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ELIMINATION OF APPLE LATENT VIRUSES BY *IN VITRO* CHEMOTHERAPY AND MERISTEM-CULTURING

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INTRODUCTION

Apples trees are infected by a large number of viruses. Latent viruses such as *Apple stem grooving virus* (ASGV), *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem pitting virus* (ASPV) are widespread and economically outstanding, causing yield losses and increased predisposition of apple plants to attack by other pathogens (GUERRA et al., 2012). Traditionally *in vivo* thermotherapy and meristem culture has been used with considerable success for apple virus elimination. However it is a labor-intensive and costly procedure. Restrictions are limited place, absence of sterility conditions, occurrence of pests and diseases and the long time required. Meristem culture combined with heat therapy may reduce virus titers, but often fails due to invasion of meristematic tissues by viruses (WANG et al., 2008). Chemotherapy, as a systemic, invasive method, could offer an alternative. Ribavirin, a nitrogenated adenosine or guanosine analog, pairing with cytosine and uracil may induce hypermutations, inactivate viruses, and inhibit several processes of viral RNA synthesis (PANATTONI et al., 2007). Ribavirin has been reported as an efficient agent for elimination of latent apple viruses (JAMES et al., 1997; CIESLINSKA, 2002). This work evaluates its efficacy alone and combined with meristem culture for elimination of apple viruses from commercial apple cultivars.

MATERIAL AND METHODS

The apple cultivars used were Royal Gala, Cripps Pink, Castel Gala and Fuji Select. Explants from plants *in vitro*, were transferred to MS medium with 1 $mg.L^{-1}$ de benzilamino-purina (BAP), 30 $g.L^{-1}$ sucrose and agar (MS/BAP) containing ribavirin (1, 5, 7.5 and 10 µg/mL); photoperiod of 16:8 hours; 23°C ± 3°C. Plants were treated 12 weeks (experiments I and II) or 8 weeks (experiment III), with a transfer to fresh MS medium after 6 or 4 weeks, respectively and rooted in MS medium. In experiment I, meristems were removed from sprouts growing on medium containing ribavirin and simultaneously from control plantlets cultivated without ribavirin. Treatments consisted of 6 plants per repetition, 3-4 independent repetitions. For RT-PCR leaves from 6 explants of each repetition were pooled. RNA extractions, primer and cycling were as

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reported (NICKEL et al., 2001; RADAELLI et al., 2006). Commercial plant substrate was used for acclimation. Part of the obtained data were analyzed by RT-PCR for residual infections two years after treatment.

RESULTS AND DISCUSSION

Plants regenerated from meristems excised from ribavirin-treated plants were virus-free while those from meristems obtained from plants not previously ribavirin-treated remained virus-infected. That means that the chemical pre-treatment appeared to be decisive for the complete elimination of ACLSV/ASPV from all treated plants of both cultivars (Table 1). It was observed that plants after 12 weeks of ribavirin treatment (WRT) alone were free of single and multiple virus infections. Both cultivars were still virus infected at the transfer to fresh medium at 6 WRT (data not shown). The presented results indicate that the addition of chemotherapy to culture of meristematic apices did not increase virus removal from cvs. Royal Gala and Cripps Pink over the cleaning capacity of the chemical treatment alone. Surprisingly, ASGV, which is considered difficult to be eliminated (WANG et al., 2006), was removed from ASGV-infected cvs. Royal Gala and Cripps Pink after 6 WRT (Table 2). There is experimental evidence that virus elimination from woody fruit plants *in vitro* cultures is highly host/virus-dependent (KNAPP et al., 1995; PUPOLA et al., 2009). Different difficulty degrees to remove certain viruses from plants may be explained also by differing capacities of viruses to invade meristematic tissues (WANG et al., 2008), besides the uneven virus distribution in woody plants (KNAPP et al., 1995).

Table 1 - Elimination of latent apple viruses by chemotherapy, meristem culture and a combination of both methods (Experiment I).

Cultivar	Accession	Virus Infections	Infected/treated plants - 12 weeks ribavirin treatment		
			Meristems	Meristems + ribavirin	Ribavirin
Royal Gala	M073	ACLSV	4/4	0/4	0/3
Cripps Pink	M080	ACLSV/ASPV	2/4	0/4	0/3

Table 2 - Elimination of latent apple viruses by ribavirin treatments (Experiment II)

Cultivars	Accession Number	Virus infections	Infected plants/ treated plants								
			6 weeks/ribavirin µg/mL					12 weeks/ribavirin µg/mL			
			1	5	7.5	10	0 [a]	1	5	7.5	10
Royal Gala	M053	ASGV	-	0/4	0/4	0/4	4/4	-	0/4	0/4	0/4
Castel Gala	M193	ACLSV/ASGV/ASPV	-	4/4	4/4	4/4	4/4	-	0/4	0/4	1/4
Cripps Pink	M075	ASGV	-	0/4	0/4	0/4	4/4	-	0/4	0/4	0/4
Fuji Select	M184	ASPV	-	4/4	4/4	4/4	4/4	-	1/4	1/3	1/3

(-) not tested; [a]greenhouse controls.

The attempt to reduce the duration of the treatment with the antiviral compound to 8 weeks still produced 50 to 100% virus free plants immediately after treatment (Table 3). However, when these

plants were retested two years later, 23% and 74%, respectively of Castel Gala and Royal Gala plants propagated from treated plants were virus-infected (data not shown). This observation is relevant in the frame of plant certification programs, showing that plants from virus elimination procedures require retesting for long periods at regular intervals after the virus removal treatments.

Table 3 - Elimination of latent apple viruses by ribavirin treatments (Experiment III)

Cultivars	Accession Number	Virus infections	Infected plants/ treated plants								
			4 weeks/ribavirin µg/mL				8 weeks/ribavirin µg/mL				
			1	5	7,5	10	0	1	5	7,5	10
Royal Gala	M053	ASGV	-	-	-	-	2/4[a]	4/4	0/4	-	0/4
Castel Gala	M193	ACLSV/ASGV/ASPV	-	-	-	-	4/4	2/4	1/4	-	2/4
Cripps	M080	ACLSV/ASPV	-	-	-	-	4/4	3/4	2/4	-	2/4

(-) not tested; [a]greenhouse controls.

Knapp et al. (1995) report that while only 8.3% of apple plants regenerated from meristems were clearly ASGV-positive immediately after treatment, after 6 months over 60% of plants retested were virus-positive. Also antiviral chemicals such as ribavirin, quercetin and glycyrrhizin may show just a virus titer depressing effect. James et al. (1997) observed that while the ribavirin treatment of *Malus* spp. resulted, individually, in 67.5% of ASGV elimination, all plants treated were free of ASGV after its combination with quercetin. It was observed that some ribavirin-untreated *in vitro* control plants of cultivar Royal Gala were virus-free (Table 3). Despite repeated testing no viruses could be detected in these plants, although their mother plants kept in a greenhouse reacted consistently positive. Hansen and Lane (1985) reported spontaneous loss of ACLSV from apples *in vitro*. Knapp et al. (1995) observed that ASGV was not lost after 4 years *in vitro* culture from apples but ApMV was lost after two and a half years. Quak (1977) reported that meristems in which viruses were detected, developed into virus-free plants. Overall number of plants obtained free of viruses in this study varied from 25 to 100% of treated plants.

CONCLUSIONS

Ribavirin treatment of plants before removal of meristems did not increase virus removal over the cleaning capacity of the chemical treatment alone. Ribavirin treatments allowed a workable degree of virus elimination efficiency of latent viruses from several apple cultivars. The observed longterm virus freedom of regenerated plants is of particular interest for plant certification programs. No phytotoxic or other durable, deleterious effects caused by ribavirin were observed. The procedure is simple to perform and may be combined with other virus elimination methods when required for specific host-virus pathosystems.

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