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High incidence of *Pelargonium line pattern virus* infecting asymptomatic *Pelargonium* spp. in Spain

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Abstract

Geraniums (*Pelargonium* spp.) are traditional ornamental plants largely cultivated in Europe and northern America. Vegetative propagation makes them prone to viral infections, which have detrimental effects on crop production and quality. Asymptomatic samples collected in Spain were tested for a range of viruses using ELISA. The tobamovirus, *Tobacco mosaic virus* (TMV), the cucumovirus, *Cucumber mosaic virus* (CMV), and several viruses in the family *Tombusviridae*, namely, *Pelargonium line pattern virus* (PLPV), *Pelargonium flower break virus* (PFBV), and *Pelargonium leaf curl virus* (PLCV), were detected either singly or in combination in 59.2% of 800 samples. PLPV and PFBV infections were confirmed by dot-blot hybridisation. The most relevant viral infection found on Spanish asymptomatic geraniums was by *Pelargonium line pattern virus* (PLPV). Symptoms did not develop for 3 years on most of the PLPV infected geranium plants under greenhouse conditions.

Introduction

Geraniums (*Pelargonium* spp.) are one of the most popular plants for indoor and outdoor use. However, their production often is affected negatively by different factors, prominent among which is damage provoked by diverse viral pathogens. These infectious agents usually do not kill the plants but they reduce their growth and quality by inducing distortions of inflorescences, white flower streaking, chlorotic/necrotic spotting of leaves and stunting (Stone, 1980; Welvaert and Samyn, 1985). This is also the case for other vegetatively propagated ornamentals such as *Alstroemeria* (Spence et al., 2000), petunia (Spence et al., 2001; Sánchez-Cuevas and Nameth, 2002), *Dianthus* (Sánchez-Navarro et al., 1999; Fraga et al., 2004a) or *Phlox* (Fraga et al., 2004b). In recent years a progressive increase in viral infections of geranium has been observed in western Europe. Viruses infecting geraniums have been described by Nameth and Adkins (1993). This increase may have been facilitated by the practice of vegetative propagation and by the symptomless condition of many plants yielding viral isolates. Viruses infecting *Pelargonium* spp. have been described in Canada (Kemp, 1967), Spain (Peña-Iglesias et al., 1974), Denmark (Paludan, 1976), Greece (Vovlas and Grigoriu, 1977), the United Kingdom (Stone, 1980), Belgium (Welvaert et al., 1982), The Netherlands (Bouwen and Maat, 1992) and Israel (Franck and Loebenstein, 1994). In Spain's temperate climate, geraniums are kept as perennials and can serve as reservoirs for new infections in both ornamental geraniums and horticultural plants. Cuttings are usually obtained from mother-stock plants free of viral pathogens.

The European Directive 93/49/EE indicates that the *Pelargonium* spp. propagating material and plants must, at least on visual inspection, be substantially free from any harmful organisms and diseases, which impair quality or any signs or

symptoms. In particular they also must be free from *Xanthomonas pelargonii*, and viruses and virus-like organisms, specifically the carmoviruses *Pelargonium flower break virus* (PFBV), *Pelargonium line pattern virus* (PLPV), the *tombusvirus Pelargonium leaf curl virus* (PLCV), and the tospoviruses *Tomato spotted wilt virus, Impatiens necrotic spot virus*.

In order to evaluate the spread of these diseases a survey of asymptomatic cultivated ornamental geraniums was made throughout the 11 continental regions of Spain over 4 years (2000–2003). We report on the virus frequency and distribution using an ELISA screening of 800 samples.

Materials and methods

Sampling

Pelargonium zonale (*Pelargonium* × *hortorum* Bailey), *P. peltatum* or *P. grandiflorum* cuttings without symptoms (apparently healthy) were collected in 2001, 2002 and 2003 throughout rural areas of Spain representing different bioclimatic zones. Andalucía, Extremadura and Castilla-La Mancha are dry regions with very hot summers and milder winters. Asturias, Euskadi and Galicia are humid regions with mild summers and mild winters. Cataluña and Valencia have mild weather throughout the year and Madrid, Navarra and Castilla-León have hot dry summers with cold winters. Sample collection was performed between April and September when the geranium plants were in full bloom for all but 47 samples, which were collected in November from Andalucía and Galicia in 2003. Cuttings were rooted in 4 cm plugs of blond turf. These plants were maintained under greenhouse conditions with a day temperature of 20 ± 4 °C and night temperature of 16 ± 4 °C in the winter and 32 ± 4 and 20 ± 4 °C, respectively, in the summer. Leaves for disease detection were harvested immediately prior to ELISA testing between 1 and 2 weeks after sample collection. If the plants were positive for PLPV, frozen leaves of the same plants were used for the dot-blot hybridisation assay. Rooted cuttings were potted in 17 cm pots and kept under greenhouse conditions. A new ELISA test was performed between 6 months and a year later after the first sample collection.

ELISA

Double direct antibody (DAS) sandwich ELISA was performed following the recommendations of the manufacture and using commercially available antibodies against *Cucumber mosaic virus* (CMV), *Tomato ringspot virus* (ToRSV), *Tobacco ringspot virus* (TRSV), PFBV, PLCV, *Pelargonium zonate spot virus* (PZSV), PLPV, *Tobacco mosaic virus* (TMV), and the tospoviruses, with their derived alkaline phosphatase-conjugates (BIOREBA). Tissue from petioles and apical blade regions of fully expanded leaves was squeezed in 10 volumes (w/v) of extraction buffer (PBST with 2% polyvinylpyrrolidone (PVP), and 100 μl of the resulting homogenate was added to each well of the plate. Three healthy controls and two positive controls were added per plate. Recordings were performed at 405 nm in a microtiter plate reader Biotek-Lx800 and samples were considered positive when the absorbance values were at least two times higher than those of the uninfected control sap. A universal potyvirus-detecting monoclonal antibody (Agdia, USA) was used in plate trapped antigen (PTA) ELISA for determining infections by potyviruses according to the manufacturer's instructions.

Dot-blot hybridisation

Tissues from petioles and apical blade regions of fully expanded leaves were squeezed in 20 volumes (w/v) of extraction buffer (50 mM sodium citrate, pH 8.5), and the homogenates were clarified by centrifugation at 6000 rpm for 5 min. The clarified extracts were spotted (4 μl) onto positively charged nylon membranes (Roche). Dot-blot hybridisations and the preparation of PLPV and PFBV probes were done as described previously (Ivars et al., 2004) using a chemiluminescent detection kit for the DIG-RNA probe as described by the reagents supplier (Roche).

Results and discussion

Virus surveys and symptoms

A total of 800 samples was collected from outdoor geranium pots in both private and public gardens. Samples were taken from a wide range of

geranium varieties (including commercial varieties like Ecco, Greco, Carol, Isabell, Palais, Glacis, Americana, Eclipse, Patriot or Global) produced and maintained using vegetative propagation. It is important to emphasize that samples were only collected from symptomless plants. If any virus symptoms, such as leaf mosaic, chlorotic mottling, vein clearing and distortion of leaves and stems appeared on the plant, no sample was collected. Geranium samples of different ages were tested using ELISA. Negative ELISA values ranged from 0.112 to 0.164, whereas positive values ranged from 0.351 to 2.992. The mean value of the PLPV negative controls was 0.131 with a standard deviation of 0.021.

Samples from 474 (59.2%) plants were found to be virus-infected (Table 1). The majority of samples in which virus was detected was infected with at least one tombus-like virus, either alone or in combination with other viruses, with PLPV being the most common (Table 1). PLPV and PFBV infections were confirmed by dot-blot hybridisation. As described previously (Ivars et al., 2004), even for samples where the ELISA values were as low as between two and three times the negative control, the dot-blot assay was positive. For the cases where lower ELISA values were observed, the ELISA was repeated between six months and a year later. Similar results were again obtained using both methods. In some of the samples, the ELISA values increased a year later, but in most of the samples the levels remained low, especially for PLPV-infected geraniums. Samples were never seen to be infected by potyviruses, tospoviruses, ToRSV, TRSV or PZSV although the potyvirus *Carnation vein mottle virus* has been described as infecting *P. grandiflorum* (Brunt et al., 1996), and the tospoviruses, ToRSV, TRSV and PZSV have been described as infecting *Pelargonium zonale* (Nameth and Adkins, 1993). The lack of evidence for these viruses infecting tested plants could also be due to the fact that these viruses might always be symptomatic when present. TMV and PLCV infections were infrequent (1.8% and 0.3% respectively). TMV- and CMV-infected plants were destroyed so no further symptoms could be evaluated. PLPV infected plants remained symptomless, even 3 years after sampling under controlled greenhouse conditions. This is not surprising, since sampling was performed in order to select less virulent virus strains. Sometimes during the summer when the temperature was higher, very mild symptoms such as chlorotic mottle appeared later when *P. zonale* plants were infected with PFBV alone (Figure 1a) or in combination with PLPV. The described petal-break symptoms in flowers, characteristic of this carmovirus, only appeared in one of the PFBV-infected plants (Figure 1b) when the plant was water-stressed. In the spring and early summer (greenhouse mean temperatures between 25 and 30 °C), an even milder chlorotic mottle appeared in very young leaves when *P. zonale* plants were infected with PLPV (Figure 1c). There was no correlation between variety and the symptoms that appeared in 17 of the 435 infected plants.

A previous preliminary study in Spain identified PLCV- and CMV-infected geraniums, but only 11 symptomatic plants were analysed (Peña-Iglesias et al., 1974). A Spanish isolate was characterised (Diez et al., 1999) from nursery-grown *P. zonale* plants (PFBV-m). A survey of geraniums in Denmark found CMV, PFBV, several little-studied viruses and yellow net vein, a virus-like disease, for which no causal agent has been determined. Those researchers concluded that these viruses had no practical importance to Danish geranium growers (Paludan, 1976). Similar surveys in Belgian nurseries found that about 70% of the

Table 1. A summary of viruses identified singly or in combination as identified by ELISA in 800 *Pelargonium* spp. samples collected in surveys in Spain

Viruses	Number of samples	%
PLPV only	369	46.1
PFBV only	24	3.0
CMV only	11	1.4
TMV only	2	0.3
PLCV only	1	0.1
PFBV and PLPV	38	4.8
CMV and PLPV	16	2.0
PLPV and TMV	5	0.6
CMV, PLPV and TMV	4	0.5
PLPV and PLCV	1	0.1
CMV and TMV	1	0.1
PFBV, PLPV and TMV	1	0.1
CMV, PFBV, PLPV and TMV	1	0.1
Total not infected	326	40.8

Carmoviruses *Pelargonium flower break virus* (PFBV) and *Pelargonium line pattern virus* (PLPV), tombusvirus *Pelargonium leaf curl virus* (PLCV), tobamovirus *Tobacco mosaic virus* (TMV), and cucumovirus *Cucumber mosaic virus* (CMV).

Figure 1. Symptoms developed in greenhouse conditions by *Pelargonium zonale* plants infected by PFBV (a and b) or PLPV (c).

P. zonale plants tested were virus-infected, which is more consistent with our results. They found 80% of their samples were infected with PFBV, 10% with CMV, 10% with TMV and 1.2% with TRSV (Welvaert et al., 1982). As in Spain, ornamental geraniums can be a reservoir for CMV or TMV, which infect other important vegetable crops. In the United Kingdom, Stone (1980) found nine different isometric viruses infecting geraniums including ToRSV, TRSV, PFBV and PLPV.

It is interesting that the most prevalent virus in Spain, PLPV, has been described as being of relatively low economic importance (Nameth and Adkins, 1993). Seasonal fluctuations in the PLPV content of leaf blades and petioles have been reported (Bouwen and Maat, 1992) and this erratic distribution might have led to an underestimation of the true infection rate of this virus. Previous results highlighted the petioles and apical regions of blades of fully expanded leaves as the best material to use for detection assays, since the highest viral accumulation levels for PLPV were observed in these parts of the infected plants (Ivars et al., 2004). The use of these parts of the plant in our study might have helped to increase our detection rate. The fact that most of the infected plants remained asymptomatic for at least 3 years is very important for virus control, since plant material coming from outside the EU is only inspected visually at the Phytosanitary entry control, according to the European Directive 93/49/EE.

Geographical and species distribution

PLPV, CMV and TMV infected all three ornamental geranium species analysed (Table 2). PFBV was not found in *P. grandiflorum*, in which, on the other hand, CMV was more frequent (25%) than in *P. peltatum* or in *P. zonale*. The level of uninfected plants was higher in *P. grandiflorum* (60%) and in *P. peltatum* (61.9%) than in *P. zonale* (only 34.5%). Asymptomatic infections were more frequent in the garden geranium (*P. zonale*), which represents 80% of the market. Therefore, it is likely that a more intensive selection against symptomatic virus has been achieved in the cutting

Table 2. The total incidence of each individual virus as identified by ELISA in 800 samples from three ornamental *Pelargonium* spp. collected in surveys in Spain

Viruses	*Pelargonium zonale*		*Pelargonium peltatum*		*Pelargonium grandiflorum*		Total	
	Number of samples	%	Number of samples	%	Number of samples	%	Number of samples	%
PLPV	385	50.2	48	33.8	2	10.0	435	47.6
PFBV	62	9.4	2	1.4	0	0	64	8.0
CMV	25	3.8	3	2.2	5	25.0	33	4.1
TMV	12	1.8	1	0.7	1	5.0	14	1.8
PLCV	2	0.3	0	0.0	0	0	2	0.3
Not infected	228	34.5	86	61.9	12	60.0	326	40.8

Carmoviruses *Pelargonium flower break virus* (PFBV) and *Pelargonium line pattern virus* (PLPV), tombusvirus *Pelargonium leaf curl virus* (PLCV), tobamovirus *Tobacco mosaic virus* (TMV), and cucumovirus *Cucumber mosaic virus* (CMV).

propagation process. More than half of the *P. zonale* samples were infected with PLPV. In order to evaluate if the sampling season was important for symptom development, 47 samples were collected in November 2003, from Andalucía and Galicia, the main Spanish pot geranium producing areas. However, no significant differences were observed in these samples. All samples collected in Galicia were virus-infected, and 50% (20/40) of the asymptomatic samples from Andalucía were infected with PLPV. CMV and TMV were mainly found in Galicia, Extremadura, Andalucía and Euskadi, although the highest incidence of these viruses was detected in plants collected in Madrid. The lowest occurrence was found in central Spain (Castilla-León, Castilla-La Mancha and Navarra) an area of high cereal production. We did not find any incidence of CMV on the Mediterranean coast. PLPV incidence was high when detected, except in Madrid and Cataluña (Table 3), two of the main geranium producing areas. In these two regions the most important infections were due to PFBV. In the regions where sampling was performed over a period of several years (Andalucía, Euskadi, Extremadura, Castilla-León and Castilla La Mancha) the percentage of virus detection per year was constant over the years with no significant differences observed.

The identification of virus-free plants in the case of geranium is hampered by many failures in the detection procedures that have been utilised for this crop (Peña Iglesias et al., 1974; Welvaert, 1974; Abo El-Nil et al., 1976; Albouy and Poutier, 1980; Stone, 1980; Stone et al., 1981; Paludan and Begtrup, 1987). The availability of reliable and sensitive virus detection methods is essential for the effective control of the transmission to uninfected plants of viral particles from asymptomatic infected plants. In this work the global disease infection rate in Spain is underestimated, because unlike other surveys, only asymptomatic plants were sampled. It is very important that growers do not rely on symptoms to determine infections in *Pelargonium* spp. since, as shown by our results, very often, symptomless plants are indeed infected. In countries with milder climates, where plants are kept as perennials, these plants might be an important PLPV reservoir. This is, to our knowledge, the first extensive survey of geranium diseases in Spain and the only one carried out in a European geranium-producing country focused on asymptomatic geranium plants.

Table 3. The incidence of viruses in *Pelargonium* spp. in different regions of Spain, as determined by ELISA

Viruses	Andalucia		Asturias		Cataluña		Euskadi		Extrema-dura		Galicia		Castilla-León		Madrid		Castilla La Mancha		Navarra		Valencia	
	N[a]	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
PLPV	80	51.6	4	80.0	1	5.3	57	41.3	59	58.4	20	80.0	162	52.6	0	na	35	38.0	4	50.0	13	86.7
PFBV	18	11.6	0	na	6	31.6	27	19.6	1	1.0	1	4.0	3	1.0	3	37.5	4	4.3	0	na	1	6.7
CMV	6	3.9	0	na	0	na	4	2.9	8	7.9	3	12.0	6	1.9	1	12.5	4	4.3	1	12.5	0	na
TMV	3	1.9	0	na	1	5.3	3	2.2	4	4.0	1	4.0	1	0.3	1	12.5	0	na	0	na	0	na
PLCV	0	na[b]	0	na	0	na	0	na	0	na	0	na	1	0.3	0	na	1	1.1	0	na	0	na
Not infected	48	31.0	1	20.0	11	57.9	47	34.1	29	28.7	0	na	135	43.8	3	37.5	48	52.2	3	37.5	1	6.7

[a] N: number of samples.
[b] na: does not apply.
Carmoviruses *Pelargonium flower break virus* (PFBV) and *Pelargonium line pattern virus* (PLPV), tombusvirus *Pelargonium leaf curl virus* (PLCV), tobamovirus *Tobacco mosaic virus* (TMV), and cucumovirus *Cucumber mosaic virus* (CMV).

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