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Validation of Diagnostic Protocols for the Detection of Grapevine Viruses Covered by Phytosanitary Rules

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INTRODUCTION

The Italian Ministry of Agriculture funded the Finalized Project "ARNADIA", aimed at producing validated reference diagnostic protocols for the control and monitoring of plant pathogens of phytosanitary interest and, among them, grapevine viruses. In this framework, the "Working group ARNADIA – grapevine viruses (WG)", composed of 8 Universities and Research Bodies, 3 accredited Private Laboratories, one Plant Health Service and one Association of Grapevine Nurseries was established. Moreover, 5 additional Italian Plant Protection Services took part in an inter-laboratory ring test.

The aim of the WG was to produce reference and validated serological and molecular protocols allowing for the harmonization of the diagnosis of 8 grapevine viruses, namely, *Grapevine leafroll-associated virus-1,-2,-3*, (GLRaV 1, 2, 3) *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Arabis mosaic virus* (ArMV), *Grapevine fanleaf virus*(GFLV) and *Grapevine fleck virus* (GFkV). Accordingly, the validation of the protocol consists in the evaluation of the processes aimed at determining their fitness for the particular use, and the validation of the assay yields test results that identify the presence of a specific target. The parameters that influence the capability of the test result to accurately predict the sample's infection status are: diagnostic sensitivity (ability of the used method to detect the presence of the pathogen in the samples truely infected by the pathogen in question - true positive) and diagnostic specificity (ability of the used method NOT to detect the presence of the pathogen in samples not infected by the pathogen in question - true negative). Other parameters that must be considered and which determine the efficiency of a protocol are: the analytical sensitivity (the smallest amount of infectious entities that can be identified by the diagnostic method), repeatability or accordance (degree of conformity of the results obtained in replications of the process, made at short time intervals, using the same reference sample and in the same working conditions i.e. equipment, operator, laboratory) and reproducibility or concordance (degree of conformity of the results obtained using the same method with the same reference samples in different laboratories). We reported the parameters obtained in the validation of a serological (ELISA) and molecular (Multiplex RT-PCR) protocols for the diagnosis of eight grapevine viruses.

MATERIALS AND METHODS

122 grapevine samples (varieties, rootstocks and pools of 5 plants, of which only one infected) have been analyzed by serological (ELISA - using 25 antisera of three commercial Companies: Agritest (8), Bioreba (9), Sediag (8) for GLRaV 1, 2, 3, GVA, GVB, GFLV, ArMV, GFkV, GLRaV 1+3, ArMV + GFLV) and molecular (multiplex RT-PCR) protocols. For ELISA, the tests were conducted carefully following instructions provided by the Companies; multiplex RT-PCR was performed using the protocol described by Gambino and Gribaudo, 2006. Moreover, three extraction methods (use of plastic bags and homogenizer, use of mortar and pestle with or without liquid nitrogen and use of milling machine) have been compared, starting from phloem tissue obtained from the bark. The tests were performed in 13 laboratories using the same samples (analyzed in blind conditions) and reagents; in each laboratory, the results have been obtained using the same threshold value calculated on

the basis of the spectrophotometer readings for ELISA and by analyzing the electrophoretic gels for the multiplex RT-PCR.

The processing of the obtained results (about 24,000 data points) has led to the definition of the validation parameters according to UNI/EN/ISO 16140 and 17025 and EPPO standards PM7/76 and PM7/98.

RESULTS AND DISCUSSION

As reported in Table 1, ELISA has proven to be a highly effective technique, comparable to the molecular method, although the latter turned out, as expected, to be more efficient for some viruses and on some specific samples (rootstocks and pool). In detail, regarding the extraction method, the use of plastic bags and homogenizer resulted less sensitive (5-8%) than the other two methods in detecting GFLV, ArMV and (2-4%) GVA. Concerning the different kind of samples, no differences have been highlighted for GLRaV-1, -2, -3 and GFkV between European varieties and rootstocks. Small and not always statistically significant differences (negative for rootstocks) were observed for ArMV, GVA and GFLV and generally good results were obtained in analyzing the pool samples, even if the accuracy was found to be lower (10-15 percentage points) for GLRaV-1, GLRaV-2 and GFkV compared to individual samples. No statistically significant differences were observed for the other viruses. Concerning the ELISA kits, all behaved absolutely equivalently in the diagnosis of GLRaV-1,-2,-3, GFLV, ArMV. Only two kits (GFkV from Sediag and GVA from Bioreba) performed worse than the respective ones from other Companies. Good results were obtained through the use of kits using mixed antisera (GLRaV-1 + -3 and GFLV + ArMV) by Bioreba, while the corresponding mixed kit GFLV + ArMV by Sediag performed worse.

In conclusion, harmonized and validated reference diagnostic protocols for grapevine viruses subjected to phytosanitary rules are, for the first time, available. The efficiency and robustness of the protocols have been proven using a large number of samples in a variety of laboratories. On the basis of this, both serological and molecular protocols resulted valid, and their use could be as a function of different specific applications.

Virus	Diagnostic protocol	Sensitivity	Specificity	Accuracy	Analytical sensitivity	Repeatability	Reproducibility
ArMV	*Multiplex*	92 %	99 %	98 %	10^{-2}	100%	100 %
	ELISA – A/B/S	64/48/50%	85/95/96%	74/72/72%	10^{-2}	100%	95%
GFLV	*Multiplex*	68 %	100%	90 %	10^{-3}	100%	76%
	ELISA – A/B/S	75/82/77%	96/92/92%	80/84/81%	10^{-2}	100%	90%
GFkV	*Multiplex*	95%	95%	95%	10^{-2}	100%	95%
	ELISA – A/B/S	90/90/30%	100%	92/92/46%	10^{-1}	98%	88%
GVA	*Multiplex*	96 %	99 %	98 %	10^{-2}	100%	94 %
	ELISA – A/B/S	77/45/87%	100/100/96%	83/58/89%	10^{-1}	98%	82%
GVB	*Multiplex*	100%	100%	100%	10^{-2}	100%	100%
	ELISA – A/B/S	86/nt/nt%	100%	92%	10^{0} (2^{-2})	100%	85%
GLRaV 1	*Multiplex*	74 %	100 %	94 %	10^{-2}	100%	70 %
	ELISA – A/B/S	89/94/96%	100%	93/96/98%	10^{-2}	100%	92%
GLRaV 2	*Multiplex*	84%	98%	85%	10^{-2}	95%	83%
	ELISA – A/B/S	86/67/87%	100%	93/96/98%	10^{0} (2^{-2})	93%	84%
GLRaV 3	*Multiplex*	100 %	93 %	95 %	10^{-3}	100%	100 %
	ELISA – A/B/S	81/90/97%	100%	84/92/97%	10^{-3}	100%	94%

Table 1. Summary of validation parameters obtained by the ELISA test for each virus and antiserum and comparison with those obtained with the molecular protocol. A= Agritest; B= Bioreba; S= Sediag

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