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Evidence of non-seed transmission of viruses in grapevine breeding material

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Summary

The vertical transmission of viruses is an important phenomenon affecting a wide range of viruses and host plants. Nevertheless, the presence of virus in a seed does not always lead to seedling infection. In grapevine, seed transmission has been reported for many nepoviruses, but little is known about Leafroll, Rugose wood complex and Fleck diseases. Thus, the aim of this study is to monitor the virological condition of seedlings obtained by crosses between infected parents, analyzing the sanitary status of seedlings after the transfer in experimental fields. It was observed that, although the viral state of parents was quite compromised, viruses were not detected in any of the 150 progeny plants, demonstrating that the main grapevine viruses are at low risk for seed transmission.

K e y w o r d s : grapevine viruses; vertical transmission; multiplex RT-PCR.

Introduction

Seed transmission plays an important role in the dissemination of some viruses. In fact, seed and pollen are the natural pathway through which some plant viruses are transmitted to progeny of hosts (vertical transmission) and spread to environment (Bassi and Martelli 2003, Amari *et al.* 2009). Nevertheless, the presence of virus in a seed, even in an embryo, does not always lead to seedling infection: in many cases, in fact, during metabolic processes associated with germination, the virus is degraded and loses its infectivity (de Assis Filho and Sherwood 2000).

In case of fruit trees, the presence of viruses in many variety collections used as source of breeding material necessitates the development of specific protocols, that exclude or control the presence of agents that might compromise the sanitary status and therefore the spread in the propagation material. Furthermore, it is known that "healthy" plants (in which viruses have not been detected) offer better performance in terms of vegetative growth, lower sensitivity to other diseases, increased productivity and fruit quality (Bassi and Martelli 2003).

In particular, seed transmission in grapevine has been reported for many nepoviruses and specially for *Grapevine fanleaf virus* (Lazar *et al.* 1990), the most widespread and important cause of infectious degeneration disease worldwide. However, *Grapevine rupestris stem pitting associated virus* has been reported to be present in pollen (Rowhani *et al.* 2000) and seeds (Stewart and Nassuth 2001), but it has not been proved to be seed-transmitted. Finally, little is known about the other viral diseases that affect grapevine as Leafroll, Rugose wood complex and Fleck diseases, that are considered by the European legislation (Directive 2005/43/EC of June 23, 2005) on certification of grapevine propagation material, together with infectious degeneration, implemented in Italian law (Decrees of February 2, 2005 and July 7, 2006).

In conclusion, much research has focused on the routes by which seeds become infected and on the progress of viral infection in reproductive organs during their development up until the seedling stage (de Assis Filho and Sherwood 2000, Amari *et al.* 2009). Few researchers have addressed the problem of seed transmission during breeding programs. Thus, the purpose of this study is to monitor virological condition of seedlings obtained by crosses between infected parents, analyzing the sanitary status of seedlings after the transfer in experimental fields.

Material and Methods

In this study, 50 seedlings each from three different crosses of seeded and seedless table grape cultivars 'Almeria' x 'Supernova', 'Ceresa' x 'Carati', 'Red Globe' x 'Regal' were investigated. The grapevine breeding material monitored were collected in the experimental conservation vineyards of the Council for Agricultural Research and Economics (CREA) in Turi (Bari, Italy).

T h e c r o s s e s w e r e d o n e i n s p r i n g o f 2 0 1 0 : emasculation was conducted few days before anthesis, followed by immediate bagging of the inflorescences to avoid contamination with pollen. Artificial pollination was carried out for each cross-combination using the designated male pollen and the emasculated inflorescences were protected by bags until fertilization. Bunches were left on the plant until ripening. At harvesting, the seeds were extracted from

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the berries, washed and stratified in a mixture of soil and sand (2:1) for a period of about 6 months, at a temperature of 4 °C (Janick and Moore 1996). Germination was carried out in May 2011 in polystyrene containers, finally seedlings were transplanted and transferred first in greenhouse and in spring of 2012 in the CREA experimental field of Turi. Before the transfer to the field, the seedlings were analyzed by Marker-Assisted Selection approach (MAS) to verify the supposed paternity and to exclude the possibility of auto-pollination (Bergamini *et al.* 2013).

Total RNA was extracted from phloem scraped from mature canes collected during winter pruning of 2013. Considering the possible uneven distribution of viruses, samples from at least two different canes of the same plant were mixed. All samples were immediately frozen in liquid nitrogen and homogenized. Total RNA was extracted using the Agilent Plant RNA Isolation Mini Kit. RNA purity and concentration were assessed using a NanoDrop 2000 UV-Vis Spectrophotometer.

A multiplex reverse transcription-polymerase chain reaction (mRT-PCR) was performed for simultaneous detection of nine grapevine viruses: *Grapevine leafroll-associated virus-1, -2 and -3* (GLRaV-1, -2, -3)*, Arabis mosaic virus* (ArMV)*, Grapevine fanleaf virus* (GFLV)*, Grapevine virus A* (GVA)*, Grapevine virus B* (GVB)*, Grapevine rupestris stem pitting associated virus* (GRSPaV)*, Grapevine fleck virus* (GFkV)*,* in combination with a plant RNA internal control (18S rRNA) used as an indicator of the effectiveness of RNA extraction and RT-PCR (Gambino and Gribaudo 2006). The mRT-PCR products were analyzed by gel electrophoresis.

Results and Discussion

Before performing the crosses, we analyzed the virological condition of the parents. As reported in the Table, viral status of both seed and pollen stock plants was quite compromised.

In order to investigate if seedlings obtained by a cross between infected parents were also infected, we examined 50 progeny plants for each of the three crosses: none of the nine screened viruses were detected in the 150 progeny plants.

The nepoviruses we analyzed were ArMV and GFLV; while in literature ArMV is not regarded as seed-transmitted in grapevines as we reported in the current study, seed transmission for GFLV did not occur in our study, contradicting previous reports (Lazar *et al.* 1990). The reason might be the use of a more specific molecular technique as the RT-PCR in our research, compared to the serological test (ELISA), and the larger number of seedlings we analyzed (over 50). In the cross 'Ceresa' x 'Carati' (Table), two 'Ceresa' mother plants and some 'Carati' plants used for the production of

Table

Virological condition of parents chosen for three crosses (**a, b, c**) screened by multiplex reverse transcription-polymerase chain reaction for simultaneous detection of nine grapevine viruses: *Grapevine leafroll-associated virus*-1, -2 and -3 (GLRaV-1, -2, -3), *Arabis mosaic virus* (ArMV), *Grapevine fanleaf virus* (GFLV), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Rupestris stem pitting-associated virus* (RSPaV), *Grapevine fleck virus* (GFkV)

a. *Almeria* x *Supernova*

Parent	No. of plants	Virus
♀Almeria	2	GLRaV-1, GLRaV-2, GLRaV-3, GFkV, RSPaV
♂Supernova	2	RSPaV
	5	GFkV, RSPaV
	9	GLRaV-1, RSPaV
	1	GVB, GLRaV-1, RSPaV
	20	GLRaV-1, GFkV, RSPaV
	1	GVB, GLRaV-1, GFkV, RSPaV
	2	GVA, GLRaV-1, GFkV, RSPaV
	2	GVA, GVB, GLRaV-1, GFkV, RSPaV

b. *Ceresa* x *Carati*

Parent	No. of plants	Virus
♀Ceresa	2	GVA, RSPaV, GFlV
♂Carati	1	GFkV, RSPaV
	29	GLRaV-1, GLRaV-3, RSPaV
	3	GLRaV-1, GLRaV-3, RSPaV, GFlV
	1	GVA, GLRaV-1, RSPaV
	3	GVB, GLRaV-1, GLRaV-3, RSPaV
	1	GLRaV-1, GFkV, RSPaV, GFlV
	3	GVA, GVB, GLRaV-1, GLRaV-3, RSPaV
	1	GVB, GLRaV-1, GLRaV-3, RSPaV, GFlV
	1	GVA, GLRaV-1, GLRaV-3, RSPaV, GFlV

c. *Red Globe* x *Regal*

Parent	No. of plants	Virus
♀Red Globe	19	NONE
	2	GFkV
	3	RSPaV
♂Regal	4	NONE
	1	GLRaV-3
	1	RSPaV
	3	GLRaV-2, GLRaV-3
	2	GVB, GLRaV-3
	7	GLRaV-3, GFkV
	3	GLRaV-3, RSPaV
	1	GLRaV-2, GFkV, RSPaV
	1	GVB, ArMV, GLRaV-3
	1	ArMV, GLRaV-3, RSPaV
	1	ArMV, GFkV, RSPaV
	16	GLRaV-3, GFkV, RSPaV
	2	GLRaV-3, RSPaV, GFlV
	1	GLRaV-1, GFkV, RSPaV
	1	GVA, GVB, ArMV, GLRaV-3
	3	ArMV, GLRaV-3, GFkV, RSPaV
	2	GVA, GVB, GLRaV-1, GLRaV-3, RSPaV
	2	GVA, GLRaV-1, GLRaV-3, GFkV, RSPaV
	1	GVA, GVB, GLRaV-1, GLRaV-2, GFkV, RSPaV

pollen were affected by GFLV, but none of the seedlings. Perhaps, GFLV is inactivated during seedling development; we analyzed the sanitary status of one year old progeny plants, not seeds like in most previous studies.

Regarding GRSPaV, our results confirm its no seed borne property as reported in literature (Meng *et al.* 2003; Morelli *et al.* 2009). In effect, for 'Almeria' x 'Supernova' and 'Ceresa' x 'Carati' crosses all parents used as seed or pollen plants were affected by GRSPaV and even for the cross 'Red Globe' x 'Regal' GRSPaV infected plants were employed. Nevertheless, GRSPaV has never been detected in any seedling plant analyzed.

Furthermore Leafroll viruses, GFkV, GVA and GVB have never been detected in our screening although they are present in different combinations in the parents of the three crosses, strongly indicating the general hypothesis that these viruses are not seed transmitted; this is likely the case as they are phloem restricted (Martelli 1993, Laimer *et al.* 2009).

It's known that most plants prevent virus invasion due to the high level of protection offered by embryos of their seeds (Salaudeen 2012) or during metabolic processes associated with germination, where virus is degraded and loses its infectivity (Nakamura *et al.* 2011).

In conclusion, our study supports the hypothesis that grapevine viruses associated with the most relevant diseases considered by the Italian legislation on certification of grapevine propagating material are at low risk for seed transmission. Therefore, this work facilitates the path leading from cross breeding to the distribution of a new variety, as it is no longer necessary to evaluate the virus status of parent plants. Despite these findings, a need for sanitary selection and use of certified propagation material remain important issues to ensure healthy vines.

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