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Phytoplasma infection of tomato in central Italy

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Summary. Tomato plants (*Lycopersicon esculentum* Mill.) showing leaf yellowing or reddening, proliferation of lateral shoots, hypertrophied calyces, and greening of petals were found in four tomato-growing areas of central Italy during surveys carried out in 1999–2000. The highest disease incidence was recorded in 1999 (78%) and 2000 (82%) near Ronciglione (Viterbo province). The phytoplasmas associated with the disease were detected and characterised using PCR and RFLP. They belonged to 16S rRNA groups I, III, V and XII (*sensu* Lee *et al*., 1998. *Int. J. Syst. Bacteriol.*, 48, 1153). Forty-five percent of tomato plants were infected with group I (max. 52% near Frascati and Ronciglione), 31% with group XII (max. 39% near Ronciglione) and 24% with other groups (max. 33% near Tarquinia and 23% near Fondi). Seven percent of tomato plants were infected with both group I and group XII. This is the first report of a group-III and a group-V phytoplasma found in tomato.

Key words: stolbur, 16Sr-I, 16Sr-III, 16Sr-V and 16Sr-XII rRNA group.

Introduction

Tomato (*Lycopersicon esculentum* Mill) is one of the most economically important crops in Italy, but its production is limited by many insect pests and disease problems, including phytoplasma diseases, which cause heavy economic losses.

Phytoplasma infections of tomato have been reported from several areas in the Mediterranean basin (Zimmermann-Gries and Klein, 1978; Alivizatos, 1989; Vibio *et al*., 1996), the USA (Dale and Smith, 1975) and Australia (Samuel *et al*., 1933; Gibb *et al*., 1996). Different phytoplasmas have been identified using PCR with universal, group- or species-specific primers and RFLP analysis of 16S rDNA amplicons (Gibb *et al*., 1995; Marcone and Ragozzino, 1995; Boudon-Padieu *et al*., 1996).

Phytoplasma diseases of tomato occur in all the main tomato-growing regions in Italy such as Apulia (Martelli *et al*., 1969), Calabria (Albanese *et al*., 1998), Campania (Marcone and Ragozzino, 1995), Basilicata, (Marcone *et al*., 1997), Sicily (Polizzi *et al*., 1990), Sardinia (Lovisolo *et al*., 1982; Lisa *et al*., 1983; Minucci and Boccardo, 1997), Veneto, Piedmont (Marzachì *et al*., 2000), and Emilia Romagna (Favali *et al*., 2000). Symptoms of phytoplasma diseases in tomatoes have also been reported in Latium (Ciccarone, 1951) but the precise aetiology was not determined at that time.

In Latium tomatoes are grown mainly in three provinces: Latina, Viterbo and Rome. In northern

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Latina about 400 ha of processing tomatoes and about 300 ha of fresh market tomatoes are cultivated, while in southern Latina about 650 ha of fresh market tomatoes are grown. Near Rome the acreage is about 75 ha for undetermined fresh market tomatoes, about 65 ha for determined fresh market tomatoes and about 100 ha for processing tomatoes. In Viterbo almost all tomatoes (about 1,500 ha) are grown for processing, especially near Tarquinia. The aim of this study was to determine the losses due to phytoplasmas and identify the phytoplasmas involved. Field surveys of symptomatic plants were carried out followed by molecular diagnosis to identify the phytoplasmas involved. Disease incidence in 1999–2000 was monitored.

Materials and methods

Field surveys

Tomato plants were collected in the summer of 1999 and 2000 from four tomato-growing areas near Frascati (Rome), Tarquinia and Ronciglione (Viterbo), and Fondi (Latina). One field with symptomatic plants was surveyed in each area.

Twenty-eight tomato plants were randomly collected from each field and rated for phytoplasma disease symptoms. The percentage of diseased plants for 1999 and 2000 was calculated. Values recorded in 1999 and 2000 were compared using the χ^2 test (Lison, 1968).

Phytoplasma diagnosis and characterisation

DNA was extracted from 1 g of freshly diced leaf midribs, following the enrichment procedure described by Ahrens and Seemüller (1992). DNA was extracted from both asymptomatic and symptomatic plants. DNA extracted from healthy periwinkle (*Catharanthus roseus*) and from periwinkle infected with chrysanthemum yellows (16Sr-I), stolbur (16Sr-XII), apple proliferation (16Sr-X), Western X-disease (16Sr-III), Italian clover phyllody (16Sr-III), and elm yellows (16Sr-V) (*sensu* Lee *et al*., 1998) from the Istituto Sperimentale per la Patologia Vegetale (Roma, Italy) phytoplasma-collection was used as control.

Thermocycling parameters used for all primer pairs were identical to those reported in the literature. The PCR products were analysed by electrophoresis in horizontal (1%) agarose gels buffered in TBE (45 mM Tris-borate, 1 mM ethylenediaminetetracetate Na salt (EDTA), pH 8.0). DNA bands were then visualised using a UV (254 nm) transilluminator after staining with 0.5 µg/ml ethidium bromide.

P1/P7 PCR amplification products from symptomatic tomatoes collected in summer 2000 as well as the reference periwinkle strains were digested with *Alu*I and *Rsa*I restriction endonucleases according to the manufacturer's instructions (Promega, Madison, WI, USA) in 20 µl reaction mixture containing 5 units of endonuclease for 3 h at 37°C. The resulting digests were separated by electrophoresis through 0.8% agarose gels with ΦX174/*Hinf*I (Gibco-BRL, UK) and 100 bp (New England BioLabs, Beverly, MA, USA) molecular markers in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Restriction fragments were stained with ethidium bromide and visualised by UV transillumination as described above.

For tissue printing freshly cut surfaces of tomato stems or petioles were pressed onto nylon membrane (N^+ Hybond, Amersham Pharmacia Biotec, Little Chalfont, UK) which was then UV-crosslinked. *In situ* denaturation with 0.5 M NaOH and neutralisation with 1 M Tris-HCl of the tissue print, both for 5 min, was done before adding the hybridisation solution. A digoxigenin-labelled riboprobe, cloned from a random fragment of the stolbur phytoplasma genome, was used. The oligonucleotide pair fM1/rP8 were designed on its sequence. Pre-hybridisation, hybridisation and post-hybridisation washing conditions were as described by Marzachì *et al.* (2000).

Results

Tomato plants generally showed stunting, yellowing or reddening of leaves, proliferation of lateral shoots and adventitious roots, hypertrophied calyces, greening of flower petals, inhibition of anther and ovary formation, flower buds with fused and enlarged sepals, plant apices generally lacking leaves, and with the youngest leaves very small, distorted, and yellowish-green. A reduced number of fruits was present on old branches (Fig. 1).

Surveyed fields showed up to 60% of infected plants. Seventy-seven percent (87/112) and 82% (92/112) of samples showed symptoms of phytoplasma disease in 1999 and 2000 respectively.

PCR amplified products were not obtained from asymptomatic tomatoes, healthy periwinkles or reaction mixtures without template used as controls. PCR amplification gave products of the expected molecular size, according to the primer pairs used, from phytoplasma-infected periwinkle and infected tomato samples.

P1/P7 primers (Schneider *et al*., 1995) revealed the presence of phytoplasma DNA in 63% (71/112) of diseased tomato plants collected in the 1999 and in 77% (86/112) in 2000.

The highest disease incidence, recorded near Ronciglione, was 78% (22/28) in 1999 and 82% (23/28) in 2000. Disease incidence was greater by 17% in 2000. The greatest year-one-year increase of disease incidence (50%) was found near Tarquinia (Table 1).

P1/P7 PCR-positive samples were amplified with group-specific primers. The group-specific P1/Aint (Smart *et al*., 1996) and the species-specific fStol/rStol primers (Maixner *et al*., 1995) were used to detect aster yellows and stolbur phytoplasmas. Forty-five percent (39/86) of diseased tomato plants were infected with phytoplasmas of the aster yellows group, (max. 52%, in Frascati and Ronciglione) while 31% (27/86) were infected with phytoplasmas of the stolbur group, (max. 39% in Ronciglione). fM1/rP8 was more sensitive than fStol/rStol, as also reported by Marzachì *et al*. (2000).

*Alu*I and *Rsa*I RFLP of P1/P7-amplified products (Fig. 2) revealed mixed infection by phytoplasmas that belonged to different 16S rRNA groups. Seven percent (6/86) of tomato plants from Ronciglione, Frascati and Tarquinia had mixed infections of phytoplasmas from groups I and XII. Twenty four percent (20/86) of PCR-positive tomato samples showed restriction patterns typical of phytoplasmas belonging to groups 16Sr-III and V (Table 2).

DNA extracted from tomato samples infected with group III were further analysed by PCR

Table 1. Incidence of phytoplasma disease in four fields in Latium, Italy, in 1999 and 2000.

Tomato fields	Diseased plants		Disease increase	
	1999	2000	(%)	$\chi^2_{(0.05)}$
Tarquinia	(14/28)[a] 50[b]	(21/28)[a] 75[b]	50	3.7
Frascati	(18/28) 64	(21/28) 75	17	0.03
Ronciglione	(22/28) 78	(23/28) 82	5	5.88
Fondi	(17/28) 60	(21/28) 75	24	0.74
Total	(71/112) 63	(86/112) 77	21	2.86

[a] Number of diseased over total plants.
[b] Percentage of diseased over total plants.

Table 2. Percentage of phytoplasmas from different 16S rRNA groups detected in tomato plants in Latium, Italy, in the summer 2000. It was not possible to characterise the phytoplasmas present in some positive samples because of the unclear RFLP patterns.

Tomato fields	Phytoplasmal agents					
	Diseased plants	Group I	Group XII	Mixed infection	Group III	Group V
Tarquinia	(21/28)[a] 75[b]	(7/21)[a] 33[b]	(6/21)[a] 29[b]	(2/21)[a] 10[b]	(3/21)[a] 14[b]	(4/21)[a] 19[b]
Frascati	(21/28) 75	(11/21) 52	(5/21) 24	(1/21) 5	(3/21) 14	(2/21) 9
Ronciglione	(23/28) 82	(12/23) 52	(9/23) 39	(3/23) 13	(2/23) 9	(1/23) 4
Fondi	(21/28) 75	(9/21) 43	(7/21) 33	n.d.	(2/21) 9	(3/21) 14
Total	(86/112) 77	(39/86) 45	(27/86) 31	(6/86) 7	(10/86) 12	(10/86) 12

[a], [b], See Table 1.
n.d., not determined.

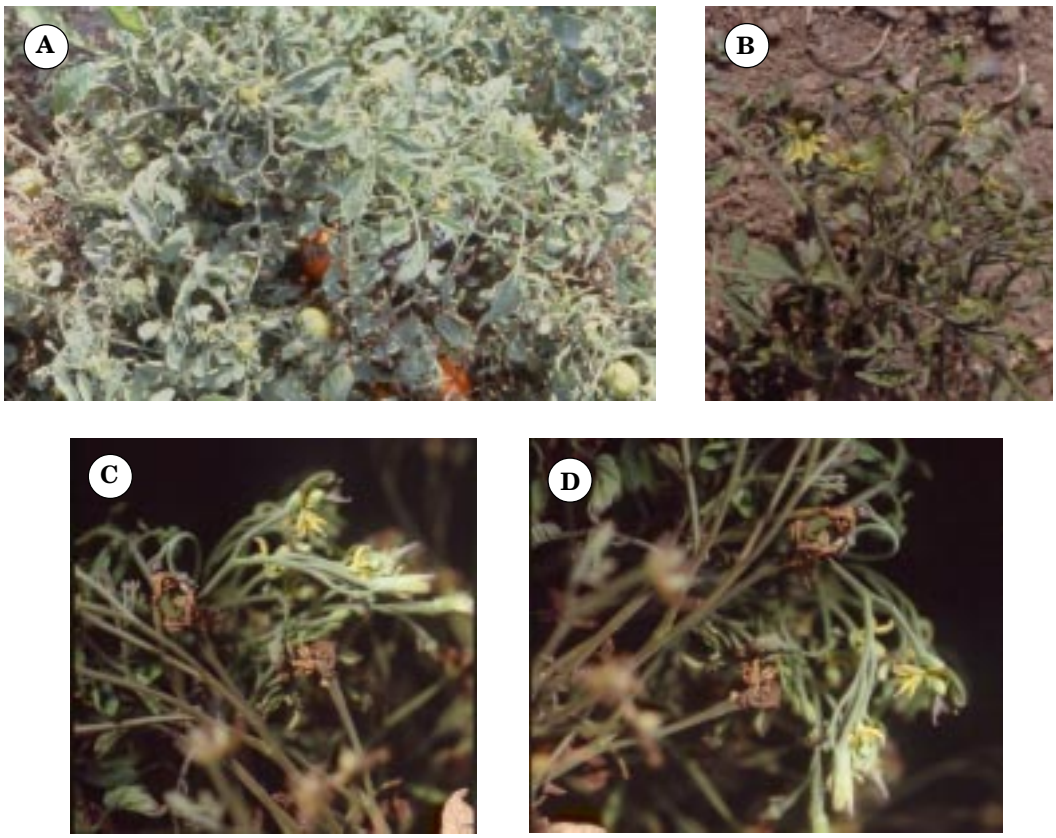

Fig. 1. Healthy (A) and phytoplasma infected tomato plant showing stunting (B), virescent flower (C), abnormally enlarged calices with completely joined sepals and abort of flowers (D).

with P1/P4 primers followed by RFLP analysis of *Alu*I-digested PCR products (Firrao *et al*., 1996). A restriction pattern profile similar to that for Italian clover phyllody was found (data not shown).

DNA extracted from tomato samples infected with group-V phytoplasmas were also amplified with P1/Ulws (Schneider *et al*., 1995), and amplicons of the expected size (1500 bp) were obtained (data not shown).

Tissue printing was nearly as sensitive as PCR for stolbur detection. The stolbur-specific riboprobe hybridised with 89% of samples that tested positive for stolbur with PCR using primers fM1/rP8.

Discussion

Tomato plants showing big-bud symptoms were infected with phytoplasmas from different groups, either in single-group infections or in mixed-group infections. Phytoplasmas from groups III and V were found for the first time in tomato.

Subgroup IB phytoplasmas are the most widespread in Italy (Marzachì *et al*., 2000) and are vectored by different leafhoppers (Fos *et al.,* 1992). Stolbur has also been detected in other solanaceous crops besides tomato, in grape and in a number of wild species (Maixner *et al*., 1995; Marzachì *et al*., 2000).

The Italian clover phyllody phytoplasma has also been linked to clover phyllody disease (Del Serrone *et al*., 2000) in northern Italy, and to decline of the Judas tree (Del Serrone and Pilotti, 1999) in Latium.

Group-V phytoplasmas have been found in wild rubus (Marcone *et al*., 1994; Poggi Pollini and Del Serrone 2000, unpublished data). The vector of this phytoplasma is not known.

Phytoplasmas cannot be controlled by the classical chemical treatments and at present there is no method to cure phytoplasma-infected plants.

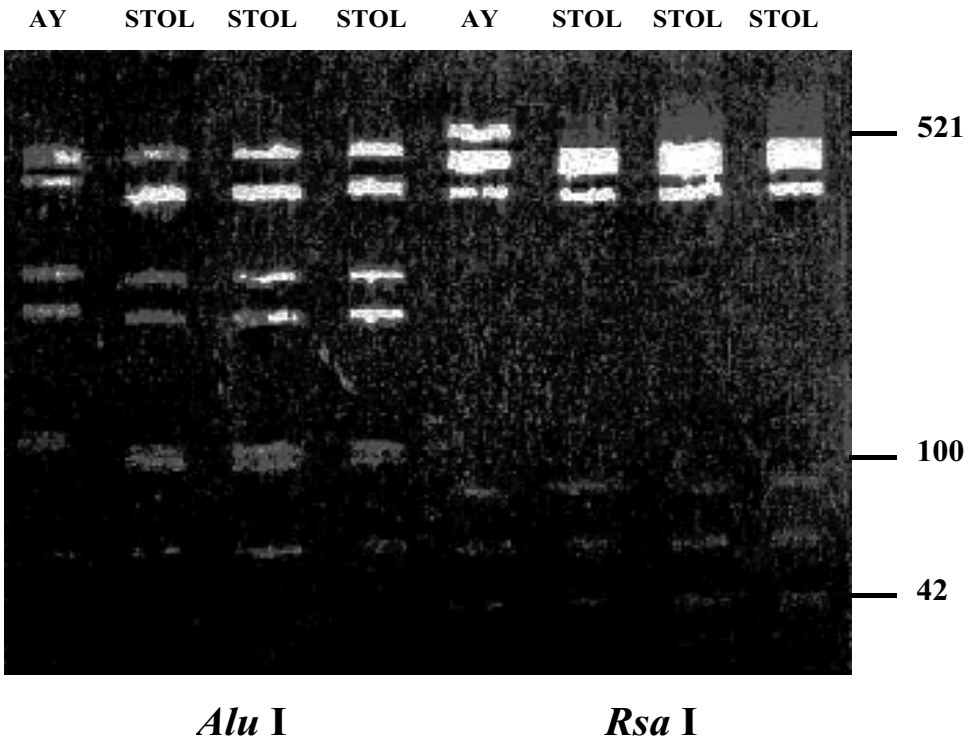

Fig. 2. *Alu*I (A) and *Rsa*I (B) restriction banding patterns of polymerase chain reaction amplified rDNA (with primers P1/P7) of tomato big bud (group I) and stolbur phytoplasmas (group XII) in naturally infected tomato plants. AY = aster yellows phytoplasma (tomato big bud), STOL = stolbur.

Phytoplasma-infection of tomato in Latium was greater in the second year studied. This highlights the need to control insect vectors as well as weeds, as both constitute a reservoir for infection. Our results also show that stolbur infection of tomato can be routinely assayed by tissue printing with a specific riboprobe and this is an important finding that will speed up diagnosis.

It was not possible to correlate specific symptoms with the occurrence of particular phytoplasmas. Failure to amplify DNA from the symptomatic samples using PCR, may be due to inhibitors and/or the low titre of the pathogen in some plants. The role of the genetic background of tomatoes and the influence of biotic or abiotic stresses on tomato phytoplasma disease expression still deserve further investigation.

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