

SHORT COMMUNICATION
USE OF ONE STEP RT-PCR FOR DETECTION OF *ASPARAGUS VIRUS 1*

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SUMMARY

Two viruses are responsible for most of the decline in asparagus crops (*Asparagus officinalis* L.) in the world: *Asparagus virus 1* (AV-1) and *Asparagus virus 2* (AV-2). AV-1 is a member of genus *Potyvirus* and has so far been identified in plant and tissue-culture material of asparagus using biological assays on indicator plants, electron microscopy and, rarely, by serodiagnosis. In this study, degenerate potyvirus primers were used in RT-PCR to amplify a cDNA product from an asparagus potyvirus, previously identified as AV-1 on the basis of host range results. RT-PCR products were sequenced, and the nucleotide sequences obtained were aligned with those of other members of the genus *Potyvirus* to devise primer pairs specific for AV-1. The use of one primer pair in a one-step RT-PCR procedure was found to yield an amplicon with RNA from an AV-1 infected plant, but not with RNA from plants infected with *Turnip mosaic virus* (TuMV), *Pea seed-borne mosaic virus* (PSbMV), or *Lettuce mosaic virus* (LMV). The method was sensitive and reproducible for the detection of AV-1 in leaves and spears of asparagus samples collected from fields and from tissue-culture plantlets.

Key words: potyvirus, *Asparagus officinalis*, molecular identification, RT-PCR, diagnosis.

Asparagus (*Asparagus officinalis* L.), a worldwide grown vegetable, is affected in many countries by a decline syndrome (Elmer *et al.*, 1996) that reduces the production age of the plantings, as well as the yield and quality of the spears. Virus infection is thought to contribute significantly to this decline and to increase the susceptibility of asparagus plants to other abiotic and biotic stresses.

Asparagus virus 1 (AV-1) (genus *Potyvirus*, family *Potyviridae*) and *Asparagus virus 2* (AV-2) (genus *Ilarvirus*,

family *Bromoviridae*) (Mink and Uyeda, 1977; Uyeda and Mink, 1981) are considered to be the most important of the viruses that infect asparagus in all continents. *Tobacco streak virus* (TSV, genus *Ilarvirus*) (Mink and Uyeda, 1977), *Cucumber mosaic virus* (CMV, genus *Cucumovirus*) (Phillips and Brunt, 1985), and *Asparagus virus 3* (AV-3, genus *Potexvirus*) (Fujisawa, 1986) have been found to infect asparagus crops but only in restricted areas of Europe, North America, Japan, and New Zealand.

In the past, studies have been made on field incidence of AV-1 and AV-2 (Bertaccini *et al.*, 1990; Falloon *et al.*, 1986; Jaspers and Falloon, 1996), on the effects of virus infection on spear and fern growth and productivity (Jaspers *et al.*, 1999), and on the susceptibility of plants to *Fusarium* rot (Evans and Stephens, 1989). Specific detection assays have been developed only for AV-2 using serological (ELISA) and, later, molecular (RT-PCR) methods (Roose *et al.*, 2002). AV-1 detection has so far been done either by biological assay on *Chenopodium* spp., electron microscope observations of filamentous (potyvirus-like) particles, or, in a few cases, by immunosorbent electron microscopy (ISEM) (Falloon *et al.*, 1986) and ELISA (Kegler *et al.*, 1991) with antisera that are not commercially available. In Italy, AV-1 and AV-2 were first recorded in 1982 (Bertaccini *et al.*, 1982). They occur at high incidence in production fields of northern Italy (Bertaccini *et al.*, 1990).

Recently, we have started a survey to assess the phytosanitary status of commercial asparagus fields in southern Italian regions. Because no standardised diagnostic tools for AV-1 were available, a primary objective of our study was to develop a method for rapid and sensitive detection of this virus for use in our investigation. Consequently, a one-step RT-PCR-based assay was developed. A potyvirus from field asparagus was first identified as AV-1 on the basis of host range and particle morphology (isolate AV-1/1770). For this purpose, crude sap of several asparagus samples, collected from different commercial fields, were mechanically inoculated to a variety of plants in the family *Solanaceae*, *Cucurbitaceae* and *Chenopodiaceae*. Necrotic local lesions appeared on inoculated leaves of *C. amaranticolor* and *C. quinoa* only after 7 to 9 days after inoculation and no

systemic infection was detected.

Electron microscope observations (Philips CM 10) of sap preparations from asparagus and *C. amaranticolor* plants detected potyvirus-like particles 710-770 nm in length. Afterwards, total plant RNA was extracted from 100 mg of the infected asparagus tissue using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), and used in RT-PCR with a universal primer pair as described by van der Vlugt *et al.* (1999), designed to amplify sequence from the 3' terminal region of potyvirus genomes. After amplification, the asparagus sample yielded a product of about 720 bp (Fig. 1). This fragment was sequenced (MWG Biotech AG, Germany) and the analysis (FASTA program) of the sequence (Accession No. EF576991) confirmed its relationship to the N-terminal CP-encoding region of potyviruses with 70% to 73.8% homology to sequences in the RNA of five species.

To improve a specific diagnostic protocol for AV-1, after multiple sequence alignment (CLUSTAL W Program) of AV-1/1770 sequence with those of other potyviruses from the GenBank database, primers (AV-1-F62: 5'-TCATCGAAAATGCCAAACCCACG-3' and AV-1-R550: 65 5'-CGAGATACTCGTGGGAAGCC-CAC-3') were designed in the CP region of the genome and tested in a one-step RT-PCR procedure. This was done in a 25 µl volume containing 2.5 µl 10x buffer (Promega, Madison, WI, USA), 1.5 mM MgCl₂, 2.5 µM of each dNTP, 0.5 µM of each primer, 20 U of RNase-OUT (Invitrogen, California USA), 1.25 U of AMV RT (Promega), 0.75 U of *Taq* polymerase (Promega) and 2 µl of total RNA. RT incubation was at 42°C for 60 min

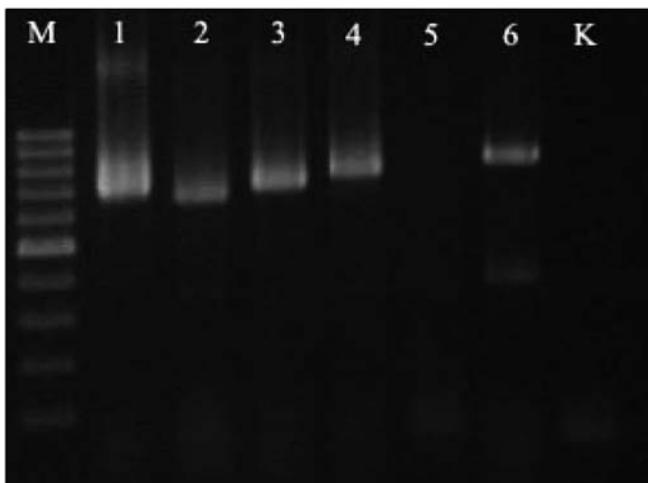


Fig. 1. Agarose gel electrophoresis of DNA products derived from amplification of total RNAs by RT-PCR using *Potyvirus* genus primers. *Turnip mosaic virus* (TuMV-DSMZ PV0104) (lane 1); *Pea seed-borne mosaic virus* (PSbMV-DSMZ PV0316) (lane 2); *Lettuce mosaic virus* (LMV-DSMZ PV0405) (lane 3); potyvirus infected asparagus (AV-1) (lane 4); healthy asparagus (lane 5); *Potato virus Y* (PVY-ISPave) (lane 6). M, 100 bp molecular marker; K, water control.

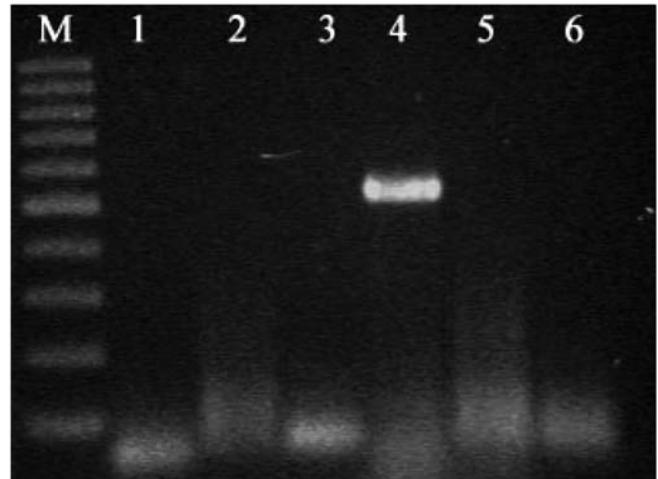


Fig. 2. Agarose gel electrophoresis of one step RT-PCR amplified products obtained with primers F62 and R550 that yielded a 511 bp amplicon from total RNAs of AV-1. M 100bp molecular marker; *Turnip mosaic virus* (lane 1); *Pea seed-borne mosaic virus* (lane 2); *Lettuce mosaic virus* (lane 3)-LMV; *Asparagus virus 1* (lane 4); healthy asparagus (lane 5); water control (lane 6).

and followed by denaturation at 95°C for 5 min and by 33 cycles of amplification comprising 30 sec at 94°C, 30 sec at 60°C and 1 min 72°C, with a final extension of the amplification products for 10 min at 72°C. The DNA products were analyzed by electrophoresis in 1.5 % agarose gels and detected by ethidium bromide staining.

A fragment of the expected size (511 bp) was amplified from AV-1-infected asparagus and no products were obtained from healthy asparagus control and from other members of the genus *Potyvirus* (Fig. 2). To determine primer effectiveness at different concentrations of virus target, RT-PCR analysis was done using dilutions of 1:10 to 1:10⁴ of 100 ng of tRNA from infected asparagus in RNase-free water. The 511 bp product was amplified up to 10⁻⁴ dilution.

More than 650 asparagus samples collected in different areas of Sicily and Campania were analysed by RT-PCR. AV-1 was detected in 54% of them and, in total, 86% of the sites visited were found to contain plants infected by the virus. The highest incidence was found in older plantings (data not shown). AV-1 detection was confirmed by sequencing (Bio-Fab, Italy) amplicons derived from AV-1/1770 and three samples from different fields, and comparing them against the published nucleotide sequences of potyviruses. The sequences of the four samples were almost identical to one other (98%) and most resembled (75 %) sequences of *Turnip mosaic virus* (TuMV- Acc. No. AB188972).

In conclusion, the RT-PCR method developed here was sensitive and reproducible in detecting AV-1 in field samples and in tissue culture material. Tissue culture

techniques efficiently assist in the commercial production of asparagus varieties and hybrids and the constitution and maintenance of nuclear stocks free from viruses is essential for producing healthy plantlets to be used in new plantings.

Although AV-1 appears to have a worldwide distribution and is considered one of the agents responsible for asparagus decline, its molecular properties have not been studied yet. To our knowledge, this is the first communication of a partial CP-encoding nucleotide sequence of AV-1 and of the development of a molecular diagnostic assay for AV-1.

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