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Comparison of ELISA and RT-PCR for the detection of PNRSV and PDV in Australian almond trees

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SUMMARY – This three-year study compared the use of ELISA and RT-PCR for identifying *Prunus* necrotic ringspot virus (PNRSV) and prune dwarf virus (PDV) on 175 leaf samples taken from trees maintained as a budwood repository for almond growers. The primers used for RT-PCR were based on DNA sequences that code for coat protein, and both viruses could be detected in the same reaction. For PNRSV, both ELISA and RT-PCR produced similar results, although RT-PCR was more consistent, and had the added advantage that plant material could be tested at any time throughout the growing season. For PDV, virus particles were not detected by ELISA, but were detected in low titre using a nested PCR technique. RT-PCR is used routinely now to index progeny developed each year by the Australian almond improvement program in place of both graft incompatibility using woody indicator species and ELISA.

Key words: *Prunus*, almond, PNRSV, PDV, ELISA, RT-PCR.

RESUME – *"Comparaison d'ELISA et RT-PCR pour la détection de PNRSV et PDV chez les amandiers australiens". Cette étude de trois ans a comparé l'utilisation d'ELISA et de RT-PCR pour identifier le virus des taches annulaires nécrotiques des Prunus (PNRSV) et le virus du nanisme du prunier (PDV) sur 175 échantillons de feuilles prélevés sur des arbres maintenus comme dépôt d'écussons pour des cultivateurs d'amandiers. Les amorces utilisées pour RT-PCR ont été basées sur les séquences d'ADN qui codent pour la protéine enveloppe, et les deux virus pourraient être détectés dans la même réaction. Pour PNRSV, ELISA et RT-PCR ont produit des résultats semblables, bien que RT-PCR ait été plus conformé, et ont eu l'avantage supplémentaire que la matière végétale pourrait être examinée à tout moment tout au long de la saison de croissance. Pour PDV, des particules de virus n'ont pas été détectées par ELISA, mais ont été détectées à faible titrage en utilisant une technique de PCR nichée. RT-PCR est maintenant employée en routine pour classer la descendance développée tous les ans par le programme d'amélioration australien d'amandier au lieu de l'incompatibilité de greffe en utilisant des indicateurs ligneux et ELISA.*

Mots-clés **:** Prunus*, amande, PNRSV, PDV, ELISA, RT-PCR.*

Introduction

Prunus necrotic ringspot virus (PNRSV) is known to infect every species of *Prunus* (Spiegel *et al.*, 1996), and may be transmitted either by pollen, seed, or vegetative propagation (Greber *et al.*, 1992; Uyemoto *et al.*, 1992). The symptoms in almond trees include chlorotic and necrotic leaf spots, mosaics, ringspots, and line patterns (Nyland *et al.*, 1976). Routine detection is usually by the enzyme-linked immunosorbent assay (ELISA), but the efficiency of the assay depends on the age of the leaves (Bertozzi *et al.*, 2002), and seasonal fluctuations of viral concentrations, which are particularly low at temperatures over 38°C (Heleguera *et al.*, 2001).

Prune dwarf virus (PDV) is the causal pathogen of various diseases of stone fruits, depending upon the type of host infected. PDV is transmitted through pollen and grafting cuts (Brunt *et al.*, 1996), and

the symptoms include chlorosis or chlorotic ringspots, leaf distortions, and overall size reduction in many species (Brunt *et al*., 1996). The main diagnostic test used in Australia is graft compatibility using woody indicator species, such as *Prunus serrulata* cv. 'Shirofugen', because PDV cannot be detected in the almond cultivars grown in Australia using ELISA (Sedgley and Collins, 2002). However, graft compatibility cannot distinguish between PNRSV and PDV (Rampitsch *et al*., 1995).

A method based on RT-PCR was used to detect the presence of both these viruses in almond leaves of the most important almond cultivars grown commercially in Australia, and the results were compared with those obtained from using ELISA.

Materials and methods

The trees used are part of the Monash bud wood repository (Riverland Vine Improvement Centre, Monash, South Australia). Leaf samples were collected randomly from around the canopy of 8 trees of 'Nonpareil' (5 - 67 samples per tree) and one tree of 'Sauret' (5 samples) in each of the three seasons from 1999 - 2001. Leaves of cherry (*Prunus avium*), known to be infected with PDV, were used as a positive control for PDV. Leaves of 'CEBAS 1' were supplied by CSIC-CEBAS, Murcia, Spain.

Double body sandwich ELISA was performed using the commercially available PNRSV and PDV alkaline phosphatase compound ELISA test kits (Agdia Inc., Elkhart, IN) according to the manufacturer's instructions (Bertozzi *et al*. 2002).

Total RNA was isolated from 50-100 mg of fresh or frozen leaves as described by Channuntapipat *et a*l. (2001). Alternatively, RNA was extracted using the RNeasy plant mini kit (Qiagen) by grinding 200 mg of fresh or frozen leaf tissue in liquid nitrogen and mixing the powder with 2.0 ml of either RLT or RLC extraction buffer containing 4.4% (w/v) polyvinylpyrollidone (PVP-40) and 1% (w/v) sodium metabisulphite. A 500 µl aliquot of the homogenate was mixed with 60 µl of 20% (w/v) N-lauroyl-sarcosine (sarkosyl), and incubated at 70°C with agitation for 10 min. The contents were then transferred to a QIAshredder mini column and centrifuged at 14,000 rpm for 5 min. The column flowthrough was mixed with 315 µl of 95% ethanol, and the remainder of the protocol was carried out according to the manufacturer's instructions.

Oligonucleotide primer sequences reported by Raquel *et al*. (1999) were used to detect PDV. These were PDVF 5'-CCAATTTACTTCCAACTTTCGA and PDVR 5'-GCACAATCAAATGATGGATCA, which produce a PCR product of 722 bp. Primer sequences to detect PNRSV were developed using the nucleotide sequences of the coat protein published on the GenBank database, National Center for Biotechnology Information (NCBI). These were PNRSVF 5'-CTTGAAGGACCAACCGAG and PNRSVR 5'-ATCTGCTAACGCAGGTAAG, which produce a PCR product of 351 bp.

RT-PCR was carried out in a volume of 20 µl containing 1 µl of total RNA, 1.5 mM $MgCl_2$, 0.5 µM of appropriate primers, 200 µM each of dNTPs, 1 x PCR buffer, 1 x sucrose-cresol red dye (20% (w/v) sucrose containing 1 mM cresol red, 1 U/µl RNase inhibitor, 0.5 U/µl Superscript II and 1.1 U Taq DNA polymerase. The PCR cycle consisted of cDNA synthesis at 50°C for 45 minutes, followed by 94°C for 1 minute, 34 cycles for 30 seconds at 94°C, 45 seconds at 56°C**,** 1 minute at 72°C and a final extension step of 5 minutes at 72°C.

Because no PCR products were detected with the primers for PDV, a second set of primers, internal to the primers used in the first-round of RT-PCR, were designed to perform nested PCR. These were PDV1F 5'-GTATGATATCTCGTACCGAG and PDV1R 5'-CTGGCTTGTTTCGCTGTGAA, which produce a PCR product of 241 bp. The PCR products from RT-PCR were diluted 1:100, and 2 µl were subjected to nested PCR with primers PDV1F / PDV1R.

The RT-PCR products for PNRSV (351 bp) and PDV (722 bp), and the PDV nested PCR product (241 bp) were inserted into the pCR 2.1-TOPO 3.9 kb vector using the TOPO TA Cloning kit (Invitrogen) following the manufacturer's instructions, and sequenced to confirm that the amplified products were from the coat proteins of PNRSV and PDV.

Results and discussion

Nine of the 175 samples gave a positive response with ELISA for PNRSV in the year 1999, and eight in the year 2000. Using RT-PCR, PNRSV was detected in nine out of 175 leaf samples in both the years 2000 and 2001. The 351 bp product used as a marker for the presence of PNRSV matched the sequence of the viral coat protein located between the primers.

Sanchez-Navarro *et al*. (1998) reported that RT-PCR can surpass ELISA in sensitivity for the detection of PNRSV. The detection of PNRSV by ELISA was shown by Bertozzi *et al*. (2002) to be most sensitive when plant material was collected in spring. Heleguera *et al*. (2001) reported that samples, collected in summer from chronically infected plants, showed a positive result for PNRSV with IC-RT-Nested-PCR and RT-PCR but produced a negative result with ELISA. In the present study, PNRSV could be detected using RT-PCR in the leaves of infected almond trees collected from early spring to late autumn.

RNA extracted from cherry leaves infected with PDV, and from the leaf sample of CEBAS 1 imported from Spain, produced an amplification product of 722 bp after RT-PCR, but no amplification products were detected for PDV in any of the Australian almond samples. After nested PCR, amplification products of 241 bp were detected for 168 of the 175 almond samples tested in both 2000 and 2001. The sequences of the amplified products matched the sequences of the viral coat protein located between the primers.

ELISA has been used successfully to detect PDV in almond in Europe (Di Terlizzi *et al*., 1994) and cherry in Australia (Johnstone *et al*., 1995), but gave negative results with almond trees in Australia (Bertozzi *et al*., 2002). RT-PCR also produced negative results for PDV, but the combination of RT-PCR with nested PCR developed in this study resulted in the detection of PDV in 96% of the 175 samples tested in 2000 and 2001.

Conclusions

The results of this study show that RT-PCR produces similar results to ELISA for the detection of PNRSV, but it is probably more consistent, and has the advantage of a wider window for testing during the season. For PDV, ELISA produced negative results for all leaves tested, but the presence of the virus was confirmed in Australian trees by using nested PCR. Leaves of infected trees from Spain, supplied by CSIC-CEBAS, produced a strong positive product for PDV after RT-PCR, suggesting that the titre of this virus may be low in Australian almond trees, and this may be an important factor in the lack of success with ELISA for detecting PDV in Australian almonds.

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