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Grapevine vitivirus A eradication in *Vitis vinifera* explants by antiviral drugs and thermotherapy

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Abstract

Grapevine shoot cultures infected by *Grapevine vitivirus* A (GVA) were grown on Quorin–Lepoivre basic medium and submitted to *in vitro* chemotherapy and thermotherapy sanitation techniques. Ribavirin (Rb) at 20 g ml^{-1}, dihydroxypropyladenine (DHPA) at 60 g ml^{-1} and their combination (RbDH) were added to the proliferating medium for three subsequent subcultures of 30 days each. Phytotoxicity was observed on drug-treated plantlets, which displayed a high percentage of mortality for each drug at doses higher than those aforementioned. Sequential ELISA were performed at the end of each subculture and ELISA-negative explants were submitted to RT-PCR. ELISA showed no antiviral activity following DHPA administration. Rb and RbDH treatment produced ELISA-negative explants which were assayed by RT-PCR and nested PCR. Biomolecular results showed no virus eradication in Rb treated explants but RbDH administration generated a percentage (40.0%) of GVA-free plantlets that permitted restoration of a new healthy generation of explants.

Sixty percent (60%) of GVA eradication as confirmed by RT-PCR was obtained by *in vitro* thermotherapy at 36 °C for 57 days.

Keywords: Chemotherapy; Dihydroxypropyladenine; Ribavirin; Thermotherapy; *Vitis vinifera* explant; GVA

1. Introduction

The detrimental effects of virus infections on the quantity and quality yield highlight the importance of virus-free propagation material and underline the need to produce virus-free plant material for the growth industry in agriculture, in accordance with the European Union directives for grapevine. Thus a number of virus elimination protocols have been set up, in experimental conditions only, using several different techniques to repair infected plants. So far, success rates have been variable.

The ability to control plant viral disease with chemicals has a great potential for agriculture where viral diseases caused significant economic losses. However, difficulties have been encountered in developing effective drugs that eliminate or substantially reduce replication of phytoviruses. In medical therapy, research into the most rational strategy for the design of drugs against specific viral targets appears as an expanding approach in the search for specific antiviral compounds for viral agents. All the steps included in the viral replication cycles can represent a target for chemotherapeutic intervention; and there are a number of host enzymes involved in viral DNA and RNA synthesis which may be considered targets for antiviral drugs. In one of the reviews on molecular targets for antiviral drugs in medical research, De Clercq (2001) selected eight potential types of enzymes, including inosine monophosphate dehydrogenase (IMPDH) and *S*-adenosilhomocysteine (SAH) hydrolase, as targets for inhibitors with broad-spectrum antiviral activity.

IMPDH inhibitors are some of the most extensively investigated classes. IMP-dehydrogenase is an enzyme which catalyzes the conversion of inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP), in the metabolic branch point of the purine synthetic pathway. By blocking conversion of IMP to XMP, IMPDH inhibitors constitute a mechanism involved in the interruption of DNA and RNA synthesis (Franchetti *et al.*, 1996). Ribavirin (1-β-D-ribofuranosil-1,2,4-triazolo-3-

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carbossamide) (Rb) is the first IMPDH inhibitor drug. Ribavirin is known as a potent chemotherapeutic agent with a multifaceted mode of action, inhibiting both the capping and elongation of viral mRNA. In particular, it prevents guanosine 5′-phosphate pool synthesis and the methylation reaction of the synthesized mRNA cap (Smith, 1984; Lerch, 1987), thereby inhibiting synthesis of viral nucleic acid.

S-Adenosilhomocysteine (SAH) hydrolase is another key enzyme in methylation reactions depending on adenosylmethionine (SAM) as the methyl donor, including those methylations that are required for the maturation of viral mRNAs. SAH hydrolase inhibitors can block those reactions involved in the capping of viral mRNA (Borchardt, 1980; De Clercq, 2004). Dihydroxypropyladenine ((*R*,*S*)-9-(2,3-dihydroxypropyl) adenine) (DHPA) is one of the oldest drugs belonging to this group (De Clercq *et al.*, 1978).

Monette (1983), Stevenson and Monette (1983), Barba *et al.* (1990) and Mainardi (1993) described the experiences of chemotherapy with Rb and DHPA, separately, on *Vitis vinifera* L. explants infected by *Grapevine leafroll associated virus* (GLRaV).

The combination of thermotherapy and shoot tip techniques has been used successfully to eliminate harmful viruses in grape, but meristematic culture regeneration after heat treatment is time-consuming and at times associated with a low success rate. *In vitro* infected explants are generally characterized by a higher virus titre compared with *in vivo* mother plants; thus they represent a rapid and also space-saving technique for obtaining a higher number of infected plantlets, which can then be treated.

Thermotherapy associated with shoot tips on in vitro *V. vinifera* was reported by Monette (1983), Leonhardt *et al.* (1998), Malossini *et al.* (2003) and Panattoni *et al.* (2004). Its efficacy for control of GLRaV and *Grapevine fanleaf nepovirus* (GFLV) varied, with different healing percentages observed in these studies.

In a field monitoring trial in central Italy, an exceedingly high level of infection by different viruses was observed in *V. vinifera* cv Sagrantino, which excluded the detection of healthy plants. *Grapevine vitivirus* A (GVA) was one of the most frequent single infections observed in this cultivar (Materazzi *et al.*, 2004).

GVA is implicated in the aetiology of Kober stem grooving (KSG), one of the four economically important grapevine diseases of the rugose wood complex (Saldarelli *et al.*, 2000) causing severe reduction in growth and yield of affected plants (Garau *et al.*, 1994; Boscia *et al.*, 2001). GVA, which is a type member of the *Vitivirus* genus (Martelli, 1997; Dovas and Katis, 2003) is considered as a phloem-associated virus with filamentous particles of about 800 nm in length, and contains positive sense single-stranded RNA that is capped at the 5′ terminus and polyadenylated at the 3′ end.

Natural sources of resistance to GVA are unknown. Thermotherapy and meristematic cultures of infected grapevine have yielded few promising results (Minafra and Boscia, 2003). Wang *et al.* (2003) obtained GVA eradication in shoot tips during cryopreservation treatment originally designed as a means of long-term storage of *V. vinifera* germoplasm. Recently, Gambino *et al.* (2006) obtained GVA-free explants through somatic embryogenesis. In addition, no chemotherapic treatment has been reported to date on GVA-infected grapevine.

In previous trials treatment with Rb on *Nicotiana benthamiana* explants was effective against GVA (Panattoni *et al.*, 2000) and with Tiazofurin (IMPDH inhibitors) administration on *in vitro V. vinifera* explants infected by GLRaV-3 (Panattoni *et al.*, 2007).

The aim of the present research was to eradicate GVA in *V. vinifera* L. cv Sagrantino explants by chemotherapy with IMPDH, SAH hydrolase inhibitors and their combination, and thermotherapy treatment using molecular assays as reverse transcription-polymerase chain reaction (RT-PCR) and nested PCR for virus identification.

2. Materials and methods

2.1. Sources of in vitro material

In vitro grapevine explants were obtained from field-grown *V. vinifera* cv Sagrantino naturally infected by GVA. Selected plants were transferred to an insect-proof green-house and assayed by enzime-linked immunosorbent assay (ELISA) for 1 year, testing for GLRaV-1 to -8; *Grapevine fleck maculavirus* (GFKV), GFLV, GVA, *Grapevine vitivirus* B (GVB), *Arabic mosaic nepovirus* (ArMV), each test being conducted at the appropriate time for each virus. ELISA was followed by RT-PCR, discarding material with mixed infections in order to use explants characterized by GVA single infection only.

Internodes from non-GVA-infected Sagrantino were used to obtain *in vitro* explants as negative controls for GVA diagnostic tests. Internodes from selected plants were surface sterilized before transfer to culture tubes with fresh Quorin–Lepoivre basic medium (1977). All explants were kept in a controlled environment chamber that assured maintenance of virus-free conditions. Environmental parameters were: temperature regime of $22 \pm 1\,^{\circ}C$, 16 h photoperiod and $50\,Em^{-2}\,s^{-1}$ light intensity provided by cool-white fluorescent tubes (Philips TLD 18 W33) according to Stevenson and Monette (1983) and Barba *et al.* (1990). Explants were transferred to QL modified medium (Bertoni *et al.*, 2000) at 30 days intervals. After an acclimatization period, each plantlet was assayed by ELISA and maintained on fresh medium.

2.2. Antiviral drugs

Dihydroxypropyladenine was kindly provided by Prof. E. De Clercq (Rega Institute, Leuven, Belgium); ribavirin was purchased from Sigma Chemical (Milan, Italy).

Drugs were hydrated in stock solution and, immediately prior to use, ultra-filtered and added to the proliferation medium.

In previous experiments, a preliminary screening of healthy *V. vinifera* cv Sagrantino explants was carried out to determine drug-induced phytotoxicity. Explants were submitted to 30-day administration at four concentrations for each drug (20, 40, 60, $80\,g\,ml^{-1}$). For drug-treatment either alone or in combination $20\,g\,ml^{-1}$ Rb and $60\,g\,ml^{-1}$ DHPA were chosen with very few detrimental effects. To define phytotoxicity levels, the number