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Molecular diagnosis of *Chrysanthemum stunt viroid* for routine indexing

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Summary. In 2002, a two-year study was started to check for *Chrysanthemum stunt viroid* (CSVd) during the production and selective breeding of new chrysanthemum varieties in a central-Italy flower-growing farm. Two molecular techniques, one-tube RT-PCR and tissue printing for hybridization assays, were improved for their effectiveness in viroid detection at different stages of plant selection. Both molecular techniques proved sensitive, reliable and easy to apply in a programme of routine indexing for the production of new and healthy chrysanthemum varieties.

Key words: *Dendranthema* × *grandiflorum*, CSVd-free material, RT-PCR, tissue blot hybridization assay.

Introduction

Chrysanthemum (*Dendranthema* × *grandiflorum*), family *Asteraceae*, is one of the leading cut flower and potted plants in the international market. In Italy, chrysanthemum is grown in all regions and ranks second after carnation, in terms of mean production output, which amounts to 650 million cut-flowers and 9 million potted plants of chrysanthemum annually. Although a great expansion of the Italian chrysanthemum industry has taken place (259.8% increase 2000/1999), chrysanthemum imports to the country amounted to 16.400,00 euro (2% increase 2000/1999), exclusively from The Netherlands (data from Pathfast Publishing Archives [AAVV, 2000]; and Ismea [AAVV, 2002]).

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Over the last few years, an interest in improving Italian chrysanthemum varieties has favoured public and private breeding activity. To further this activity, genetic and phytosanitary quality of the germplasm must be guaranteed. International agencies (IPPC, WTO) promote measures to prevent the introduction and spread of plant pests. In addition, specific regulations are issued at the national and international level (e.g.: Directive 98/56/EC; Directive 2000/29/EC) regarding the marketing of healthy propagating material for ornamentals. Plant production is further regulated by a guaranteed phytosanitary certification scheme for the commercial trade of ornamentals, including chrysanthemum (EPPO Standards [OEPP/EPPO, 2002]).

Chrysanthemum is reported to be susceptible to nine viruses and two viroids (Verma *et al*., 2003). In particular, *Chrysanthemum stunt viroid* (CSVd), a member of the genus *Pospiviroid*, family *Pospi-*

viroidae (Bouwen and Zaayen, 2003), is an organism of quarantine significance causing "stunt" disease in most chrysanthemum-growing areas of the world (OEPP/EPPO, 1997). The disease appeared in Europe in the 1950s, via infected varieties imported from the USA. Typical symptoms are stunting with a reduction of up to 50% in the height of mature plants, smaller leaves and reduced root growth. Stems break easily, flowers are reduced in size and number and show premature flowering by a few days to a few weeks. Some varieties are more severely damaged with the leaves spotted, flecked, distorted or curled, and with flower break or bleaching. Symptoms are extremely dependent on environmental conditions, especially temperature and light, so that current chrysanthemum varieties commonly are often symptomless. CSVd is transmitted by propagation from infected mother plants. Afterwards, the viroid is spread in the nursery and in crops by mechanical means causing considerable quality decline due to the difficulty in detecting infection by visual inspection.

Since 2002, a central Italian flower farm has had a phytosanitary scheme to check for CSVd during the production and selection of new CSVd-free chrysanthemum varieties and hybrids. Since plants frequently carry the viroid latently, laboratory-based assays were improved to routinely test material at various stages of selection and propagation. Two of these assays, one-tube RT-PCR and tissue blot by hybridization, were examined for their effectiveness in detecting CSVd.

Materials and methods

Plant materials

Thirty-nine seasonal and 93 year-round chrysanthemum varieties were selected for the breeding programme; five plants per variety were tested for CSVd. The health status of the candidate material was then examined at the different stages of production scheme i.e. either as nuclear stocks maintained *in vitro*, or as selecting stocks, generated *in vitro* propagation and planted in a glasshouse.

Naturally CSVd-infected and healthy plants were maintained separately in the glasshouse and *in vitro* as positive and negative controls for the laboratory assays.

Tissue blot hybridization assay

A nucleic acid hybridization assay was done according to Loreti *et al.* (1999). A full-length cDNA copy of CSVd was cloned in *pSK* plasmid vector (Stratagene, La Jolla, CA, USA) and linearized with *Xba* I. Then, specific full-lenght cRNA probe was transcribed *in vitro* with T7 RNA polymerase (Dig RNA labelling kit SP6/T7, Boehringer Mannheim, Indianapolis, IN, USA) and stored at -20°C. Freshly cut rolled leaves, collected from *in vivo* stocks, were carefully pressed onto a nylon membrane and UV fixed. Imprinted membranes were pre-hybridized in 50% formamide, 5× SSC (0.3 M NaCl, 0.03 M tri-sodium citrate pH 7), 2% blocking reagent, 0.1% N-lauryl sarkosine, 0.02% SDS and 100 μg ml^{-1} denatured herring-sperm DNA. Hybridization was at 68°C with a solution containing the CSVd cRNA-specific probe labelled with digoxigenin. Membranes were finally treated for chemiluminescent detection following manufacturer's instructions (DIG Luminescent Detection kit for Nucleic Acids, Boehringer Mannheim). To evaluate the best concentration of the probe, time of exposure for chemiluminescent detection and detection of non-specific reactions, different concentrations of the probe (100 ng ml^{-1}; 50 ng ml^{-1} and 25 ng ml^{-1}) and three serial tenfold dilutions of the CSVd RNA extract were first tested by dot blot hybridization following the same hybridization protocol.

One tube – one step RT-PCR

Leaves from *in vitro* plantlets were collected to extract the total nucleic acid (TNAs) according to Faggioli *et al.* (2001). Fresh tissue (0.2 g) was powdered in liquid nitrogen and ground in 900 μl of 0.2 M Tris-HCl buffer (pH 8.2) containing 17.5 μl of 5 M NaCl, 8.5 μl of 10% Triton X-100 and 1.7 μl of 2-mercaptoethanol. After centrifugation, the supernatant was mixed with 0.5 ml of water-saturated phenol pH 7.0, 100 μl of 5% SDS and 100 μl of 0.1 M EDTA pH 7.0 and, after a further centrifugation, TNAs were recovered by ethanol precipitation and resuspended in 500 μl distilled sterile water. Oligonucleotide primers CSVd 1 (5'-ATCCCCGGGGAAACCTGGAGGAAGT-3'), and CSVd 2 (5'-CCCTGAAGGACTTCTTCGC-3'), corresponding to nucleotides 88–112 and 87–68 respectively, were designed on a highly conserved region of the CSVd sequences retrieved from GenBank Database. One tube-one step RT-PCR (Mumford *et al.*,

2000; Ragozzino *et al*., 2004) consisted of a preheat-denaturation step at 95°C for 5 min and quick cooling on ice of target RNA (1.5 μl of TNAs) mixed with 1 μg of complementary primer. Then, 57.5 μl of the reaction mixture, containing 1× of HotStarTaq® DNA polymerase buffer (Qiagen GmbH, Hilden, Germany), 1.5 mM $MgCl_2$; 125 μM dNTPs each, 80 U transcriptase M-MLV (Invitrogen, UK), 2 U HotStarTaq® DNA polymerase (Qiagen), 40 U Rnase OUT inhibitor (Invitrogen) and 0.15 μg forward primer, was added to the tube. Reverse transcription was performed at 42°C for 45 min followed by 15 min at 95°C to activate HotStarTaq® polymerase, and by PCR cycling as follows: denaturation at 95°C for 45 sec, annealing at 60°C for 45 sec, extension at 72°C for 1 min, for a total of 35 cycles, followed by final extension for 7 min at 72°C. To determine primer effectiveness at different concentrations of viroid target, the one-step RT-PCR assay was carried out at the ratios of 1:10, $1:10^2$ and $1:10^3$ of the RNA extract. Amplified products were analysed by electrophoresis on 1.5% agarose gel, and stained with ethidium bromide.

Results and discussion

The digoxigenin labelled CSVd-cRNA probe reacted with complementary nucleic acids up to a concentration of 25 ng ml^{-1} with the 10^{-3} dilution of CSVd-RNA (Fig. 1). A probe concentration of 50 ng ml^{-1} was therefore chosen for the tissue blot hybridization assays. A clear positive signal of hybridization reaction was obtained with naturally infected leaves after 30 min of exposure (Fig. 2). As regards RT-PCR, the dilution series of RNA showed the presence of the 356 bp fragment up to a 10^{-3} dilution in RNase-free water (Fig. 3).

Both techniques were sensitive and reliable for CSVd detection, and suitable for routine controls in the two-year breeding study.

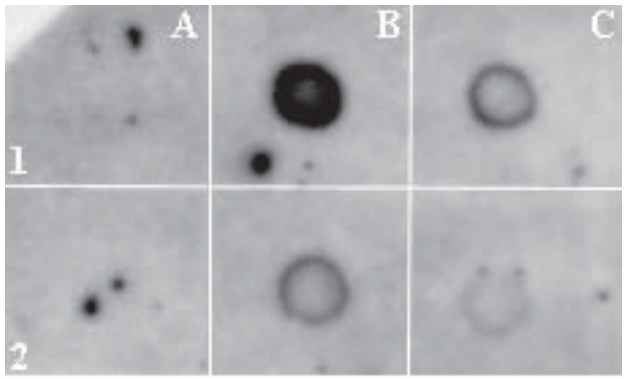

Fig. 1. Dot blot hybridization assay of tenfold dilutions of CSVd-RNA using a digoxigenin labelled probe at 25 μg ml^{-1} concentration: 1A, water control; 2A, negative control; 1B, positive control; 1C, 10^{-1} dilution of positive control; 2B, 10^{-2} dilution; 2C, 10^{-3} dilution.

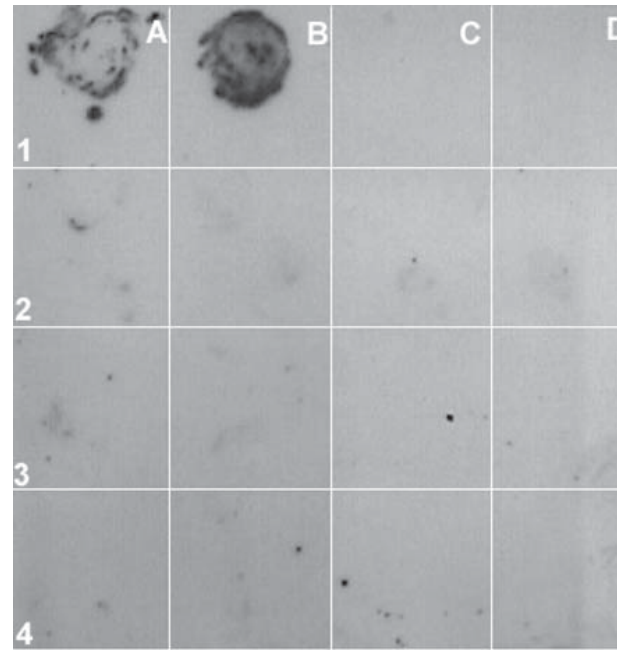

Fig. 2. Tissue blot hybridization assay of fresh leaves of chrysanthemum samples collected during *in vivo* screening: 1A, 1B, positive controls; 1C, 1D, negative controls; 2A–4D, samples.

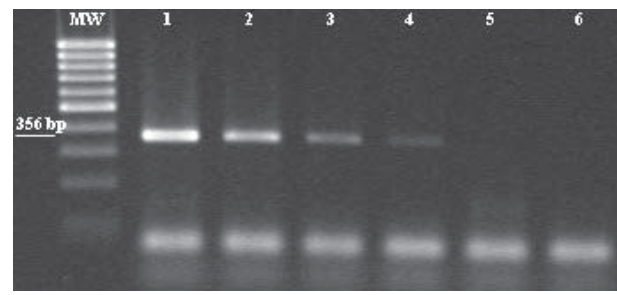

Fig. 3. Agarose gel electrophoresis of RT-PCR products obtained in an assay for CSVd detection of three serial tenfold dilutions of a RNA extract: MW, molecular weight marker (100 bp, Fermentas MBI); 1–4, dilution series from undiluted to 10^{-3} dilution; 5, healthy control; 6, no -template control.

On the basis of the results obtained and the characteristics of the two methods, the tissue blot hybridization assay was preferable when a large number of samples had to be tested. This technique was therefore better at the start of the testing activity, when 39 seasonal varieties and 93 parents for year-round breeding had to be selected as candidate for their genetic and phenotypic characteristics, and five plants of each were tested. In addition, stocks of candidate seasonal varieties and of year-round parent filiations were grown in plots under a glasshouse for phenotypic evaluation. As all these screening stages occurred in a short period of the year, the phytosanitary assessment of the propagating material required a massive sampling of plant material, so that the requisite number of cuttings was collected depending on the population size of each clone. At these stages the tissue blot hybridization assay had an important advantage in rapidly processing fresh leaf samples and storing imprinted membranes at 4°C before subsequent viroid detection. This method was also easy to handle and low-cost, while maintaining adequate sensitivity and specificity.

The one tube-one step RT-PCR method, on the other hand, was more reliable for ascertaining the viroid-free status of cuttings for the *in vitro* nuclear stock arrangement of each new candidate variety and hybrid. When working on a limited number of samples, this method had several advantages: it requires only a small amount of tissue, which is all what *in vitro* plantlets can provide, the extraction and PCR procedures were both easy, the test took only one-day, and, lastly, it was sensitive and specific.

The two-years programme to select and propagate chrysanthemum candidate varieties and hybrids required analysis of 267 plantlets belonging to 84 *in vitro* nuclear stocks (39 seasonal varieties and 45 hybrids) and of more than 2500 samples of leaf cuttings collected during the phenotypic evaluations in the glasshouse. Only one seasonal variety was found infected and eliminated at the beginning of the two-year testing period, whereas, as expected, no CSVd detection occurred in the samples during the screening stages, showing that the phytosanitary measures applied during the cultivation avoided viroid contamination and spread in vegetative material.

In conclusion, the study carried out in collaboration with a private firm, established that both molecular detection methods are reliable and easy to apply in the routine indexing for the production of new and healthy chrysanthemum varieties, being rapid, sensitive, and at least one of them being able to cope with a great number of samples.

Acknowledgements

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Literarure cited

AAVV, 2000. *International Statistics, Flowers and Plants 2000*. Pathfast Publishing UK. http://www.pathfast publishing.com

AAVV, 2002. *Filiera: Fiori e Piante*. Ismea, Roma, 153 pp.

Bouwen I. and A. van Zaayen, 2003. Chrysanthemum stunt viroid. In: *Viroids* (A. Hadidi, R. Flores, J.W. Randles, Js. Semancik, ed.), CSIRO Publishing, Collingwood, Australia, 218–223.

Faggioli F., E. Ragozzino and M. Barba, 2001. Simultaneous detection of stone or pome fruit viroid by single tube-RT-PCR. *Acta Horticulturae* 550, 59–63.

Loreti S., F. Faggioli, M. Cardoni, G. Mordenti, A.R. Babini, C. Poggi Pollini and M. Barba, 1999. Comparison of different diagnostic methods for detection of peach latent mosaic viroid. *EPPO Bulletin* 29, 433–438.

Mumford R.A., K. Walsh and N. Boonham, 2000. A comparison of molecular methods for the routine detection of viroids. *EPPO Bulletin* 30, 431–435.

OEPP/EPPO, 1997. Chrysanthemum stunt viroid, Data sheets on Quarantine Pests. In: *Quarantine Pests for Europe*, 2nd ed., 1227–1230.

OEPP/EPPO, 2002. EPPO Standards PM 4/6 (2). Certification scheme for chrysanthemum. *EPPO Bulletin* 32, 105–114.

Ragozzino E., Faggioli F. and Barba M., 2004. Development of a one tube–one step RT-PCR protocol for the detection of seven viroids in four genera: *Apscaviroid*, *Hostunviroid*, *Pelamoviroid* and *Pospiviroid*. *Journal of Virological Methods* (submitted).

Verma N., A. Sharma, R. Ram, V. Hallan, A.A. Zaidi and I.D. Garg, 2003. Detection, identification and incidence of *Chrysanthemum B carlavirus* in chrysanthemum in India. *Crop Protection* 22, 425–429.

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