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Short communication

Elimination of a new ampelovirus (GLRaV-Pr) and *Grapevine rupestris stem pitting associated virus* (GRSPaV) from two *Vitis vinifera* cultivars combining *in vitro* thermotherapy with shoot tip culture

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ABSTRACT

A new virus species designated as *Grapevine leafroll associated virus*-Pr (GLRaV-Pr), which is classified in a distinct phylogenetic group of the genus *Ampelovirus* (*Closteroviridae*), was recently characterized from Greek grapevine cultivars. Elimination studies of GLRaV-Pr were carried out in two grapevine cultivars, 'Mantilaria' and 'Prevezaniko', co-infected with *Grapevine rupestris stem pitting associated virus* (GRSPaV, *Flexiviridae*). Both viruses were detected by nested RT-PCR assays. Virus elimination was achieved by combining *in vitro* thermotherapy with meristem (≤0.2 mm) or shoot tip culture (≤0.5 cm). The survival and regeneration rate of meristems was very low. On the other hand, high survival rates were observed in the cultured shoot tips accompanied with high elimination rates for both viruses. Data obtained in this study indicate that virus elimination depends on the genotype of grapevine. The results confirmed that sanitation is easier for species of the *Closteroviridae* family than for GRSPaV, whereas it seems that eradication of GLRaV-Pr and GRSPaV is feasible even with larger plant tissue parts if combined with an appropriate thermotherapy profile *in vitro*.

1. Introduction

The use of healthy propagating material is an important measure for the control of grapevine viruses. Among the most widely applied methods for virus elimination from infected grapevine clones is meristem or shoot tip culture, often combined with thermotherapy (Milkus et al., 2000). The outcome of virus eradication depends on the type and number of virus species that exist in a certain grapevine variety, the cultivar and the protocol used. Meristem culture and somatic embryogenesis have been proven to be the most effective procedures for eradication of phloem-associated viruses such as those of the family *Closteroviridae* (Goussard et al., 1991; Gribaudo et al., 1997; Popescu et al., 2003; Gambino et al., 2006). The combination of thermotherapy with *in vitro* meristem or shoot tip culture was successfully applied for the elimination of viruses that are located on parenchymatic cells and enter meristematic regions just like nepoviruses (Milkus et al., 2000; Valero et al., 2003).

Recently a newly identified *Ampelovirus* species, *Grapevine leafroll associated virus*-Pr (GLRaV-Pr), isolated from Greek grapevine cultivars, has been characterized. This virus along with GLRaV-4, -5, -6 and GLRaV-9 are classified in a distinct phylogenetic group (subgroup I) within the genus *Ampelovirus* (Maliogka et al., 2008a). To our knowledge there are no studies concerning the grapevine sanitation from subgroup I ampeloviruses. Furthermore, little is known about the efficacy of different techniques on the eradication of *Grapevine rupestris stem pitting associated virus* (GRSPaV) (Gribaudo et al., 2006; Gambino et al., 2006), a foveavirus associated with rugose wood syndrome of grapevine (Zhang et al., 1998). The sanitation of propagating material from GRSPaV applying meristem tip culture or *in vivo* and *in vitro* thermotherapy is particularly difficult (Minafra and Boscia, 2003; Gribaudo et al., 2006; Skiada et al., 2009). This is probably related to the ability of the virus to readily invade meristem cells (Rowhani et al., 2000). Somatic embryogenesis represents so far the most promising method for the eradication of GRSPaV (Gambino et al., 2006; Gribaudo et al., 2006). However, it is technically difficult, enables the risk of somaclonal variation, time-demanding and much more genotype-dependent compared to other techniques.

The target of this study was the development of a simple and effective protocol for the elimination of GLRaV-Pr and GRSPaV from the grapevine cultivars 'Mantilaria' and 'Prevezaniko'. To achieve this, *in vitro* thermotherapy combined with meristem and shoot tip culture was applied.

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2. Materials and methods

2.1. Plant material and establishment of in vitro cultures

In vitro cultures were initiated from two Greek cultivars of *Vitis vinifera* L., 'Mantilaria' and 'Prevezaniko' originating from the collection of the private company Vitro Hellas S.A. 'Mantilaria' is a red cultivar collected from the area of Irakleio (Island of Crete), whereas 'Prevezaniko' is a pink one originating from Zitsa (Epirus, north-western Greece). The mother plants were infected with GLRaV-Pr and GRSPaV as was revealed by nested RT-PCR assays (Dovas and Katis, 2003; Maliogka et al., 2008b).

Actively growing shoots from infected grapevine plants sprouting in laboratory conditions were stripped of leaves and washed with tap water. Single node segments were disinfected using 2% (w/v) NaOCl for 15 min with three drops of Tween 20, shaken on a rotary shaker and rinsed three times with sterile distilled water. Explants were cultured in 0.5 QL–MS (Quoirin and Lepoivre, 1977; Murashige and Skoog, 1962; Skiada et al., 2009) medium supplemented with 0.3 μM BA (6-benzyladenine), 0.005 μM NAA (α-naphthaleneacetic acid), 3% (w/v) sucrose and 0.6% agar (B&V S.r.L., type S 1000), while pH was adjusted to 5.75 prior to autoclaving. Cultures were kept at 22 ± 2 °C and 16 h photoperiod under cool white fluorescent light (40 μmol m^{-2} s^{-1}). Forty days later the explants were transferred to a new proliferation medium composed of QL–MS (Skiada et al., 2009) supplemented with 0.3 μM BA and 0.005 μM NAA, pH 5.9 in the case of 'Prevezaniko' and a QL supplemented with 0.01 μM BA and 0.01 μM NAA, pH 5.9 for 'Mantilaria'. Subcultures were carried out every 6 weeks.

2.2. Thermotherapy

Heat treatment was carried out in *in vitro* cultures in a heat chamber (Percival Scientific, I-36LLVL) for 6 weeks. The initial temperature was 26 ± 0.5 °C for the day and 23 ± 0.5 °C for the night and it was gradually increased by 3° per week. The final temperatures, which were retained for 1 week, were 40 ± 0.5 °C (day) and 37 ± 0.5 °C (night). The procedure was repeated twice and a total number of 160 explants originating from 'Mantilaria' and 172 from 'Prevezaniko' were used.

When the heat treatment period ended, meristems (0.1–0.2 mm) and shoot tips (0.5 cm) were excised from the apical bud of the plantlets. Meristems of both cultivars were cultured in 0.5 MS medium, while shoot tips were established in the appropriate medium for each cultivar. After 6–8 weeks the plantlets derived from meristems were 2 cm or more in length and were transferred in the proliferation medium of each cultivar.

2.3. Nested RT-PCR detection of GLRaV-Pr and GRSPaV

Regenerated shootlets were tested for virus presence approximately 40 days and 12 weeks after the transfer of shoot tips and meristems, respectively, from the heat treated plantlets. The detection of GLRaV-Pr was based on a nested RT-PCR assay that was recently developed (Maliogka et al., 2008b), while for the GRSPaV diagnosis a nested RT-PCR for the generic detection of foveaviruses was applied (Dovas and Katis, 2003). For this purpose, total RNA was extracted from 0.2 g of grapevine explants (stems, petioles and leaves) according to a method described previously (Rott and Jelkmann, 2001), modified by adding 6% PVP and 0.2 M β-mercaptoethanol in the grinding buffer. The tests were repeated after 6 and 12 months.

2.4. Transfer to ex vitro conditions and repeated testing

In vitro clones that were found free of GLRaV-Pr and GRSPaV were transferred to *ex vitro* conditions. The plantlets were placed in a greenhouse, under a 90% relative humidity (RH) fog-system and 50% shading for 10 days. The next 20 days RH was reduced (5%/day), while light intensity was gradually increasing. Three months later the acclimated plants were transferred to pots and cultivated in greenhouse conditions. Tests for virus presence were repeated every 6 months for over a year using samples of stems coming from the basal leaves.

3. Results and discussion

In this study we evaluated protocols for the eradication of the newly assigned subgroup I *Ampelovirus* species (GLRaV-Pr) (Maliogka et al., 2008a) and GRSPaV from two grapevine cultivars. The combination of *in vitro* thermotherapy with shoot tip culture was effective for the elimination of both viruses. A percentage of 59.37% of 'Mantilaria' and 41.86% of 'Prevezaniko' explants that were subjected to thermotherapy survived the heat stress (Table 1). The majority of the survived plantlets showed no shoot tip or other kind of necrosis. Of the explants originating from 'Mantilaria' and 'Prevezaniko' shoot tips, 96.36 and 77.77%, respectively, survived and regenerated (Table 1). For the 'Mantilaria' cultivar, 92.45 and 39.62% of the shoot tips were GLRaV-Pr and GRSPaV-free, respectively; overall, both viruses were eradicated from 37.73% of the regenerated plantlets. As far as 'Prevezaniko' is concerned, 89.29% of the plantlets originating from shoot tips were GLRaV-Pr-free, 92.85% were GRSPaV-free and 89.29% were free from both viruses. These results were consistently confirmed by all the nested RT-PCR assays conducted both in *in vitro* and *ex vitro* conditions.

The experiments reported here revealed that virus elimination from 'Prevezaniko' was easier. It seems that the success of thermotherapy depends not only on the virus species involved but also on the specific interaction between pathogen and grapevine genotype. Even though efforts for virus elimination were also made using the apical meristem region, the survival rates of the explants were extremely low (5% for 'Mantilaria', 0% for 'Prevezaniko'). Interestingly, both of the 'Mantilaria' plantlets that survived and regenerated (Table 1) were found to be infected with GRSPaV, thus indicating the difficulty to eradicate this virus. The apical meristems are usually preferred because they are more likely to be virus-free, especially from phloem-limited viruses which do not enter these regions (Gambino et al., 2006). In our study the eradication of GLRaV-Pr and GRSPaV using larger parts of

Table 1
Survival rates and virus elimination post-thermotherapy.

Cultivar	Number of survived explants post-thermotherapy	Meristems				Shoot tips			
		Regenerated plantlets from meristems	Virus elimination			Regenerated plantlets from shoot tips	Virus elimination		
			GLRaV-Pr	GRSPaV	GLRaV-Pr + GRSPaV		GLRaV-Pr	GRSPaV	GLRaV-Pr + GRSPaV
Mantilaria	95/160 (59.37%)	2/40 (5%)	2/2	0/2	0/2	53/55 (96.36%)	49/53 (92.45%)	21/53 (39.62%)	20/53 (37.73%)
Prevezaniko	72/172 (41.86%)	0/36 (0%)	–	–	–	28/36 (77.77%)	25/28 (89.29%)	26/28 (92.85%)	25/28 (89.29%)

plant tissue is attributed to the combined application of *in vitro* thermotherapy. Long duration of the thermotherapy (42 days) at high temperatures (up to 40 °C) was selected for elimination of GRSPaV, as this virus is hardly eliminated (Minafra and Boscia, 2003; Gribaudo et al., 2006). In previous studies, in which the eradication rates from GRSPaV were very low, the temperatures applied were much lower (34 °C) (Gribaudo et al., 1997, 2006). The profile of repeated cycles with high and low temperatures (day-night) that was applied here seems to be crucial for the eradication of GRSPaV confirming previous elimination study of the virus that was conducted on a different grapevine cultivar (Skiada et al., 2009).

It has been reported that heat treatment hampers virus replication and enhances its disorganization, thus leading to the eradication of the virus from the shoot tips (Cooper and Walkey, 1978). Recently, it was shown that viral RNA of *Raspberry bushy dwarf virus* (RBDV), a virus that enters meristem tissues, was disorganized in leaves and shoot tips in plants growing at high temperatures (38 °C) (Wang et al., 2008). This was attributed to the enhancement of RNA silencing, that is the cellular defence mechanism of plants acting against viral RNA (Wang et al., 2008), a mechanism significantly reinforced in higher temperatures (Chellappan et al., 2005).

The application of reliable and sensitive detection assays is also an important step in the process of plant sanitation. In this study, diagnosis of GRSPaV was based on a generic nested RT-PCR assay, which shows a high detection range and increased sensitivity (Dovas and Katis, 2003). In addition, the nested RT-PCR which was recently developed for the detection of the novel ampelovirus species (GLRaV-Pr) (Maliogka et al., 2008b) was successfully applied for testing plantlets of the two grapevine cultivars studied. In both cases, the nested PCR step offers increased specificity and sensitivity compared to a simple PCR assay.

Our results confirmed that virus elimination in grapevine is easier on virus species of the *Closteroviridae* family than on GRSPaV (Minafra and Boscia, 2003; Gribaudo et al., 2006) and eradication of both GLRaV-Pr and GRSPaV is feasible even with larger plant tissue parts if combined with *in vitro* thermotherapy. The same conclusion was recently reached with the grapevine cultivar 'Agiorgitiko' (Skiada et al., 2009). This is particularly important since the application of a proper temperature profile in combination with the culture of a large number of shoot tips can lead to an adequate number of healthy plantlets, thus avoiding the problems of meristem culture even for difficult to eradicate viruses such as GRSPaV.

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