Machado A., Weiss B., Wang Q., Katinger H., Laimer da Câmara Machado M. 1995. New aspects of virus elimination in fruit trees. ACTA HORTIC. 386: 409-418.

Koubouris G., Maliogka V., Efthimiou K., Katis N., Vasilakakis M. 2007. Elimination of plum pox virus through *in vitro* thermotherapy and shoot tip culture compared to conventional heat treatment in apricot cultivar Bebecou. J. GEN. PLANT. PATHOL. 73(5): 370-373.

Laimer da Mâchado M., Heinrich M., Hanzer V., Arthofer W., Strommer S., Paltrinieri S., Martini M., Bertaccini A., Kummert J., Davies D. 2001. Improved detection of viruses and phytoplasmas in fruit tree tissue culture. ACTA HORTIC. 550: 463-470.

Lloyd G., McCown B. 1980. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia* by use of shoot tip culture. PROC. INT. PLANT. PROPAGATORS SOC. 30: 421-427.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. PHYSIOL. PLANT 15: 473-479.

Postman J.D., Hadidi A. 1995. Elimination of apple scar skin viroid from pears by *in vitro* thermotherapy and apical meristem culture. ACTA HORTIC. 386: 536-543.

Rizqi A., Zemzami M. Spiegel S. 2001. Recovery of virus-free almond plants by improved *in vitro* shoot-tip grafting. ACTA HORTIC. 550: 447-454.

Snir I., Stein A. 1985. *In vitro* detection and elimination of prunus necrotic ringspot virus in sweet cherry (*Prunus avium*). RIV. ORTIFLORO-FRUTT. IT. 69: 191-194.

Spiegel S., Stein A., Tam Y. 1995. In vitro thermotherapy of *Rosaceae* fruit trees. ACTA HORTIC. 386: 419-420.

Stein A., Spiegel S., Faingersh G., Levy S. 1991. Responses of micropropagated peach cultivars to thermotherapy for elimination of *Prunus necrotic ringspot virus*. ANN. APPL. BIOL. 119: 265-271.

Wang L., Wang G., Hong N., Tang R., Deng X., Zhang H. 2006. Effect of thermotherapy on elimination of apple stem grooving virus and *apple chlorotic leaf spot virus* for *in vitro*-cultured pear shoot tips. HORTSCIENCE 41(3): 729-732.

UZYSKIWANIE WOLNYCH OD WIRUSA NEKROTYCZNEJ PLAMISTOŚCI PIERŚCIENIOWEJ WIŚNI (PNRSV) PĘDÓW ŚLIWY ODMIANY ‘EARLIBLUE’ METODĄ TERMOTERAPII *IN VITRO*

Ewa Dziedzic

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

Badania miały na celu uwolnienie od wirusa nekrotycznej plamistości pierścieniowej wiśni (PNRSV) śliwy odmiany ‘Earliblue’ metodą termoterapii z wykorzystaniem kultur *in vitro*. Obecność wirusa w pędach kultury śliwy przed zabiegiem oraz brak wirusa po termoterapii zostały udowodnione za pomocą testu serologicznego – ELISA. Zabieg termoterapii przeprowadzono z uwzględnieniem różnych kombinacji zakresów temperatury (38°C/36°C, 38°C/36°C+35°C/35°C, 37°C/36°C) oraz zróżnicowanego okresu czasu zabiegu od 10 do 29 dni. Pożywki do zabiegu były przygotowywane na podstawie zmodyfikowanego składu mineralnego skład pożywki Murashige i Skoog – MS (1962) oraz Lloyd i McCown – WPM (1980). Pożywki nie zawierały związków hormonalnych. Wyższy procent przeżycia pędów śliwy odmiany ‘Earliblue’ uzyskano po zastosowaniu pożywek uboższych w składniki mineralne, niższego zakresu oraz krótszego okresu działania temperatury oraz lepszych warunków świetlnych. Wysoka skuteczność termoterapii polegająca na uwolnieniu testowanych pędów w większości zastosowanych kombinacji była potwierdzona testem ELISA.

Słowa kluczowe: PNRSV, termoterapia *in vitro,* ELISA