



Elimination of cherry virus A from *Prunus domestica* ‘Besztercei Bt. 2’ using in vitro techniques

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Abstract

Cherry virus A, a capillovirus, can infect different *Prunus* species and be present as a latent infection in orchards. CVA infection was detected in a Hungarian stock collection of *Prunus domestica* ‘Besztercei Bt. 2’. In our study, different in vitro virus elimination techniques (thermotherapy combined with shoot tip culture and chemotherapy alone or combined with thermotherapy) were used and compared for their efficiency in eliminating CVA from ‘Besztercei Bt. 2’ plum cultivar. Thermotherapy was carried out at 38/36°C (day/night) in a heat chamber followed by the excision of 1–2 mm long shoot tips for plant regeneration. As a chemotherapy agent, ribavirin or zidovudine was added to the multiplication medium at two different concentrations with or without two weeks of thermotherapy pretreatment. The plum shoots tolerated 14–18 days of heat treatment, 64% of the plants regenerated from shoot tips, and 75% of the tested regenerated plants were confirmed by RT–PCR to be CVA-free. Ribavirin and zidovudine did not negatively affect the survival of the plum shoots at any applied concentrations. Zidovudine alone was not able to efficiently eradicate virus from the treated plantlets, but when zidovudine was combined with heat treatment, the number of CVA-positive plants decreased to 60%. In contrast, ribavirin alone was very efficient at eliminating CVA from all the tested plants when used at a concentration of 25 mg L⁻¹. To our knowledge, this is the first report of eliminating CVA from *Prunus* sp. via in vitro techniques.

Key message

The aim was to compare and evaluate the effectiveness of well-known in vitro techniques for eradicating CVA from plum shoot cultures.

Keywords CVA · Virus elimination · *Prunus* · In vitro thermotherapy · Shoot tip culture · Chemotherapy · Ribavirin · Zidovudine

Abbreviations

ACLSV	Apple chlorotic leaf spot trichovirus
CVA	Cherry capillovirus A
PDV	Prune dwarf ilarvirus
PNRSV	Prunus necrotic ringspot ilarvirus
PPV	Plum pox potyvirus
RT–PCR	Reverse transcription–polymerase chain reaction
SAM	Shoot apical meristem

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Introduction

Cherry virus A (CVA) – a virus belonging to the genus *Capillovirus*, family *Betaflexiviridae* – has been increasingly reported worldwide since its first discovery. CVA

was first reported in Germany in sweet cherry (Jelkmann 1995). Since then, it has been detected in several *Prunus* hosts not only in sweet and sour cherry (Komorowska and Cieślińska 2004; Grimová et al. 2010) but also in apricot, peach, plum, Japanese apricot (*Prunus mume*) and myrobalan (James and Jelkmann 1997; Barone et al. 2006; Marais et al. 2008; Kesanakurti et al. 2017; Baráth et al. 2018; Ben Mansour et al. 2023; Wani et al. 2024). CVA is graft-transmissible, and although its vector is still unknown, its presence has been reported in pollen (Beaver-Kanuya and Harper 2019). The virus does not appear to cause any obvious symptoms in host plants but is very widespread and is often reported to be present in mixed infections. Together with other viruses e.g., little cherry virus-1, *Prunus* necrotic ringspot virus, cherry green ring mottle virus, cherry necrotic rusty mottle virus, it may affect the severity of symptoms, or it may have some influence on graft incompatibility in susceptible combinations of scions and rootstocks (James and Jelkmann 1997; Marais et al. 2012).

To create and maintain healthy fruit tree plantations, it is important to use certified virus-free propagation material. In *Prunus* species, the most widespread methods for eliminating viruses are based on in vitro shoot cultures (Szabó et al. 2024). Meristem culture has been used for a long time to produce virus-free plants (Knapp et al. 1998), but the molecular mechanism of the virus exclusion from the meristem has been only investigated recently. In their research, Wu and coworkers have shown in the *Arabidopsis* model plant that a transcription factor WUSCHEL controls the invasion of the meristem by several viruses and block their vertical transmission into the shoot apical meristem (SAM) (Wu et al. 2020). There is evidence that nepoviruses and tobnaviruses can enter into the meristem in different hosts, but it seems that the viral movement into the SAM could happen through several different mechanisms which can be different in different host/virus interactions ((Bradamante et al. 2021). During thermotherapy, the replication of the virus is inhibited and the degradation of the RNA increases, so the movement of the virus towards the meristematic cells can slow down, and the newly formed shoot tips can remain virus-free, allowing the excision of a larger portion of the shoot tips, which can increase the probability of successful plant regeneration (Bettoni et al. 2022). In general, the size of excised shoot tips is positively correlated with survival and shoot regeneration but negatively correlated with successful virus eradication (Wang 2018).

Stone fruits are known to be especially sensitive to high temperatures and have a low regeneration capacity from meristems (Lenz et al. 1983; Manganaris et al. 2003; Polák and Hauptmanová 2009; Şekerz et al. 2015).

In vitro thermotherapy followed by shoot tip excision and culture can shorten the treatment time compared to in vivo thermotherapy and increase the regeneration capacity compared to meristem isolation, thereby increasing the efficiency of the elimination process (Stein et al. 1991; Koubouris et al. 2007). Peach and cherry cultivars are extremely heat sensitive (Lenz et al. 1983; Polák and Hauptmanová 2009), while plums can tolerate long-term high temperatures (Dziedzic 2008). The duration and degree of heat treatment should be optimized for each genotype because of the different heat tolerances of individual plant species and cultivars and the resilience of virus species (Gella and Errea 1998; Cieślińska 2007).

Chemotherapy is also used for plant virus elimination. The addition of antiviral agents to culture media can inhibit virus replication in host plants. The most common antiviral agent for plant virus eradication is ribavirin (Virazol). It is a synthetic analogue of guanosine and inhibits viral RNA synthesis by integrating it into newly synthesized RNA (Thomas et al. 2012). Ribavirin has been employed to eliminate the most common viruses from infected sweet cherry, myrobalan, plum, apricot, pear and apple cultivars (Deogratias et al. 1989; Cieślińska 2007; Hauptmanová and Polák 2011; Hu et al. 2012, 2015; Şekerz et al. 2015; Mazeikiene et al. 2019; Zare Khafri et al. 2024). Zidovudine (azidothymidine) has been proven to be a more effective chemotherapeutic agent than ribavirin for virus eradication in peach plants, without causing phytotoxic effects (Pavelkova et al. 2015). No phytotoxic effects of zidovudine were visible on shoot growth of apple cultures even in a wide concentration range up to 1280 mg L⁻¹ (Sedlák et al. 2023). As a thymidine analogue, zidovudine inhibits reverse transcriptase and prevents the reproduction of retroviruses in the host genome (Veal and Back 1995). Chemotherapy alone is often ineffective or only eliminates viruses with little efficacy, but after preliminary heat treatment, it results in many virus-free plants (Spiegel et al. 1999; Cieślińska 2007; Hu et al. 2012, 2015). The combination of in vitro heat treatment, meristem culture and ribavirin has been reported to be effective against plum pox virus (PPV) despite the reduction in the regeneration capacity of plum plants (Jakab-Ilyefalvi and Pamfil 2011).

In this study, we used different methods to eliminate latent CVA from the plum cultivar ‘Besztercei Bt. 2’ investigating the effects of heat treatment length, different concentrations of ribavirin and zidovudine and the combinations of such factors. The objective of this study was to compare and evaluate the effectiveness of well-known in vitro techniques for successfully eradicating CVA from plum shoots.

Materials and methods

Plant material and culture conditions

CVA-infected (preliminarily tested by RT–PCR) *Prunus domestica* cultivar ‘Besztercei Bt. 2’ was selected from the stock plantation of the Research Centre for Fruit Growing, Hungarian University of Agriculture and Life Sciences (MATE), Érd, Hungary, to establish in vitro shoot cultures. Shoots were surface sterilized in HgCl₂ solution (4 g L⁻¹, 6 min) and then rinsed in sterile distilled water three times. Two-node-long shoot segments were cultured on multiplication media: modified MS media (Murashige and Skoog 1962) supplemented with MS macroelements, FeNaEDTA (20 mg L⁻¹), microelements according to Balla and Vertesy (2001), myo-inositol (500 mg L⁻¹), Jacquot vitamins (Jacquot 1955), phloroglucinol (10 mg L⁻¹), 6-benzylaminopurine (BAP, 0.25 mg L⁻¹), indole-3-butyric acid (IBA, 0.1 mg L⁻¹), gibberellic acid (GA₃, 0.3 mg L⁻¹), sucrose (30 g L⁻¹) and agar (6 g L⁻¹). pH of the medium was adjusted to 5.2 before autoclaving at 121 °C for 20 min. In vitro plants were kept in a growth room at 24 ± 1 °C under 16 h photoperiod with a light intensity of 60 μM m⁻² s⁻¹ provided by warm and cool white fluorescent lamps, and subcultured every four weeks. After successful shoot culture establishment, the virus presence was confirmed by RT–PCR, and virus-infected lines were selected and propagated for virus elimination treatments.

Virus elimination methods

Combination of in vitro thermotherapy and shoot tip culture

Two-week-old shoot cultures were transferred into a climate chamber for heat treatment at 38/36°C (day/night) and 12 h of light (160 μmol m⁻² s⁻¹). After 14–23 days of heat treatment, 1–2 mm long shoot tips (containing 2–4 leaf primordia) were cut off under a stereomicroscope under sterile conditions and maintained on multiplication media in Petri dishes for regeneration. Shoot tips were subcultured to fresh medium every 4 weeks.

Chemotherapy

After autoclaving, ribavirin (Duchefa, The Netherlands) or zidovudine (Sigma–Aldrich, USA) was added to the multiplication media by filter sterilization (Millipore, 0.22 μm, PES membrane) to a final concentration of 25 or 50 mg L⁻¹. Five in vitro shoots (1 cm long) per vessel were maintained on a multiplication medium containing the antiviral agent for 2 successive subcultures each lasting four weeks (total

8 weeks). After 8 weeks, the apical parts (1 cm long) of the treated shoots were removed and cultured as separated lines on antiviral agent-free media until virus indexing.

As a control, 1 cm long shoots were maintained on multiplication media without antiviral agents for 8 weeks. For each treatment, 15 shoots were treated.

Combinations of thermotherapy and chemotherapy

After two weeks of in vitro thermotherapy, 1 cm long apical shoots were cut and transferred onto multiplication media supplemented with the antiviral agent zidovudine at concentrations of 25 or 50 mg L⁻¹ for 8 weeks with one subculture. After 8 weeks, the shoots were cultured on antiviral agent-free media until virus indexing.

As a control group, shoot cultures were treated with thermotherapy for two weeks without chemotherapy.

Virus detection

After treatment, whole micropropagated shoots without basal calli were collected to determine infection status. RNA was extracted from the in vitro plant samples via the CTAB method (Gambino et al. 2008). For virus detection, the reverse-transcription PCR method (RT–PCR) was used. cDNA synthesis was performed from the extracted RNA with a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) using random primers according to the manufacturer’s instructions. PCR was performed using Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific), and virus-specific primers CVamp_Fm (ATGTCGATCATACCAGTYAAG) and CVamp_Rm (TTACTTCTCGCAACYAC) were used (Baráth et al. 2018). The PCR results were evaluated via gel electrophoresis of the PCR products.

Results

Virus elimination using thermotherapy combined with shoot tip excision

Forty-nine shoot tips 1–2 mm long were excised after thermotherapy (Fig. 1D), 19 shoot tips (39%) died early (turned brown or undesirable calli had formed and were unable to regenerate). In total, 20 in vitro plants regenerated from shoot tips were tested via RT–PCR for the presence of CVA. Fifteen plants (75%) were CVA-free, while 25% remained infected (Table 1).

Examining the effect of heat treatment length, plum cultures were able to tolerate heat treatment at 38/36°C even

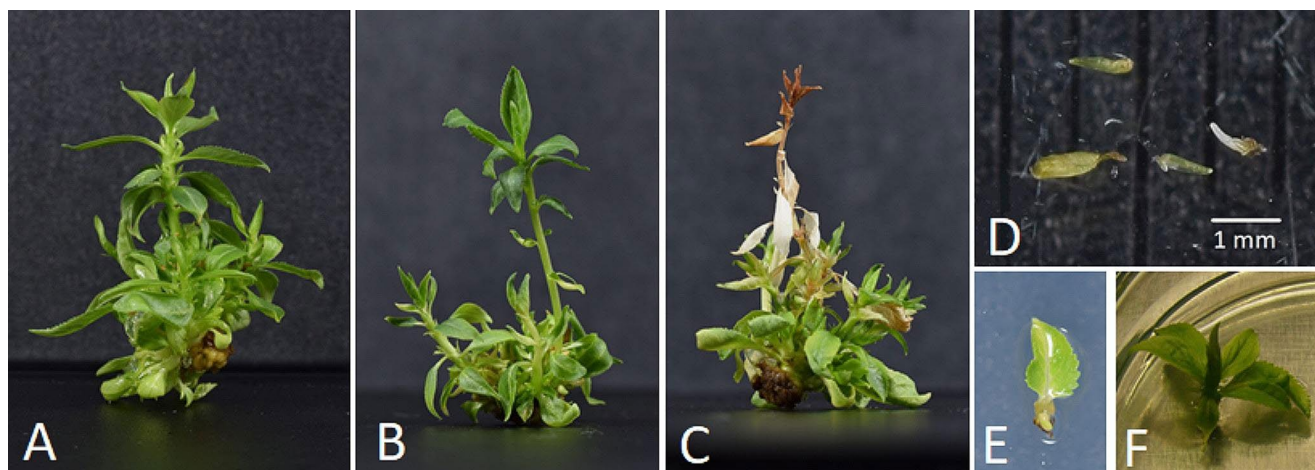


Fig. 1 *Prunus domestica* cv. 'Besztercei Bt. 2' underwent in vitro thermotherapy followed by shoot tip excision and regeneration (**A**, shoot culture without treatment; **B**, shoot culture 2 weeks after thermother-

apy; **C**, 4 weeks after thermotherapy; **D**, excised shoot tips; **E**, **F**, shoot tip regeneration)

Table 1 Effect of thermotherapy followed by shoot tip culture on plant survival and virus elimination

Length of thermotherapy (days)	Excised shoot tips (regenerated/total)	CVA-free/total tested by RT-PCR	Elimination rate (%)
14	4/6	3/4	75
15	18/26	7/9	78
17	3/7	2/3	67
18	5/8	3/4	75
23	0/2	-	-

Table 2 Effect of 8 weeks of chemotherapy alone or in combination with 14 days of thermotherapy on plant survival and the CVA elimination rate

Chemotherapy	Survival rate (%)	CVA-free (%)
NT control	100	7
Zidovudine 25 mg L ⁻¹	100	20
Zidovudine 50 mg L ⁻¹	100	20
Thermotherapy control	100	7
Thermotherapy + Zidovudine 25 mg L ⁻¹	100	40
Thermotherapy + Zidovudine 50 mg L ⁻¹	100	20
NT control	100	25
Ribavirin 25 mg L ⁻¹	100	100
Ribavirin 50 mg L ⁻¹	100	40

NT control: nontreated control group

for a long period (23 days),- but when shoot tips were excised after this long treatment period, they died quickly and were unable to regenerate. The plants tolerated the 15-day heat treatment well; they remained green and vigorous, and their shoot tips were intact and suitable for preparation (Fig. 1B). The excised shoot tips could regenerate to proliferable shoots after 6 months (Fig. 1E-F) at a high level (69%). All the excised shoot tips were labelled and the

successfully regenerated plantlets were able to be individually tested, and 78% of them became CVA-free. Our small-scale pilot test showed that the length of thermotherapy did not increase or reduce the effectiveness of virus elimination within the time interval used (14–18 days), most of CVA-free plants (78%) resulted after 15 days, but after 14 or 18 days the elimination rates were 75%. However, after 3 weeks of treatment, the harmful consequences of long-term thermotherapy became visible, as the plants significantly weakened, turned yellow, and their tips died (Fig. 1C).

Virus elimination using chemotherapy alone or in combination with heat treatment

The plant survival rate was not negatively affected by any of the antiviral agents used at concentrations of 25 or 50 mg L⁻¹. No significant phytotoxic effects were observed during these treatments. Zidovudine alone was not able to efficiently eliminate CVA at any concentration; however, treatment with zidovudine (25 mg L⁻¹) after heat treatment increased the elimination from 7 to 40%. Ribavirin applied at a lower concentration (25 mg L⁻¹) was very efficient, and no CVA was detected in the tested plants. At a higher concentration (50 mg L⁻¹) of ribavirin, 40% of the tested plants were virus-free. Surprisingly, some of the control plants (7–25%) tested negative for CVA.

Discussion

In the case of CVA-infected plum cultivar 'Besztercei Bt. 2', 1–2 mm long shoot tip isolation following in vitro thermotherapy proved to be a suitable method for eliminating CVA and producing CVA-free plants. The size of the excised tip

seemed to be a crucial factor in the elimination procedure. We tried to achieve the smallest possible size that could survive and regenerate but was free from the virus. In our work, we isolated 1- to 2-mm long shoot tips. Even using these relatively large shoot tips, not limited only to the meristematic region, 39% of the shoot tips died early (turned brown or undesirable calli had formed and were unable to regenerate). Zarghami and Ahmadi (2023) reported that the survival rate of peach cultivars decreased from 65 to 20% with decreasing meristem size (from 1.0 to 0.2 mm). The use of a larger (1–2 mm) shoot tip in combination with heat treatment was effective in eliminating viruses from apricots and nectarines (Koubouris et al. 2007; Manganaris et al. 2003). In vitro thermotherapy of plum shoots at 38/36°C for 15 days proved to be an ideal duration, as the plants could tolerate this length of time at increased temperature, and remained green and vigorous, their shoot tips were intact and suitable for excision. The shoot tips that were excised after 15 days of heat treatment and further grown on culture medium resulted in 69% regeneration, and 78% of the tested regenerated plantlets were confirmed to be CVA-free. During the examined time interval (14–18 days), the length of thermotherapy did not change the effectiveness of virus eradication. Abdullahi and Lawrence (2022) reported that 7 days of heat treatment at 37/32°C prior to meristem preparation was sufficient to eliminate apple chlorotic leaf spot virus (ACLSV) from Shiro plum, while Koubouris et al. (2007) applied heat treatment for 20 days at 35–37 °C to eliminate PPV from the apricot cultivar ‘Bebecou’. Hesari et al. (2022) applied heat treatment at 37 °C for 4 weeks and this resulted in 100% PPV-free and Prunus necrotic ring spot virus (PNRSV)-free peach plants. A 45-day-long period at 37/35°C was suitable for eliminating PPV, PNRSV and prune dwarf virus (PDV) from apricot cultivars (Křižan and Ondrušiková 2009).

The plum cultivar ‘Besztercei Bt. 2’ proved to be tolerant to the applied chemical treatments, and no significant phytotoxic effects or plant deaths occurred even at high (50 mg L⁻¹) concentrations of the agents used. Interestingly, even during treatment with ribavirin or zidovudine, a lower concentration (25 mg L⁻¹) resulted in more virus-free plants, which is not consistent with previously published results (Cieślińska 2007; Deogratias et al. 1989; Paunovic et al. 2007); however, Cieślińska (2007) obtained more ACLSV-free plants using ribavirin at concentration of 50 mg L⁻¹ than the concentration of 100 mg L⁻¹. Hauptmanová and Polák (2011) also obtained similar results when 5 mg L⁻¹ ribavirin removed PPV from apricot (57%), while using a treatment of 10 mg L⁻¹ ribavirin, all treated shoots remained infected after 9 weeks of chemotherapy, and hence they concluded that the treatment period should be extended to 12 weeks for effective virus elimination. Cieślińska (2007) showed

that ribavirin at concentrations of 10, 25, 50 and 100 mg L⁻¹ was effective in eliminating ACLSV from 37 to 85% of myrobalan and PNRSV from 38 to 100% of the ‘Empress’ plum, but elimination of PNRSV from myrobalan and PDV from ‘Early Rivers’ sweet cherry shoots was successful only if chemotherapy was combined with thermotherapy. Regenerating the apical part of the axis (approximately 3 mm in length) after 20 mg L⁻¹ ribavirin treatment for 4 weeks was suitable for obtaining the PDV-free cherry cultivars ‘Tamara’ and ‘Amid’ (Papstein et al. 2019). Ribavirin at higher concentrations (50–100 mg L⁻¹) is often phytotoxic to shoots (Cieślińska 2002, 2007; Deogratias et al. 1989; Vescan et al. 2011; Sedlák et al. 2023). Hauptmanová and Polák (2011) successfully eliminated PPV from plum and apricot plants by long-term treatment at lower ribavirin concentrations (5 or 10 mg L⁻¹). Even a very low concentration (1 mg L⁻¹) and short treatment time (4 weeks) were suitable for the in vitro elimination of PPV from apricots (Şekerz et al. 2015). Chemotherapy with lower concentrations of antiviral agents in combination with heat treatment was found to be effective in eliminating PPV from plums (Jakab-Ilyefalvi and Pamfil 2011). Zare Khafri et al. (2024) reported results similar to our work on apricot cultivars, where thermotherapy with shoot tip (1–2 mm) culture resulted in the highest eradication rate (90–100%) but lower regeneration rate (60–80%); however, with the use of chemotherapy (25 mg L⁻¹ ribavirin), more virus-free plants were obtained overall. The differences between reported survival rates and the success of the virus elimination process obtained in these studies may be due to the host species and cultivars as well as the type and strategy of the infecting virus.

The resulting CVA-free untreated control plants indicating the possibility of spontaneous loss of the virus during long-term in vitro maintenance (Nacheva et al. 2002; Chandel 2010). However, the reason could also be a very low concentration of the virus in the shoots, so the RT-PCR method was not sensitive enough to detect the presence of the virus (Jevremović et al. 2023).

In the present study, thermotherapy at 38/36°C for 14–18 days combined with shoot tip (1–2 mm) culture resulted in a high percentage of virus-free plants; however, this could be achieved with significant plant mortality. Chemotherapy (ribavirin at 25 mg L⁻¹) completely eradicated the virus from the plum shoots without causing plant mortality. Based on our results, chemotherapy can effectively eradicate CVA from plum cultivar ‘Besztercei Bt. 2’ with a high plant survival rate in a shorter treatment time compared to thermotherapy followed by shoot tip culture. To our knowledge, this is the first report of eliminating CVA from in vitro *Prunus* shoot culture(s). Although CVA infection has not been correlated with any disease, the possibility that it could influence the severity of symptoms in mixed infections must

be considered; therefore, our virus elimination study may be useful in the future.

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Author contributions ÉP and ÉV conceived the idea and designed the experiments. LKS conducted the research; ZK helped with the maintenance of plant cultures and the implementation of treatments; and DF assisted in virus testing and data analysis. All the authors discussed the results and contributed to the final manuscript.

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Data availability All the data mentioned are publicly available in this manuscript or in the cited scientific articles.

Declarations

Conflict of interest The authors have no relevant financial or nonfinancial interests to disclose.

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