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Original article

Rapid assessment of sanitary and physiological state of thermotherapy-treated apple shoots by chlorophyll content evaluation

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Summary

Production of virus-free plants is crucial for vegetative propagation. In *in vitro* conditions, thermotherapy treatments are the most common method for virus elimination. However, shoot-tip necrosis and hyperhydricity are the most common physiological problems in thermotherapy-treated shoots, causing an important loss of virus-free plants. In the present study *Apple chlorotic leaf spot virus* (ACLSV, *Trichovirus* genus), *Apple stem pitting virus* (ASPV, *Foveavirus* genus) and *Apple mosaic virus* (ApMV, *Ilarvirus* genus) infected 'Abbasi' apple (*Malus domestica* Borkh.) shoots were treated through two *in vitro* thermotherapy strategies, constant (37°C) and alternating (32/38°C) cycles. Plant growth, shoot-tip necrosis, meristem establishment, hyperhydricity and total chlorophyll content (SPAD-502 meter readings) were recorded in virus-infected and virus-free shoots. Results showed that duration of high temperatures exposure caused shoot-tip necrosis decreasing shoot-tip survival. Meristem hyperhydricity showed two different trends related to length of treatment, increasing up to 20 d and decreasing up to 70 d applying heat treatments at constant and alternating cyclic temperatures, respectively. Treatment at constant 38°C for 5–15 d led to 100% eradication of ACLSV and ASPV, while all treated plants were still infected with ApMV. The alternating cycles of 32/38°C treatment was effective in eliminating ApMV, as well as ACLSV and ASPV. Virus-free plants had 3.9-fold growth in comparison with virus-infected plants. In addition, SPAD-502 meter readings (total chlorophyll index) showed significant differences between virus-infected and virus-free plants. Suggesting that rapid assessment of physiological, growth and sanitary state of apple shoots after thermotherapy treatments, should be performed by chlorophyll content evaluation using SPAD-502 meter. Higher SPAD-502 meter readings in apple virus-free shoots correlated with better growth and reduction of shoot-tip necrosis and hyperhydricity, confirming that photosynthetic rate is affected by virus infection via decreasing leaf chlorophyll content.

Keywords
in vitro culture, shoot-tip necrosis, hyperhydricity, RT-PCR, healthy plant

Significance of this study

What is already known on this subject?

- Thermotherapy *in vitro* treatments are the most common method for virus elimination in plant species. However, shoot-tip necrosis and hyperhydricity are the most common problems associated to these thermotherapy-treated shoots, causing important loss of virus-free plants.

What are the new findings?

- This work describes the development of a rapid protocol to assess the sanitary and physiological state of thermotherapy-treated apple shoots through chlorophyll content evaluation by using SPAD-502 meter readings.

What is the expected impact on horticulture?

- The obtained results will allow the development of a rapid and routine-applied methodology for evaluation of physiological and sanitary state of thermotherapy-treated apple shoots to optimize *in vitro* protocols for virus elimination in infected apple trees.

Introduction

Apple chlorotic leaf spot virus (ACLSV, *Trichovirus* genus) and *Apple stem pitting virus* (ASPV, *Foveavirus* genus) belong to the *Betaflexiviridae* family and *Apple mosaic virus* (ApMV, *Ilarvirus* genus) to the *Bromoviridae* family. They are widely reported on apple (*Malus domestica* L. Borkh.) trees in different areas of Iran (Abtahi et al., 2013, 2017; Keshavarz and Shams-Bakhsh, 2015; Alemzadeh et al., 2016). ACLSV-infected pome fruit trees have low fruit-yield, express malformed leaves with necrotic lesions, but may be symptomless in some infected trees (Keshavarz and Shams-Bakhsh, 2015). Abtahi et al. (2013) presented the first report of ASPV in 5.12% of surveyed apple trees from different areas in Iran. All of the ASPV-infected 'Gala' apple trees showed mottled and malformed leaf symptoms. ApMV usually induces leaf yellowing, leaf necrotic spots at light infection and fruit deformation at severe infection. There are several reports indicating the importance of these pathogens in causing major crop losses in pome fruits (Hadidi et al., 1998) with up to 60% in multi-infection (Campbell, 1963; Posnette et al., 1963; Schmidt, 1972).

Table 1. List of primers used in the identification of virus through a multiplex RT-PCR.

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	269	Cho et al., 2015
	R: GTTTGGAGGGGCTTCCCACT		
NAD5	F: GATGCTTCTTGGGGCTTCTTGTT	181	Menzel et al., 2002
	R: CTCCAGTCACCAACATTGGCATAA		

Virus elimination is one of the main strategies to control systematic viruses in pome fruit trees and subsequently to guarantee sustainable agriculture. Production of virus-free plants is crucial for the successful vegetative propagation of fruit trees. Most plant viruses are normally transmitted by vegetative propagation such as grafting, and after infection they cannot be controlled by using agricultural pesticides (Barba, 1998), so establishment of healthy pre-basic stock plants is necessary to control production losses. Therefore, virus elimination strategy is the main avenue for controlling systematic virus infections and to subsequently guarantee sustainable agriculture (Barba et al., 2015).

Meristem culture eliminated ApMV and ASPV (Bhardwaj et al., 1998; Wang et al., 2016) in apple cultivars at low rate. However, treatment of infected shoots with *in vitro* thermotherapy have increased the efficiency of ACLSV, ASPV, ASGV (*Apple stem grooving virus*) and ApMV elimination in *Malus* and *Pyrus* species (Zilka et al., 2002; Cieslinska, 2002; Wang et al., 2006; Tan et al., 2010; Hu et al., 2012, 2015; Lizarraga et al., 2017). The latest reports indicate that applying thermal cycles between 32 and 42°C according were an effective thermotherapy treatment for apple and pear (Knapp et al., 1995; Zilka et al., 2002; Tan et al., 2010). In addition to heat treatment regime and size of meristem, it has noted that virus × genotype interaction, virus concentration in the initial plant, as well as the number of viruses infecting the plant also affect the success of heat treatment (Knapp et al., 1995; Da Camara Machado et al., 1998; Paprstein et al., 2008).

Virus elimination occurs when the elevated temperatures alter the balance between newly formed viruses and degraded particles in favour of the virus degradation (Kassanis, 1957; Cooper and Walker, 1978) because of a detachment of bonds from virus capsid proteins followed by the occurrence of other intercellular events that result in competition between viral RNA and mRNA for ribosome bonds. However, shoot-tip necrosis and hyperhydricity are common problems in these thermotherapy-treated plants, causing an important loss of these virus-free plants (Hu et al., 2012, 2015).

This study aims to assay a rapid assessment methodology of sanitary and physiological state. The method used is to evaluate shoots treated with different heat thermotherapy methods, and analyse them for chlorophyll content by using SPAD-502 meter readings. The intention is to find a fast method to analyse *in vitro* elimination of viruses in infected apple.

Materials and methods

Plant materials

Apple buds of the Iranian cultivar 'Abbasi' collected from an apple germplasm collection orchard (58.54N, 25.36E; alt. 1,380 m a.s.l.) of Agricultural and Natural Resources Research and Education Centre of Shahrood, Semnan Province, Iran, were examined. Following disinfestation, buds were individually numbered and tracked through proliferation for the detection of ACLSV, ASPV, ASGV and ApMV from the derived shoots.

Thermotherapy treatments

Infected shoots (3.0 cm) were cultured in MS medium (Murashige and Skoog, 1962) supplemented with 0.5 mg L^{-1} benzylaminopurine (BAP). Two thermotherapy methods were performed: A.) Constant temperature 38°C, 16/8 h light/dark photoperiod. Meristems (0.5 mm) of these shoots were dissected after incubation of 5, 10, 15 and 20 d. B.) Alternating 4-hr. cycles of 32/38°C, also on a 16/8 h light/dark photoperiod, following a temperature ramp-up period of 1 d at 28°C and 2 d at 30°C. Meristems (0.5 mm) were dissected at 55, 60, 65 and 70 d. All of the treated meristems were incubated on modified MS medium with 0.5 mg L^{-1} BAP, 0.05 mg L^{-1} IBA, 0.5 mg L^{-1} GA3, 20 g L^{-1} sucrose, and 8 g L^{-1} agar.

Evaluation of sanitary state trough RT-PCR

After five months of meristem incubation, 100 mg of leaves were collected and immediately stored in liquid nitrogen. Macerated leaves after smashing were used for RNA extraction by Qiagen RNA isolation kit (RNeasy Mini Kit). Specific primers of ACLSV, ASPV, ASGV, ApMV and *NAD-5* (internal control) were used for RT-PCR detection (Table 1). A multiplex RT-PCR was optimized according to Menzel et al. (2002) with modifications. AccuPower CycleScript RT PreMix (Bioneer) and AccuPower PCR Premix (Bioneer) tubes were used for RT and PCR. Cycling conditions were as follows: 95°C for 4 min, followed by 40 cycles of 94°C for 30 s, 52°C for 1 min, 72°C for 1.20 min and a final extension step at 72°C for 10 min. PCR products separated by electrophoresis in 1.5% agarose gels in TAE buffer, stained with GelRed™, and visualized under UV-light. In addition, in order to determinate suitable time for virus detection, we evaluated the presence of ACLSV by using RT-PCR analysis of *in vitro* plants after five and eight months of meristem culture.

Physiological and chlorophyll content evaluation

Shoot-tip necrosis, successful meristem establishment, and incidence of hyperhydricity were evaluated in all the assayed treatments. Each experiment was performed in separate completely randomized design (CRD) with three replications (4 plants for each replication). A total of 40 rooted micropropagated plants were evaluated in each thermotherapy

treatment. Plant length was recorded for virus-infected and virus-free pot plants after five months in greenhouse with 20 replicates of the total micropropagated plants evaluated. Finally, we recorded chlorophyll content using a Minolta SPAD-502 meter. The SPAD-502 meter is a non-destructive measuring device initially developed for the chlorophyll content of leaves widely used to optimise the timing and quantity of fertiliser to improve crop yield. Chlorophyll content is in general one indicator of plant health also affected by other physiological factors including growth regulators, photorespiration, oxidative stress, etc. SAS (SAS Institute Inc. 1989, v. 9.4) was used for statistical analysis of physiological and SPAD-502 measurements, and means were compared based on LSD test at 5% probability level.

Results

Efficiency of virus eradication by thermotherapy methods

Initially apple cv. 'Abbasi' shoots were infected with ACLSV, ASPV and ApMV. The efficiency of two of the thermotherapy procedures for virus elimination are given in Table 2. The RT-PCR results for virus detection showed that previously infected plants under constant 38°C temperature for 5, 10 and 15 d were free of ACLSV and ASPV, but still infected with ApMV. In contrast, applying 4 h of cycles 32/38°C, all plants at 55 d were still infected, 50% of the meristems were ACLSV-free after 60 d, 100% ACLSV-free plants after 65 and 70 d, and all plants were ASPV- and ApMV-free after 60 d. RT-PCR results for ACLSV after five and eight months of meristem culture by two thermotherapy procedures confirmed the absence of this virus in the shoots after five months of meristem culture. After eight months, ACLSV bands were however detected in some samples, indicating positive shoots not detected previously. For this reason, we have to wait until the eight months to be sure of the effect of the thermotherapy.

The effect of thermotherapy on shoot tip necrosis, meristem establishment and hyperhydricity

At constant temperature (38°C) treatment we obtained 53.33% meristem establishment after 10 d but at 15 d meristem establishment success dropped to 13.33%. With therapy method B. (4-h alternating cycles of 32/38°C), we achieved 60% successful meristem establishment at 55 d. At constant temperature (38°C) treatment (A), shoot-tip necrosis, meristem establishment and hyperhydricity were significantly ($p \leq 0.01$) affected by thermo-duration. Shoot-tip necrosis increased from day 5 (0%) to day 20 (60.99%), while the percentage of successful meristem establishment decreased from 53.33% to 0%, respectively. Meristems derived from apple shoots after 15 and 20 d of thermotherapy treatments showed 13.33% of hyperhydricity symptoms, evidencing the negative effect of thermotherapy in the *in vitro* culture of these tissues (Figure 1).

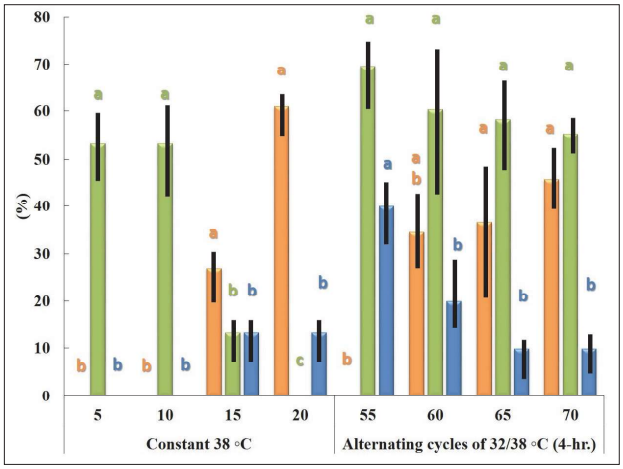

Figure 1. Shoot-tip necrosis (orange bars), meristem establishment (green bars) and meristem hyperhydricity (blue bars), in % of number of plants tested, after different days of thermotherapy procedures for *Malus domestica* Borkh. cv. 'Abbasi'. Mean differences were statistically compared using LSD, $p \leq 0.05$. Values with different letters showed significant differences. Black vertical lines indicate the standard deviations.

On the other hand, significant effects ($p \leq 0.01$) on shoot-tip necrosis, meristem establishment and hyperhydricity were also observed in treatment B (4-h, 32/38°C alternating cycles of temperature). Shoot-tip necrosis and meristem establishment showed opposite results in relation to alternating temperature. Shoots at this method survived up to the highest number of days of the experiment (70 d), and shoot proliferation was observed during thermotherapy. The highest incidence of shoot-tip necrosis (45.66%) was seen in shoots after 70 d, while the highest meristem establishment (69.66%) and hyperhydricity (40%) was observed in meristems extracted from shoots after 55 d of heat therapy (Figure 1). In contrast to the therapy method A, hyperhydricity declined with the passing of time in treatment B.

SPAD-502 meter readings

SPAD readings of total chlorophyll content in treatment were significantly different for virus-infected *vs.* virus-free plants (Figure 2). Total chlorophyll content in untreated

Table 2. Efficiency of thermotherapy at constant 38°C and alternating cycles of 32/38°C (4 h) on virus eradication from *Malus domestica* Borkh. 'Abbasi' using RT-PCR.

Thermotherapy procedure	Days in thermotherapy	Number of virus-free/tested plants		
		ACLSV	ASPV	ApMV
38°C	5	2/2	2/2	0/2
	10	2/2	2/2	0/2
	15	2/2	2/2	0/2
	20	–	–	–
32/38°C	55	0/2	0/2	2/2
	60	1/2	1/2	2/2
	65	2/2	2/2	2/2
	70	2/2	2/2	2/2

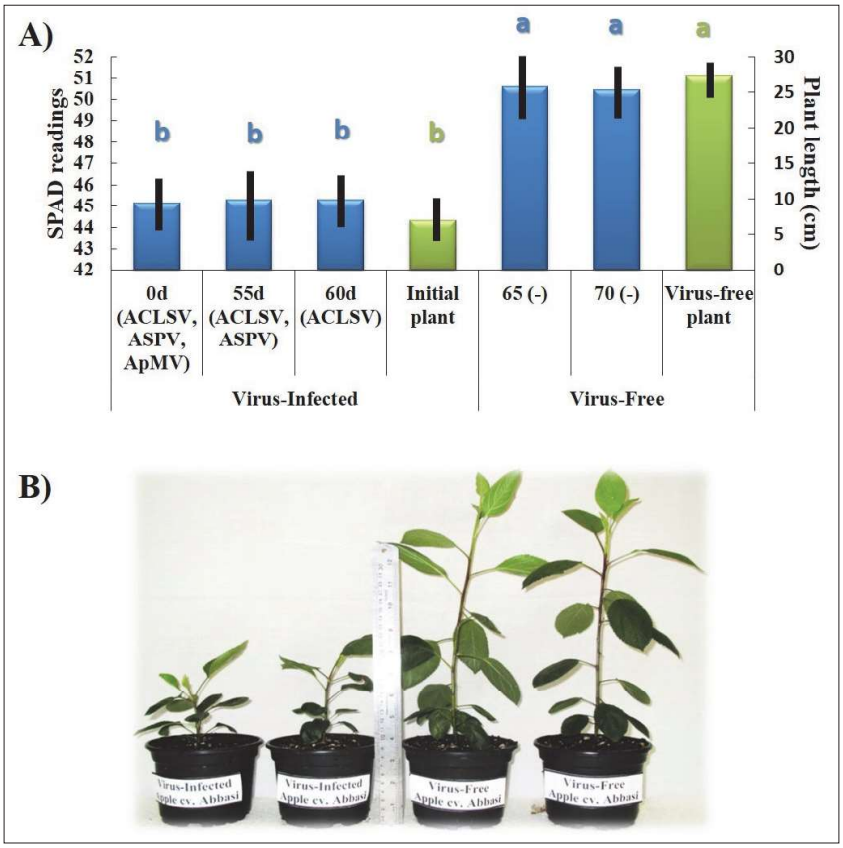

Figure 2. A) SPAD readings (blue bars) after different days of thermotherapy of alternating cycles of 32/38°C (4 h) for *Malus domestica* Borkh. cv. 'Abbasi' and plant length (green bars) in initial plants and virus-free plants. Mean differences were statistically compared using LSD, $p \leq 0.05$. Values with different letters showed significant differences. Black vertical lines indicate the standard deviations. B) Virus-infected and virus-free plants after five months adaptation in greenhouse.

plants and infected plants was lower than ACLSV-free plants derived from 65 and 70 d. After five months of greenhouse adaptation the virus-free apple cv. 'Abbasi' plants (27.25 cm) showed 3.9-fold higher growth than plants infected with ASPV, ACLSV and ApMV (7 cm). The 3.9-fold increase in growth of virus-free 'Abbasi' plants compared to virus-infected plants confirm that photosynthetic rate is affected by ACLSV via decreasing leaf chlorophyll content (Figure 2).

Discussion

'Abbasi' shoots were infected with ACLSV, ASPV and ApMV, but not ASGV. Naderpour (2013) also reported the non-infection to ASGV in apple cultivars in Iran. ACLSV and ASPV were removed after thermotherapy in 5, 10 and 15 d at constant 38°C, while all plants were still infected with ApMV. Lizarraga et al. (2017) reported that, with high temperature (40°C) and meristem (0.7–1 mm) culture, ApMV could be removed from *Malus* and *Pyrus* cultivars with 100% efficiency. Incubation of meristems smaller than 0.2 mm led to ApMV-free plants of apple 'Tydeman's Early Worcester', but the viability of the plants was less than 50% (Bhardwaj et al., 1998).

Paprstein et al. (2008) also reported 80% eradication of ACLSV and 100% for ASPV for apple 'Idared' plants, but no virus-free plants for 'Sampion' cultivar was reported after 6 d at 39°C. Tan et al. (2010) also obtained pear shoots free of ACLSV and ASPV after 30 d at 37°C, and Wang et al. (2006) obtained 67% ACLSV-free plants of pear cv. 'Huang-hua' after 35 d at 37°C. The duration of heat therapy is an important factor for virus elimination according to the literature (Tan et al., 2010; Hu et al., 2012). In our study, the 4-h, cyclic 32/38°C method required 65 d for complete elimination of ACLSV, ASPV and ApMV. The complete inactivation of these viruses in our study is confirmed by Paprstein et al. (2008).

In addition, Wang et al. (2006) reported that extended thermotherapy (37°C) for more than 35 d decreased ACLSV titer in shoot tips of *Pyrus pyrifolia* 'Huanghua'. After 33 d of thermotherapy (38/36°C, 16/8 h), no positive ACLSV apples were found (Knapp et al., 1995). Zilka et al. (2002) stated that, after 20 d at 42/31°C therapy, 'Bon Rouge' and 'Cascade' pear had no viral diseases symptoms. Two cycles of thermotherapy were compared by Tan et al. (2010) for *Pyrus pyrifolia* 'Fengshui': cycles 42/34°C led to ACLSV- and ASPV-free plants after 45 and 50 d, respectively. In contrast with our results, they reported that cycles of 38/32°C failed to yield virus free plants. This discrepancy may be related to difference in the meristem size (0.5 mm in our *vs.* ≤ 1 mm in their study), and temperature variation (16/8 h *vs.* 4/4) in our *vs.* Tan et al. (2010) studies.

The timing of virus detection after meristem culture is a factor that should be considered, as our results indicated that virus detection after eight months is more reliable than detection after five months, because the presence of virus concentration is more easily detectable then using RT-PCR. Virus detection results of rooted plants are consistent with the results derived from eight months after meristem cultures *in vitro*.

Thermotherapy is a well-known method for inactivation of viral pathogens within plants. According to our results, elimination efficiency and plant survival can be affected by choice of thermotherapy method. Maximizing of plant tolerance to high temperatures and exposure times are critical for treatment success. As with other temperate trees, *in vitro* apple shoots are sensitive to the high temperatures of thermotherapy (Hu et al., 2015), so our study was concerned with the effects of shoot-tip necrosis, meristem establishment and hyperhydricity. Paprstein et al. (2008) also reported 64.5% and 44.4% successful meristems establishment of 'Idared' and 'Sampion' apples, respectively, under heat therapy at 39°C for 10 d.

In agreement with our results, Hu et al. (2015) obtained no surviving apple shoots after 20 d of thermotherapy at 38°C. High temperatures promote free radical production and evidence has shown that increasingly high temperatures are highly injurious, causing structural breakdown, necrosis and death (Benson, 2000). Thermotherapy heat stress can induce hyperhydricity in apple shoots. It appears that the different results for hyperhydricity between the thermotherapy using constant or alternating temperature, can be explained by that constant high temperature exposure for more than 15 d prevents shoots survival, since the plants do not overcome an imbalance between photosynthesis and transpiration. Whereas in the 4-h cyclic 32/38°C method, shoots have sufficient time to adapt (during the lower temperature) and after 60 d, they do not change significantly. Heat shock (42°C) led to an accumulation of H_2O_2 in wheat leaves (Ranjeet et al., 2012) and the increasing level of H_2O_2 in leaves caused hyperhydricity (Wu et al., 2009) via oxidative stress. It is probable that the closure of stomata that was induced by elevated temperature led to a disruption in apoplastic transport causing hyperhydricity (Dries et al., 2013).

Another subject of our attention is "Somatic Stress Memory" (SSM) which is contrary to most transitory responses (e.g., chromatin features) in relation to abiotic and biotic stresses, and in some cases SSM may be accrued for short periods of time even after the removal of a particular stress. This was noted where 3 and 7 d of memory duration was reported following heat shock (Lämke et al., 2016; Brzezinka et al., 2016; Singh et al., 2014). It appears that this may be a period of "reminding" that helps meristems establish better.

Chlorophyll content evaluation showed that the total leaf chlorophyll in ACLSV-free plants was higher than in infected plants. It has been previously shown that chlorophyll content, i.e., the number, size and shape of chlorophyll molecules, and inner membrane structure can be altered in the presence of viral infection (Tu et al., 1967; Goodman et al., 1986). Moreover, chloroplast degradation was reported as a result of synthesis reduction of chloroplast-associated proteins, abnormalities in chloroplast structure and function, chlorophyll–protein complexes reduction, and inhibition of photosystem II (PSII) activity in virus-infected plants (Almasi et al., 2000; Koiwa et al., 1992). Complex linking of virus-host plant physiological process led to changes in ultrastructure of chloroplast such as their shape, inner membrane structure as well as the number, size or chlorophyll content of the chloroplasts (Tu et al., 1967; Goodman et al., 1986). These changes seem to be related to the acceleration of senescence in host plants with a chlorophyll biosynthesis inhibition (Almasi et al., 2000). The other important impact effects induced by viruses include changes in the chlorophyll protein complexes and chloroplast proteins, photosynthetic electron transport, CO_2-fixation and on some biosynthetic processes (Almasi et al., 2001).

In addition, the growth intensity of a plant reflected in the increase of SPAD readings is not only affected by chlorophyll content but several other physiological factors (growth regulators, photorespiration, oxidative stress, etc.) might be also responsible for such an effect. These factors also indicated the better physiological state of the plants.

The obtained results show the suitability of chlorophyll content evaluation by using SPAD-502 meter as a rapid method for evaluation of thermotherapy-treated apple shoots, to optimize *in vitro* protocols for virus elimination in infected apple trees. In agreement with this finding, image analyses of leaves in fruit trees are an efficient routine method for genotype classification, and are applied for that purpose (Borraz-Martínez et al., 2019).

Conclusions

At *in vitro* conditions, thermotherapy treatments are the most common method for virus elimination. However, shoot-tip necrosis and hyperhydricity are common problems in these thermotherapy-treated shoots, causing an important loss of virus-free plants. This work describes the development of a rapid methodology to assess the sanitary and physiological state of thermotherapy-treated apple shoots through chlorophyll content evaluation using SPAD-502 meter readings. In the apple assayed shoots, high temperature exposition caused shoot-tip necrosis and decreasing shoot-tip survival. Meristem hyperhydricity, however, showed two different trends related to length of thermotherapy treatments, increasing up to 20 d at constant temperature and decreasing up to 70 d when heat treatments were applied at constant and alternating cyclic temperatures. The alternating cycles of 32/38°C treatment was effective in eliminating ApMV, as well as ACLSV and ASPV. In addition, virus-free plants had a 3.9-fold growth compared to virus-infected plants. A rapid assessment of physiology, growth and sanitary state of apple shoots after thermotherapy treatments should be performed by chlorophyll content evaluation using SPAD-502 meter. Higher SPAD-502 meter readings in apple virus-free shoots correlated with a better growth and a reduction of shoot-tip necrosis and hyperhydricity, confirming that photosynthetic rate is affected by virus infection via decreasing leaf chlorophyll content. The obtained results will allow the development of a rapid and routine-applied method for evaluation of thermotherapy-treated apple shoots to optimize *in vitro* protocols for virus elimination in infected apple trees.

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