INCIDENCE OF VIRUSES INFECTING SPINACH IN GREECE, HIGHLIGHTING THE IMPORTANCE OF WEEDS AS RESERVOIR HOSTS

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SUMMARY

The aim of this survey was to identify viruses infecting spinach (*Spinacia oleracea* L.) in the most important spinach-producing areas in Greece. A total of 1074 spinach samples were collected from eleven districts belonging to the prefectures of Thessaloniki, Chalkidiki and Imathia in northern Greece, and Evia in central Greece. Samples were tested by ELISA, mechanical inoculation onto indicator plants and immunoelectron microscopy. *Beet western yellows virus* (BWYV), *Cucumber mosaic virus* (CMV) and *Turnip mosaic virus* (TuMV) were identified in 13.5%, 7% and 5.4% of samples, respectively, infected samples being detected in all regions examined. This is the first record of CMV and TuMV infecting spinach in Greece, and the first report of BWYV occurrence in any crop nationwide. Surveys were also conducted to assess the potential reservoir hosts of BWYV, CMV and TuMV in weeds collected from spinach fields. All three viruses were detected among 125 samples tested by ELISA. TuMV prevailed as it occurred in 14.4% of all weed samples.

Key words: spinach, virus incidence, BWYV, CMV, TuMV, ELISA, weeds.

INTRODUCTION

Spinach (*Spinacia oleracea* L., family Chenopodiaceae) is an annual dioecious plant, yielding a quick crop in cool, damp weather. In hot, dry weather the plants often run to seed before they have made sufficient leaf growth, but in warm climates they produce an excellent crop in late winter, performing best at 16-18°C. Spinach is an economically important vegetable crop in many countries which, in Greece, is grown annually for fresh and processed (frozen and canned) consumption on *ca.* 3,800 ha with an annual yield of 43,000 tonnes, i.e. about 7.9% of the total European Union production (FAOSTAT, 2011). Its commercial value was estimated to *ca.* 23.7 million € for 2003, according to the latest available statistics from the Hellenic Ministry of Agriculture (2011). Major spinach-producing regions of Greece include, among others, the prefectures of Thessaloniki, Chalkidiki and Imathia (northern Greece), and Evia (central Greece).

As with most agricultural commodities, diseases impose significant production constraints affecting both the yield and the overall quality of the crop. Although many diseases of spinach have been reported (Walker, 1952; Farr *et al*., 1989), only certain fungal and viral infections are generally recognised as economically important, while bacterial diseases are negligeable (Correll *et al*., 1994). Some 109 different viruses have been found in spinach (Brunt *et al*., 1996), but only 15 naturally occurring virus diseases have been reported in the USA and Europe (Wilson, 1983; Halliwell and Johnson, 1988). Only a few of these are considered to be of economic importance in Europe (Smith *et al*., 1988).

Information on viral infections of spinach in Greece is limited (Kyriakopoulou, 1998), and no specific data on their incidence are available. Therefore, an extensive survey was carried out to study the occurrence of spinach-infecting viruses in the country, both in spinach crops and in the wild flora, for in insight in their epidemiology.

MATERIALS AND METHODS

Sampling. A total of 1074 samples consisting of young leaves from plants showing virus-like symptoms such as stunting, deformation, mosaic, and yellowing were collected from spinach fields at eleven locations from Thessaloniki, Imathia and Chalkidiki (northern Greece), and Evia (central Greece) (Fig. 1). All samples were kept at 0-4°C and tested by ELISA as quick as possible, while part of them was stored at -80°C for mechanical inoculations. To fully examine the frequency of mixed infections, a more detailed survey of symptomatic plants was repeated in Thessaloniki where some mixed infections had been identified in the course of

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Fig. 1. Sampling areas of infected spinach plants in Greece.

the first survey. Finally, a total of 125 weed samples were collected randomly from the Nea Ionia area in the Thessaloniki municipality either from existing spinach fields or from fields previously cultivated with spinach. Weeds were randomly collected, irrespective of the presence of symptoms (most of them were symptomless), the sampling unit consisting mainly of youngest fully expanded leaves and flowers, when present.

ELISA Information on the antisera used in this study is given in Table 1. All spinach and weed samples were tested by double antibody sandwich (DAS), triple antibody sandwich (TAS) and antigen coated plate (ACP) ELISA, as described (Koch and Salomon, 1994; Barg, 1996). All samples were tested for the presence of *Alfalfa mosaic virus* (AMV), *Bean yellow mosaic virus* (BYMV), *Beet mosaic virus* (BtMV), *Beet western yellows virus* (BWYV), *Broad bean wilt virus* (BBWV), *Cucumber mosaic virus* (CMV), *Lettuce mosaic virus* (LMV), *Spinach latent virus* (SpLV), *Tomato spotted wilt virus* (TSWV), *Turnip mosaic virus* (TuMV) and *Watermelon mosaic virus* (WMV), while weed samples were tested only for the viruses found in spinach. Samples were homogenized in PBS-Tween using an approximate ratio of 1 g tissue/10 ml buffer. IgGs of all antisera were purified by adsorption to protein-A (Sambrook *et al*., 1989). All antisera were diluted with PBS-Tween. Alkaline phosphatase-IgG conjugates were diluted with PBS-Tween containing 2% polyvinylpyrrolidone (PVP) and 0.2% egg albumin. All incubation steps were performed at 37°C for 3 h, except for incubation following sample addition, which was overnight at 4°C. DAS-ELISA was routinely used for detection of CMV, AMV, SpLV, TSWV, BYMV, BBWV1, BBWV2, BWYV, LMV and BtMV. TuMV was detected by TAS-ELISA using culture supernatants containing the monoclonal antibody EMA 67 diluted 1/2500. ACP-ELISA was used for WMV detection (Koch and Salomon, 1994). Plates were coated by adding 100 µl of 1 µg/ml IgGs in each well. Alkaline phosphatase-conjugated IgGs were diluted as shown in Table 1. All tests were performed using 'Nunc Immunoplate II' 96 well microtitre plates (Gibco, UK) while optical density (O.D.)

Table 1. Origin and optimal dilutions of antisera used in this study. "-" indicates lack of conjugate. All antisera were polyclonal raised in rabbit, apart from that provided by Dr. C.E. Jenner against TuMV (EMA 67) which is a monoclonal raised in mouse.

Specificity	Optimal dilution of antiserum	Optimal dilution of conjugate	Source
TSWV	1:1000	1:1000	Aristotle University of Thessaloniki
SpLV	1:250	1:250	Aristotle University of Thessaloniki
CMV	1:1500	1:500	Aristotle University of Thessaloniki
WMV	1:1250	-	Aristotle University of Thessaloniki
TuMV	1:1000	-	IPO-DLO, Holland
-//-	1:30	-	Dr. C. Jenner, HRI, U.K.
LMV	1:1000	1:1000	DSMZ GmbH, Germany
BYMV	1:500	1:500	Aristotle University of Thessaloniki
AMV	1:1500	1:1500	Aristotle University of Thessaloniki
BtMV	1:500	1:500	DSMZ GmbH, Germany
BBWV1	1:1000	1:1000	DSMZ GmbH, Germany
BBWV2	1:500	1:500	DSMZ GmbH, Germany
BWYV	1:1000	1:1000	DSMZ GmbH, Germany

at 405 nm was measured with a Tosoh MPR A4i microplate reader. A sample was considered positive if the absorbance reading was more than twice that of a healthy plant sap extract.

Immunoelectron microscopy. Electron microscopy was done following the decoration technique described by Hill (1984), using a Jeol 1200X transmission electron microscope. For virus identification particles from crude leaf extracts in 0.1 M phosphate buffer, pH 7.0, were trapped on carbon-coated plastic grids for 15 min, followed by incubation at 37°C for 1 h with virus-specific antisera. Grids were stained with 2% (w/v) phosphotungstic acid solution.

Mechanical transmission. Test plants were selected mainly from known host ranges of spinach viruses reported by Brunt *et al.* (1996). All viruses recovered were back-inoculated to spinach cv. Polka to fulfill Koch's postulates. For mechanical transmission (Chatzivassiliou *et al.*, 2004), leaf tissues were ground in a mortar in the presence of 0.01 M phosphate buffer pH 7, containing 0.1% sodium sulfite (Na_2SO_3). A number of spinach samples showing virus-like symptoms but negative in ELISA were sap-inoculated to the indicator species *Chenopodium quinoa*, *Nicotiana tabacum* cv. Samsun NN and *S. oleracea* cv. Polka. to find out whether they were infected by viruses other thant those looked for by ELISA.

For CMV and TuMV detection, spinach samples infected only by CMV or TuMV and showing high ELISA readings were used for sap inoculation to a total of 18 test plants. Sap from herbaceous indicators that did not show symptoms was inoculated onto *Cucumis sativus* and *C. quinoa* to reveal latent infections by CMV and TuMV, if any. BWYV-infected spinach samples from Thessaloniki area were also sap-inoculated to the following indicator species: *S. oleracea* cv. Polka, *Beta vulgaris* cv. Ultra Moro, *Raphanus sativus* cv. Palla di Neve and *B. rapa* cv. Bency. Healthy samples were tested as negative controls.

RESULTS

In the 1074 spinach samples collected in the couse of the surveys and ELISA-tested for the presence of the viruses listed in Table 1 only BWYV, CMV and TuMV were found with an incidence of 13.5%, 7% and 5.4%, respectively (Table 2). CMV was detected in all four Greek regions inspected, while BWYV and TuMV were found only in three of them. All positive samples had O.D. readings of at least four times that of the average value of two negative controls in each test. Typical symptoms of spinach plants infected in the field by BWYV, CMV and TuMV are shown in Fig. 2 A-C. A more limited survey for mixed infections in some spinach fields in Thessaloniki revealed mixed infections with two or all three of the above viruses (Table 3), the most common being a combination of CMV and BWYV.

Table 2. Incidence (%) of CMV, BWYV and TuMV infection in spinach crops from different regions of Greece.

Region		Virus incidence (% of plants)		
	No. of samples	BWYV	CMV	TuMV
Chalkidiki	152	23.7	2	0
Thessaloniki	726	10.3	7	4.5
Imathia	83	0	1.2	1.2
Evia	113	30	17.7	21.2
Total	1074	13.5	7	5.4

Table 3. Incidence of various combinations of mixed infections of CMV, BWYV and TuMV in 96 spinach samples from Tessaloniki area.

Region	Number of mixed infection samples			
	CMV+TuMV	CMV+BWYV	TuMV+BWYV	CMV+TuMV+BWYV
Thessaloniki	5/96 (5.2%)	12/96 (12.5%)	5/96 (5.2%)	6/96 (6.2%)

Mechanical transmission. Some symptomatic plants were ELISA-negative and failed to produce infections to mechanically inoculated hosts including back-inoculated spinach, suggesting that they were not infected by any readily mechanically transmissible virus or that the symptoms shown could be due to genetic abnormalities, pesticide toxicity, or nutritional disorders (mineral excesses or deficiency)

CMV-infected spinach crops reproduced the expected symptoms on indicator plants, including chlorotic local lesions in *C. amaranticolor* and *C. quinoa*, systemic mosaic in *C. sativus* and necrotic local lesions in *Vigna unguiculata* (Table 4). In addition, all symptoms observed in CMV-infected spinach samples in the field were successfully reproduced by mechanical inoculation, including distinct stunting of the plant, progressive chlorosis starting from the younger leaves then expanding to the entire foliage, severe curling of younger leaves and necrosis of parts of the older leaves, usually progressing from the outer tips inwards Similarly, TuMV-infected spinach plants induced the expected symptoms on indicator plants (Table 4). Symptoms exhibited by field-infected spinach were successfully reproduced on mechanically inoculated spinach, i.e diffuse yellow systemic mosaic and curling of the inner young leaves (not shown).

As expected, inoculations onto herbaceous hosts from none of the BWYV-infected spinach induced symptoms, confirming the fact that BWYV is not sap-transmissible (Correll *et al*., 1994; Brunt *et al*., 1996). Vector transmission of this virus was not attempted.

Electron microscopy. Isometric particles 26 and 29 nm in diameter were detected and identified as BWYV or CMV virions based on decoration with the respective

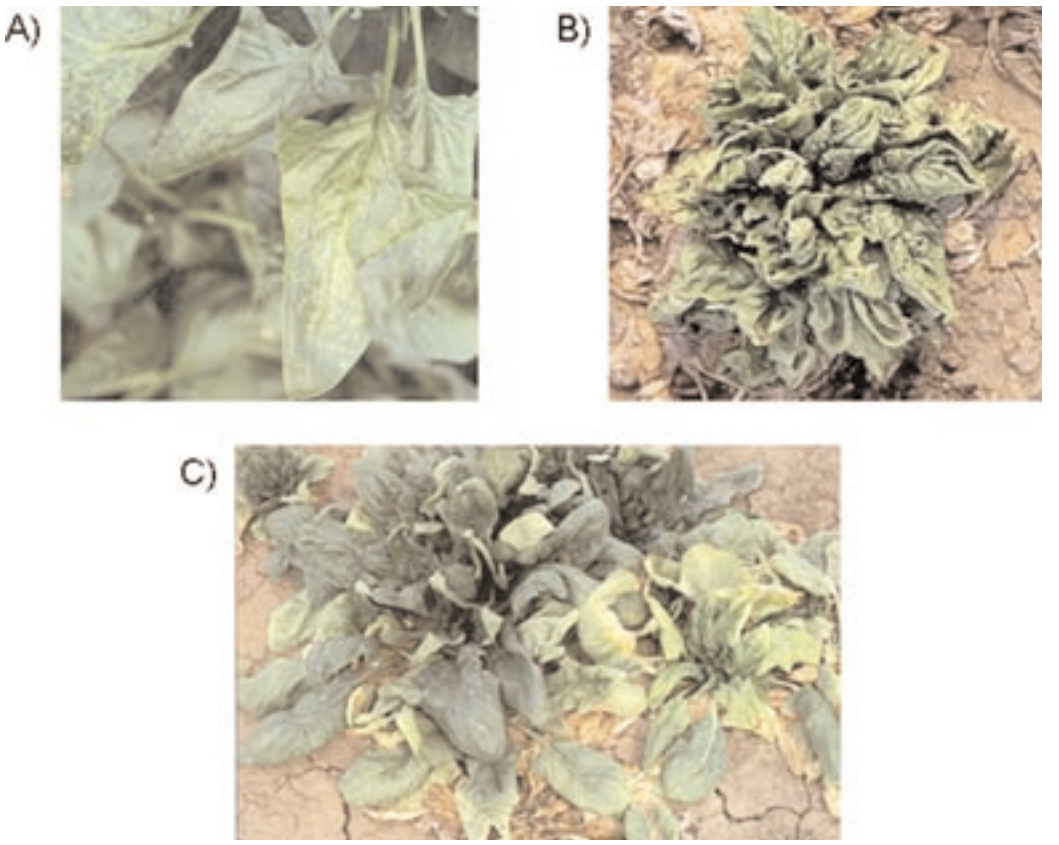

Fig. 2. Symptoms of infected spinach plants in the field. A. Spinach plant infected with TuMV and BWYV. B. BWYV-infected spinach plant showing curling of outer leaves, necrosis of crown leaves and general stunting. C. Spinach plant infected with CMV (on the right): plant is severely stunted, the entire foliage is completely chlorotic, younger leaves show an inward rolling of thee margins, while the crown leaves are necrotic. All photographs taken in fields from Thessaloniki.

Table 4. Host responses to mechanical inoculation with CMV and TuMV.

Host	CMV	TuMV
Amaranthaceae		
Beta vulgaris cv. Maribo Ultramoro	LI	NI
Chenopodium amaranticolor	CLL	CLL
C. quinoa	CLL	CLL
Spinacia oleracea cv. Polka	St, SLC, Cu	CLL, SM, Cu,
Asteraceae		
Cichorium endiva cv. President	NI	NI
Brassicaceae		
Brassica oleracea var. *gemmifera*	NI	NI
B. rapa cv. Bency	NI	SM
Raphanus sativus cv. Palla di Neve	NI	SYMo
R. sativus cv. Supremo	NI	NI
Cucurbitaceae		
Citrullus lanatus cv. Galaxy	NI	NI
Cucumis melo cv. Nobel	NI	NI
C. sativus cv. Cetriolo Lungo	SM	NI
Cucurbita pepo	SM	NI
Fabaceae		
Phaseolus vulgaris cv. Green Crop	NI	NI
Vicia faba	NI	NI
Vigna sinensis	NLL	NI
Solanaceae		
Nicotiana glutinosa	LI	NI
N. tabacum cv. Samsun NN	LI	NI

CLL: chlorotic local lesions; St: stunting; SLC: systemic leaf chlorosis; NI: not infected; SM: systemic mosaic; NLL: necrotic local lesions; SYMo: systemic yellow mottle; Cu: curling; LI: latent infection.

Table 5. Incidence of BWYV, CMV and TuMV in weeds sampled in spinach plots.

Weed species	Common name	Number of samples infected with:		
		CMV	BWYV	TuMV
Asteraceae				
Aster spp.		0/3 (0%)	1/3 (33.3%)	3/3 (100%)
Cirsium arvense	Canada thistle	0/1 (0%)	1/1 (100%)	0/1 (0%)
Conyza canadensis		1/5 (20%)	2/5 (40%)	1/5 (20%)
Sonchus oleraceus	Annual sowthistle	0/1 (0%)	1/1 (100%)	0/1 (0%)
Xanthium strumarium	Heartleaf cocklebur	0/1 (0%)	0/1 (0%)	1/1 (100%)
Chenopodiaceae				
Amaranthus spp.	Pigweed	0/1 (0%)	0/1 (0%)	0/1 (0%)
Amaranthus retroflexus	Redroot pigweed	0/38 (0%)	1/38 (2.6%)	1/38 (2.6%)
Chenopodium album	Lambsquarters	0/15 (0%)	0/15 (0%)	10/15 (66.6%)
Convolvulaceae				
Convolvulus arvensis	Field bindweed	0/2 (0%)	2/2 (100%)	0/2 (0%)
Cyperaceae				
Cyperus rotundus	Purple nutsedge	0/4 (0%)	2/4 (50%)	2/4 (50%)
Portulacaceae				
Portulaca oleracea	Common purslane	1/18 (5.5%)	0/18 (0%)	0/18 (0%)
Solanaceae				
Solanum elaeagnifolium	Silverleaf nightshade	1/6 (16.6%)	0/6 (0%)	0/6 (0%)
Solanum nigrum	Black nightshade	0/12 (0%)	0/12 (0%)	0/12 (0%)
Zygophylaceae				
Tribulus terrestris	Puncturevine	0/2 (0%)	0/2 (0%)	0/2 (0%)
Poaceae				
Setaria verticilata	Bristly foxtail	0/2 (0%)	0/2 (0%)	0/2 (0%)
Sorghum halepense	Johnsongrass	0/14 (0%)	0/14 (0%)	0/14 (0%)
Total		3/125 (2.4%)	10/125 (8%)	18/125 (14.4%)

antisera, and filamentous particles 720 nm long were detected in the case of TuMV-infected samples (not shown).

Weeds as virus reservoirs. The three viruses found in spinach (CMV, BWYV and TuMV) were also identified in a number of weed species, TuMV being the prevailing one followed by BWYV and CMV (Table 5). Infection levels were very high in the case of TuMV, which infected approximately 15% of all weeds sampled, covering four different plant families. However, mechanical transmission tests produced variable results, i.e. all three CMV-infected samples produced local chlorotic lesions on *C. quinoa*, whereas TuMV-infected samples of only two weed species (*C. album* and *Xanthium strumarium*) produced symptoms in the test plants. As expected, no mechanical transmision was obtained from any of the BWYV-infected samples.

DISCUSSION

To our knowledge, only two published reports provide epidemiological information on spinach viruses, one from the UK (Bailiss and Okonkwo, 1979) and the other from Texas (Halliwell and Johnson, 1988). Thus, the present study represents the third wide range investigation aimed at gathering information on the occurence and prevalence of viruses infecting this crop. The presence of CMV and TuMV in Greek spinach crops was reported earlier by Kyriakopoulou (1998) but virus incidence was not determined. On the other hand, no previous record of BWYV occurrence in any Greek crop seems to exist. Therefore, this represents the first report of this virus form Greece.

The only viruses infecting spinach in the surveyed areas were CMV (7% incidence), TuMV (5.4% incidence) and BWYV (13.5% incidence). Their presence in northern and central Greece, indicates that they have a nation-wide distribution in spinach crops.

TuMV had the lowest infection level, and did not occur in Chalkidiki (northern Greece). This can be explained examining the crop rotation history of this region. The fields inspected in Chalkidiki are located in an area where only tomato and spinach have been grown for many consecutive years. Tomato is a natural host of CMV and BWYV (Brunt *et al*., 1996), but not of TuMV, therefore, it does not constitute a natural source of inoculum for this virus. Conversely, the areas with the highest level of TuMV infection (Evia and Thessaloniki) have a long history of vegetable crop growing. All fields surveyed were surrounded by plots hosting a variety of vegetables, including cauliflower, turnip, eggplant, pepper and lettuce. Since most of these species are natural TuMV hosts (Brunt *et al*., 1996), more than likely they represent inoculum sources.

Mixed infections are common in some fields of Thessaloniki, where 28 of 96 samples were infected with at least two of the three viruses. This is not surprising, infection with two or even more viruses being not an uncommon phenomenon (Matthews, 1991). The most frequent combination of viruses was CMV with BWYV (in accordance with individual incidence levels), although every other possible combination of the three viruses was detected in single spinach samples. Such mixed infections may complicate diagnosis based on biological detection methods alone, especially if host responses are variable (Zink and Duffus, 1972).

In our study the presence of CMV, BWYV and TuMV was verified by ELISA in 11 species of weeds belonging to 6 families sampled from spinach-growing fields, thus leading to the conclusion that these weeds act as a source of viral inoculum. An interesting finding was that the infection rate of weeds with each of the viruses detected does not seem to tally with that observed in spinach. In fact, CMV, the prevalent spinach-infecting virus the area of Nea Ionia (Thessaloniki) with an infection rate of about 59%, occurs only in less than 2.5% of the weeds. The prevalence of this virus in spinach can be explained by the fact that it has an extremely wide host range of about 750 species, most of which are important agricultural and horticultural crops (Smith *et al*., 1988). Many of these crops are actually grown in the area of Nea Ionia, acting as inoculum sources for the virus. All the infected weed species are alleged hosts of the viruses found in them (Bourbos and Skoundridakis, 1990; Shattuck, 1992; Johnstone and Duffus, 1984).

The presence of viruses in spinach crops and associated wild flora, represent a threat for the Greek spinach industry and calls for the implemetation of measures to prevent destructive virus spread and subsequent crop losses. The elimination of weeds that constitute the main virus and vector reservoirs, is generally suggested as an effective procedure for managing viral epidemics (Thresh, 1981). Other control measures include the use of virus-free seed and modified planting and harvesting procedures (Matthews, 1991). In any case, the most efficient way currently used to control viral diseases is the use of resistant cultivars. A number of such spinach cultivars have been produced by classical breeding, espressing resistance to TuMV (Edwardson and Christie, 1991) and CMV (Pound *et al*., 1962). However, a promising stategy for virus disease control is the development of virus-resistant transgenic spinach plants (Provvidenti and Hampton, 1992; Al-Khayri e*t al*., 1993). Such plants could minimize disease control costs and at the same time produce satisfactory yields, thus maximizing growers' profit.

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Received October 19, 2010
Accepted December 31, 2010