Virus-associated Diseases of Double Petunia: Frequency and Distribution in Ohio Greenhouses

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Abstract. Double petunia plants expressing virus-like symptoms were collected in greenhouses and garden centers throughout Ohio in Spring 1997 and 1998 in an effort to determine the frequency and distribution of petunia viruses present in the state. Direct antibody-sandwich and indirect enzyme-linked immunosorbent assay (ELISA) were conducted with commercial antisera made against 13 viruses, a potyvirus kit capable of detecting 80 different potyviruses, and our antiserum raised against a tobamo-like virus inducing severe mosaic in double petunia. Viral-associated double-stranded ribonucleic acid (dsRNA) analysis and light microscopy for detection of inclusion bodies were also carried out. ELISA, dsRNA analysis, and light microscopy revealed the presence of tobacco mosaic tobamovirus, an unknown tobamo-like petunia virus, tomato ringspot nepovirus, tobacco streak ilarvirus, and tobacco ringspot nepovirus. Tomato aspermy cucumovirus, tomato spotted wilt tospovirus, impatiens necrotic spot tospovirus, alfalfa mosaic virus, cucumber mosaic cucumovirus, potato virus X potexvirus, and chrysanthemum B carlavirus were not detected. No potyviruses were identified. A number of plants with virus-like symptoms tested negative for all viruses.

Petunia ×hybrida Hort. is a one of six members of the Solanaceae grown primarily as an ornamental plant, and the most economically important due to its horticultural value (Sink, 1984). It is a popular bedding plant, the third most valuable after geranium and impatiens (Hamrick, 1997) but it is also grown in pots, hanging baskets, and balcony/window boxes (Lesemann, 1996). Petunia plants propagate by seeds in nature, but new species of vegetatively propagated petunia, such as "Supertunia" and double petunias, have been recently introduced in Europe and the United States as garden ornamentals (Lesemann, 1996; Sikron et al., 1995). Double petunia plants provide the most impressive flowers of all bedding plants (Nau, 1991) and are usually female sterile because of malformed or underdeveloped pistils (Goldsmith, 1968).

Several viruses have been reported as naturally infecting petunias (Bellardi et al. 1996; Leseman, 1996; Mavric et al., 1996; Pirone, 1978; Sikron et al., 1995), and the list of viruses capable of infecting petunias is very long (Brunt et al., 1996), because petunias are often used as indicator hosts for virus identification assays (Lane, 1992). Virus diseases constitute a threat to the production and quality of the petunia crop (Sikron et al.,

1995). Symptoms induced by these viruses in infected petunia plants are variable, as they are influenced by environmental conditions, strain of the virus, mixed infections with other viruses, genetic diversity of petunia cultivars, and cultural conditions. Some infected plants remain symptomless (Lesemann, 1996). According to Lesemann (1996), petunia virus (PV)-associated diseases became widespread after 1992, when asexually propagated hybrids were introduced from Japan without strict virus-indexing schemes. The symptoms associated with virus diseases in petunias are stunting and deformation of the foliage, light-green streaks, discolored and deformed flowers (Lindgren, 1993), irregular vein-yellowing, mottling, color breaking, and a reduction in the number and size of the flowers (Edwardson and Christie, 1997 a and b). Mixed virus infections apparently aggravate symptoms (Leseman, 1996).

The virus most often detected in petunias in the United States is tobacco mosaic tobamovirus [(TMV); Agdia, 1995; Lawson and Hsu, 1994]. Other commonly detected viruses are cucumber mosaic cucumovirus (CMV), tobacco ringspot virus nepovirus (TRSV), unknown potyviruses, tomato spotted wilt tospovirus (TSWV), tomato ringspot nepovirus (ToRSV), occasionally tobacco streak ilarvirus (TSV), tomato aspermy cucumovirus (TAV), and chrysanthemum virus B carlavirus (CVB) (Brunt et al., 1996).

The purpose of this study was to screen for the viruses infecting double petunia plants in commercial greenhouses and garden centers in Ohio and to determine their the frequency and distribution.

Plant material. Double petunia samples were collected from 15 greenhouses and 6 garden centers in Toledo, Cincinnati, Cleveland, and Columbus, Ohio, in Spring 1997 and 1998. In 1997, growers and garden center managers were requested to send a sample of 12 double petunia plants. Since the number of plants initially received was small, we collected the material directly in greenhouses and garden centers. Only plants displaying virus-like symptoms were selected. We also included in the survey all double petunia samples sent to the C.W. Ellett Plant and Pest Diagnostic Clinic, The Ohio State Univ., Columbus. Plants were collected at different stages of development, in plug trays, pots, and baskets. Plants were transplanted and maintained under insect-free greenhouse conditions, with ample distance between plants to minimize cross-contamination. Tissue (~10 g) was harvested from each plant and stored at -20 °C. Virus-like symptoms, such as mosaic, ringspots, leaf deformation, leaf puckering, spots and/or stunting were recorded one week after transplanting or whenever they were eventually observed.

Detection of viruses by ELISA. Leaves, petals, and young stems from 544 symptomatic double petunia plants were tested by direct antibody-sandwich (DAS)-enzyme-linked immunosorbent assay (ELISA) using a modification (condensation of incubation time using heat and shaking) of a previously described procedure (Clark and Adams, 1977) for detecting alfalfa mosaic virus (AMV), CMV, TAV, TSV, potato virus X potexvirus (PVX), TRSV, ToRSV, TMV, TSWV, INSV, and CVB using commercial polyclonal antisera produced in rabbits (Agdia, Elkhart, Ind.). DAS-ELISA was also conducted with polyclonal antibodies we produced against a possible new tobamo-like virus affecting double petunia tentatively named petunia virus, and a commercial indirect ELISA screen which tests for 80 potyviruses (Agdia). During the screening process with our antisera produced against PV, it was determined that our antisera did cross-react slightly with tomato mosaic virus and pepper mild mottle mosaic virus. Optical density at 405 nm was measured with an MRX microplate reader (Dynex Technologies, Chantilly, Va). Samples were considered positive when the mean absorbance of duplicate wells exceeded twice the mean absorbance of appropriate healthy controls. Each plate included a healthy tissue sample and extraction buffer as negative controls. Tissue known to be positive for a particular virus was used as the positive control.

Viral dsRNA extraction and analysis. Leaf, flower petals, and young stem tissue (5 g) was used for analysis. Viral-associated double-stranded ribonucleic acid (dsRNA) was extracted and purified using a modification of the CF-11 cellulose column chromatography method described by Morris and Dodds, (1979) and Jordan et al. (1983).

Light microscopy of inclusion bodies. Epidermal strips from the underside of healthy

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and symptomatic double petunia and Turkish tobacco plants were stained with a combination of Calcomine Orange and Luxol Brilliant Green, a general stain for proteins, and Azure A (Aldrich Chemical Co., Milwaukee), a nucleic acid-specific stain (Christie and Edwardson, 1994). The stained tissue was observed with a Zeiss Axioscope microscope, equipped with a Zeiss MC80 microscope camera (Carl Zeiss, Inc., Thornwood, N.Y.).

Results

Plants collected in greenhouses and garden centers had virus-like symptoms in leaves such as mottling, vein-clearing, rat-tailing and mosaic, puckering, leaf-curling, ringspots and oak-leaf patterning (Fig. 1A–D). Flowers also expressed symptoms such as spots, color breaking, flower deformation, and small flower size (Fig. 2 A and B). Some plants expressing chlorosis, stunting, and leaf curling grew out of the symptoms a few weeks after being transplanted and receiving fertilizer application. In contrast, some plants continued to express the same symptoms initially observed at planting. Other symptoms, such as mosaic and leaf deformation and stunting became more conspicuous. Other plants expressed severe chlorosis of the veins (vein-clearing) three to five months after transplanting.

None of the 544 double petunia plants tested in 1997 or 1998 were positive for AMV, CMV, CVB, INSV, PVX, TAV, or any of the

potyviruses detected by the potyvirus screen.

In 1997, 8.5% of the plants collected (18/212) were positive for virus infections. Of these, 12 plants were positive for ToRSV, 5 for TSWV, and 1 for TSV. No mixed infections were detected in 1997 (Table 1). The symptoms observed in TSWV positive samples varied from severe leaf puckering, leaf deformation and reduced leaf size, to inter-veinal chlorosis and small local lesions. Some of these symptoms (inter-veinal chlorosis and local lesions) were also detected in plants infected with ToRSV. Those plants also expressed ringspots or were asymptomatic. The only plant found positive for TSV expressed white spots on leaves, vein chlorosis, and leaf bronzing.

In 1997, 67% of the virus-infected plants (assayed with ELISA) belonged to the asexually propagated cultivars 'Double Sheer Madness' and 'Purple Pirouette' or 'Rose Pirouette'. In 1998, TMV and an unknown tobamovirus-like petunia virus (PV) were also found in these cultivars, but 63% of the infected plants that year were found in the asexually propagated cultivars of 'Double Sheer Madness' and 'Purple Pirouette'.

In 1998, 6% (20/332) of the plants screened were positive for virus: 16 were TMV-positive and 15 were PV-positive. One plant was positive for TMV and TRSV, 13 plants were positive for TMV and PV, 3 were positive for just TMV, and 2 samples were positive for PV and not for TMV (Table 1). Most of the virus-

infected plants identified by DAS-ELISA in 1998 were positive when tested with the commercial antibodies against the common or vulgare strain of TMV (TMV-c) and our own polyclonal antibodies raised against the unknown petunia virus (PV). All plants that tested positive for either or both of these viruses, expressed various degrees of mosaic, puckering and leaf deformation. The absorbance values for the PV samples were usually lower in those samples that tested positive for both TMV and PV (mixed infection) (Table 1).

Some of the 1998 plants that expressed virus-like symptoms, such as mosaic, severe mottling, leaf puckering, leaf curling, leaf deformation, and flower spotting or breaking, were negative for the 13 viruses screened by ELISA.

The dsRNA analysis of some these symptomatic samples produced profiles not consistent with known viruses or did not produce any viral dsRNA bands. The dsRNA analysis of samples serologically positive for a mixed infection of TMV and PV produced banding patterns consistent with infections by TMV only (data not shown).

In samples that gave a negative ELISA, no cytoplasmic inclusions were observed in stained epidermal tissue of symptomatic or asymptomatic petunia plants. Hexagonal plates and crystals (Fig. 3) were observed in epidermal strips of TMV and PV-infected plants, stained with orange-green and Azure A, only after heating the Azure A. Most of the inclu-

Fig. 1. Foliar symptoms associated with virus infection of double petunias, (A) vein-clearing, (B) leaf mosaic and deformation, (C) rat-tailing, and (D) leaf stunting.

Fig. 2. Flower symptoms associated with virus infection in double petunia, (A) mosaic and (B) speckling and deformation.

Table 1. Results of ELISA testing of double petunias for 12 common viruses in 1997 (212^a) and 1998 (332^a).

Year	Viruses detected							Negative ^c
	TMV	ToRSV	TRSV	TSWV	TSV	PV	Mixed infection ^b	
1997	0	12	0	5	1	0	0	194
1998	3	0	1 ^y	0	0	2	14	312

^aTotal samples tested that year.

^bThirteen samples as a mixed infection of TMV and PV, one sample as a mixed infection of TMV and TRSV.

^cNegative for all viruses tested.

sion bodies observed were located around stomata.

In 1997, seven plants with severe chlorosis of the veins (vein clearing) and distortion of new leaves were observed. In 1998, 11 plants with vein-clearing were tested. All 18 of these plants were negative for all viruses screened with ELISA, and no dsRNA or inclusion bodies were observed.

Discussion

The ELISA results indicated that TMV, PV, ToRSV, TSWV, TSV, and TRSV were the only viruses present in the double petunia plants tested in 1997 and 1998. ToRSV was detected in 5.7%, TSWV in 2.4%, and TSV in 0.5% of the collected plants in 1997. In 1998, TMV was found in 5.1%, PV in 4.5% and a mixed infection of TMV and TRSV in 0.3% of the samples tested.

In 1997, 67% of the virus-infected plants comprised the seed-propagated cultivars 'Double Sheer Madness' and 'Pirouette Purple' or 'Rose Pirouette'.

In 1998, 63% (12/19) of the plants infected with TMV and PV belonged to vegetatively propagated cultivars ('White Queen', 'Venice Blue', and 'Blue Ribbon'). The fact that most of the TMV and PV infected plants were asexually propagated seems to indicate that a strict virus-indexing program was not being followed in the greenhouse where the plants were collected. It is known that many viruses are transmitted by vegetative propagation (Mathews, 1992) and that virus infections in petunias have increased dramatically since vegetatively propagated cultivars have been introduced (Lesemann, 1996). This transmission could possibly be avoided by heat sterilization of the knives/cutting tools or by treating them with 2.8 g/L of sodium troclosene for

15 seconds (Cohen et al., 1991). However, this may be impractical in greenhouse production areas.

Recovery from virus symptoms may also have been the result of an environmental change (light intensity, temperature, etc.) that directly affected virus replication (Lesemann, 1996). Once the plants were transplanted into bigger containers and were cared for appropriately, the stress symptoms disappeared (Armitage, 1986). It is also possible that the viruses infecting the symptomatic plants had already reached their peak titer and then declined after transplanting with subsequent recovery from symptoms, as reported for AMV (Jaspars and Bos, 1980). None of the typical symptoms expressed by double petunia plants infected with TSWV were observed in all of the samples that were serologically positive for this virus. Some of the symptoms observed, such as interveinal chlorosis and leaf puckering, were also observed in ToRSV-infected plants.

Our results agree with the observations made by Lesemann (1996) who found great variability of virus-induced symptoms in petunias. This variability may be due to the influence of genetic diversity between petunia cultivars, virus types and strains, and cultural conditions.

All plants infected with TMV and PV expressed mosaic symptoms of variable intensity. The fact that there was variability of symptoms expressed by virus-infected plants is of great importance for growers, especially when plants are asexually propagated.

Results obtained in this study emphasize that symptoms are not a reliable tool for virus diagnosis in double petunia, and that extreme precautions have to be taken in order to avoid virus dissemination, especially in greenhouses producing other species susceptible to the same viruses as petunias.

The containers (plug flats, and pots) in which bedding plants, such as double petunias, are grown for spring sale in Ohio maintain the plants in close contact in a high humidity environment. These conditions favor infectious diseases such as those caused by *Botrytis* spp. and the spread of mechanically transmitted viruses, such as TMV and PVX. Since petunia baskets are usually hung over benches containing flats of other petunias in many greenhouses, it is also possible that drainage water may carry tobamoviruses from the infected plants in the baskets to the plants below (Koenig, 1986).

It is difficult to associate virus-infected plants with specific cultivars because many plants with the same characteristics are found in the greenhouse under different names. In addition, many greenhouses and retail stores produce and sell mixed plantings under colorful names such as Double Madness mix, Glorious mix, or Artists mix, in order to attract the attention of the consumer.

The commercial TMV-common (TMV-c) antibodies reacted with a low absorbance value with PV-infected tissue. It was interesting to observe that two samples tested negative with the PV antibodies, but were positive for TMV-c. These results were unexpected because the

Fig. 3. Cytoplasmic inclusions associated with double petunia leaf infected with an uncharacterized petunia tobamovirus (PV) (arrow).

PV antibodies also recognized all TMV isolates that we screened to some degree. It is possible that our PV antibodies do not recognize other tobamoviruses or all strains of TMV, while the commercial antibodies against TMV do. The fact that some samples were negative for TMV-c and positive for PV was not unexpected, since samples infected with PV usually have low absorbance or negative readings when infected with TMV. Since the antibodies raised against the PV also detect other tobamoviruses, such as ToMV and PMMV, it is possible that some of those samples with positive reaction for PV but negative for TMV-c are not infected with the petunia virus, but with other tobamoviruses or that PV is serologically related to ToMV, PMMV, or both.

The dsRNA analysis did not detect viruses such as ToRSV. This was probably due to low concentration of the virus in the infected plants. Luteoviruses and potyviruses are very rarely detected by this method because they yield very low quantities of dsRNA (Valverde et al., 1990). The dsRNA analysis also revealed the presence of dsRNA in several symptomatic and ELISA negative petunia plants. Unknown viruses could have induced these dsRNAs. One of the limitations of the dsRNA analysis is that it requires knowledge of the number and sizes of viral RNAs. The dsRNA of known molecular weights are not commercially available to be used as markers.

The hexagonal and angular crystals observed by light microscopy in epidermal strips, which stained green with orange-green stain and blue

when the Azure A stain was heated, are similar to those reported for the tobamoviruses. The characteristics of these inclusions and the staining reaction are considered diagnostic for tobamoviruses (Christie and Edwardson, 1994). The hexagonal plates are characteristic of TMV strains. No inclusion bodies were detected in other samples with virus-like symptoms. It is possible that these plants may not have been infected with viruses or that we did visualize inclusions due to tissue selection. Light microscopy of inclusion bodies is an art (Lane, 1992), which requires knowledge of cell anatomy, of the inclusion bodies, and plants at the right stage of virus infection (Christie and Edwardson, 1994).

It is possible that the plants expressing severe vein chlorosis and distortion of young leaves were infected by the petunia vein clearing virus (PVCV), a tentative member of the caulimovirus group (Brunt et al., 1996). This virus has been found in petunia plants in the United States (Lockhart and Lesemann, 1997). No attempts were made to determine if the symptomatic plants were, in fact, infected with PVCV because the virus is not mechanically or seed transmitted, and possible vectors have yet to be identified (Lesemann and Casper, 1973). The virus occurs at low concentration in infected plants, and its isometric particles are not clearly distinguished from cell components in negative staining. There is no commercial PVCV-antiserum available for serological tests at this time.

This is the first systematic virus survey of double petunia in Ohio. Based on this research, we now have a better idea of what viruses are important in causing disease in the bedding plant industry in Ohio. We have also identified a possible new strain or possible new tobamo-like virus associated with disease in double petunia that does not react well with the commercial antisera available. Work will need to continue to determine the identity of this virus.

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