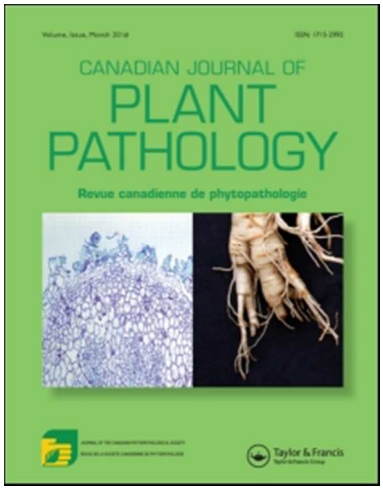

Development of a detached leaf procedure for the evaluation of seasonal susceptibility of peach to Plum pox virus infection by the green peach aphid (Myzus persicae (Sulzer)).

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Development of a detached leaf procedure for the evaluation of seasonal susceptibility of peach to *Plum pox virus* infection by the green peach aphid (*Myzus persicae* (Sulzer)).

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Abstract: A method was developed for use in a subsequent study to evaluate changes in seasonal susceptibility of orchard peach trees to *plum pox virus* (PPV*)* infection by aphids. To understand this, detached healthy leaves would need to be collected from peach trees in the field at different times over the growing season and evaluated for susceptibility to aphid transmission of PPV. This study examined whether virus multiplication could be detected in aphid-inoculated detached leaves and if transmission efficiency of PPV by green peach aphids to detached leaves was comparable with that for peach seedlings. Results demonstrated that transmission efficiencies of viruliferous aphids transferred to detached peach leaves subsequently maintained on an agar bed for three weeks was not significantly different from that for intact seedlings. Overlaying infected PPV plum or peach leaf segments on the healthy peach leaves with subsequent application of aphids to the infected leaf pieces provided a comparable transmission efficiency. Reduced handling of the aphids using this method minimized the possibility of damaging the aphids and facilitated higher throughput testing. Comparable infection rates were obtained for detached leaves using either 50 or 25 viruliferous aphids per leaf. Residue of PPV was not detected by direct quantitative reverse transcriptase polymerase chain reaction assay (DqRT-PCR) on non-host plants probed by viruliferous aphids. The effect of short term storage temperatures pre- or post-inoculation did not significantly alter the susceptibility of peaches to PPV infection and the transmission rate. Application of the leaf overlay method to evaluate seasonal changes in susceptibility is the subject of an ongoing study.

Key Words: *aphid transmission, detached leaves, plum pox virus, susceptibility*

Introduction

Plum pox virus (PPV), causal agent of plum pox or Sharka disease, is the most devastating viral disease of stone fruit (*Prunus* spp.) worldwide (Nemeth 1986). Different strains significantly limit stone fruit production in peaches, plums, apricots, nectarines, almonds and sweet and sour cherries in areas where they are established. In 1999, the Dideron strain of PPV (PPV-D) was first detected in North America in Pennsylvania in several peach and plum orchards (Levy et al. 2000; Damsteegt et al. 2001). The following year, PPV-D was subsequently detected in nectarine and peach in Ontario, Canada (Thompson et al. 2001) and an eradication program was implemented by the Canadian Food Inspection Agency (CFIA).

The efficiency of disease transmission is dependent on the frequency of the occurrence of vectors and the cultivar susceptibility to the pathogen. While numerous aphid species have been shown to transmit PPV-D in a non-persistent manner, the green peach aphid (*Myzus persicae* (Sulzer)), the spirea aphid (*Aphis spiraecola* (Patch)) and the soybean aphid (*Aphis glycines* (Matsumura)) represent the most prevalent and efficient vector species in Niagara orchards (Lowery et al. 2009). Incidence and populations of these species is variable over the growing season, linked with weather, presence of predator species, and availability of feeding hosts. The application of oil sprays was recommended by the International PPV Expert Panel in 2009 as a protectant to reduce spread of PPV by aphid vectors in *Prunus* orchards over the entire growing season. Growers have resisted using oil sprays however, due to concerns associated with cost and possible foliar phytotoxicity during the warmer summer months. Since 2007, ongoing studies in this laboratory have suggested that susceptibility of peach to aphid transmission of PPV may decrease over the summer. By studying susceptibility of peach trees to PPV over the growing

season, it may be possible to reduce the number of oil sprays needed to provide protection and use oil only during periods of elevated susceptibility.

Under the eradication program, it is not possible to conduct field studies to evaluate the susceptibility of trees to aphid transmitted PPV. Although whole plants are necessary for examining host plant resistance in some systems (Klinger et al. 2005), other systems have shown that only parts of a plant, such as detached leaves, can be used for assessing resistance or virulence (Sams et al. 1975; Rufener et al. 1987; Sharma et al. 2005; Kalleshwaraswamy & Kumar 2008). This paper reports on the development and evaluation of a detached leaf method now in use in ongoing studies evaluating foliar susceptibility to aphid transmitted PPV.

Materials and Methods

Virus source

The Canadian isolate of the Dideron strain of PPV (PPV-D), characterized by Rochon et al. (2003) that was used in this study was isolated from an infected peach tree in Niagara. Virus was maintained in plum (*Prunus domestica* L. cv. Stanley) and peach (*Prunus persica* L. cv. Elberta) seedlings for use in transmission trials. Seedlings were inoculated using the layered leaf method detailed below. Virus presence in the plants was confirmed by ELISA as described below after 3-4 weeks incubation.

Rearing of aphids

Green peach aphids (*M. persicae* Sulzer) were reared in ventilated Plexiglass cages on Bok Choy (*Brassica rapa*, subspecies *pekinensis var.* Heavy (422E), Stokes Seeds, St. Catharines, ON) and maintained under fluorescent lighting on a 16 h photoperiod. Ceramic plant watering spikes (Lee Valley Tools, Burlington, ON) were inserted in each pot to minimize exposure of the plants to outside aphid contamination through hand watering.

Manual serial aphid transfer inoculation method for seedlings

Third and fourth instar nymphs and apterous adult green peach aphids were transferred using a fine artist's brush to 5 cm Petri dishes (VWR Scientific, Mississauga, ON) with tight fitting lids and starved at ambient temperature (22°C) for a minimum of 2 h. Aphids were then transferred to leaf pieces from PPV-infected plum seedlings (*P. domestica* L. cv. Stanley) in sealed Petri dishes for a five minute acquisition access period (AAP). Following AAP, 50 aphids were transferred to the upper leaf surfaces of each of 5 or more peach seedlings (*P. persica* L. *cv. Babygold*) in separate trials (Table 1). All seedlings were at the 5 to 6 leaf stage (ca. 15 cm tall) and were pre-treated with the aphicide pirimicarb (Pirimor® WG , Syngenta Inc., Guelph, ON, Canada) before the aphids were released. This minimized aphid escape and was found to result in complete aphid mortality within 48 h, as demonstrated in other systems with different hosts (Scott & Smilowitz 1980).

Pirimicarb did not affect probing of apterous green peach aphids during the first day of transfer to leaves from treated potato (Lowery & Boiteau 1988) and had no effect on the rate of spread of *Turnip mosaic virus* under natural field conditions (Lowery et al. 1990). Following transfer of the viruliferous aphids, seedlings were placed in 20 lb polybags, sealed, and stored in the dark in plastic lidded Rubbermaid® tubs for 48 h after which time the bags were removed. The seedlings were then transferred to containment rooms and grown for an additional 3 weeks (22°C, 4100 lux halide lighting, 16 h photoperiod). Fully expanded apical leaves were macerated in ELISA extraction buffer (1:6, tissue:buffer) (Clark & Adams, 1977). The suspension was further diluted using direct plant extraction buffer (DiPEB) and assayed by direct real time reverse transcriptase Taqman probe based polymerase chain reaction assay (DqRT-PCR, Kim et al. 2008).

Seedling and detached leaf manual serial aphid inoculation trials

In order to evaluate changes in susceptibility of field trees over the growing season it is necessary to use detached leaves collected from the trees at various times during the summer (Sharma et al. 2006). To examine whether virus multiplication could be detected in detached leaves, aphid inoculated detached seedling leaves were tested by PCR and simultaneously compared with the aphid inoculated seedlings as described above. Detached leaves were supported on a 0.4% agar gel bed, midrib up, in a 24.5 x 24.5 x 2.5 cm (l x w x h) Nunclon TM polystyrene culture dish with lid (VWR Scientific, Mississauga, ON). Pirliss® 50DF (50% pirimicarb ai, Plant Products, Brampton, ON) was added to the agar (0.063% Pirliss, w/v) to minimize aphid escape and was found to result in complete aphid mortality within 48 h (Lowery & Boiteau 1988). Starved aphids were transferred onto PPV- infected peach (*P. persica* L. cv. Elberta) leaf pieces for a 5 minute AAP. Following acquisition, 50 viruliferous aphids were then transferred to each of the virus-free detached leaves in the agar plates that were then sealed with Parafilm® to minimize moisture loss. Plates were stored in the dark for 24 h and then incubated for 3 weeks in the containment room. Leaves were then assayed by DqRT-PCR as described. Fifty leaves each of apple and pear were also aphid-inoculated as described to determine whether assays detected any residual virus left in or on non PPV hosts by probing aphids. Fifty peach leaves were used as controls.

Leaf overlay inoculation method.

To reduce aphid handling, a layered leaf approach was also examined. A 1.0 x 1.0 cm piece of infected plum leaf was overlaid on the lower surface of each of 6 or more detached peach leaves supported on the agar gel bed in separate trials (Table 3). Twenty-five starved aphids were then transferred onto each of the PPV- infected plum /peach leaf pieces and the plates sealed. Loss in turgor in the infected plum or peach leaf piece usually resulted in aphids moving onto the peach

leaf within 6 h where they continued probing and feeding. Plates were stored in the dark for 24 h, incubated for 3 weeks in the containment room and leaves assayed for PPV as described. A comparison between 50 and 25 aphids per leaf using the detached leaf method was also made to compare transmission efficacies (Table 2). As a control, PPV infected peach or plum leaves layered over healthy peach leaves in the absence of aphids did not infect the healthy peach leaves. PPV is not known to be mechanically transmissible (OEPP/EPPO 1994).

Pre- and Post-inoculation temperature

The effect of short term handling storage temperature of leaves pre- and post-inoculation with PPV was examined. A total of 62 peach leaves were collected from the field during the active growing season from ten year old peach (cv. Babygold) trees and randomly assigned to three temperature regimes of 4°, 10°, 20°C (± 1.0 °C) for 48 h. Control treatments (ambient room temperature) were inoculated with PPV on the same day with 25 starved green peach aphids as described, while temperature-treated leaves were kept in the dark under the designated test temperatures prior to or after the 48 hr of exposure to viruliferous aphids. Plates were then incubated for 3 weeks in the containment room and then assayed by DqRT-PCR as described. Experiments were repeated 6 times for each handling temperature.

Statistical Analysis

SAS software was used (SAS Institute 1998). Treatment means were compared using the one way ANOVA Welch's test (Welch 1947). The chi-square test, Fisher's exact test, and contingency coefficients were used to analyze differences in transmission rates between the pre- and post-inoculation temperature treatments at the 95% confidence level.

Results and discussion

Utilizing the manual serial transfer inoculation method, inoculation of intact peach seedlings with PPV using green peach aphid as the vector resulted in higher rates of infection compared with inoculation of detached leaves maintained on agar plates, but the difference was not significant (Table 1) Demonstrating that the positive PCR tests were the result of virus replication within the detached leaves and not the residue from the initial aphid inoculations, PPV was not detected from either apple or pear leaves probed by viruliferous aphids, while 16% of peach leaves exposed at the same time tested positive. Detached peach leaves were maintained for 3-4 weeks post-inoculation on the agar beds as described without any noticeable chlorosis or degradation, allowing ample time for virus multiplication. For these experiments we relied on fungicide sprays having been applied to peach trees in the field, but the addition of antimicrobials to the agar media would help prevent the growth of fungi and perhaps extend the viability of leaves for a longer length of time. Alternatively, the excised leaves could be treated with a fungicide prior to use. If required, the viability of leaves might be lengthened further with the addition of nutrients and growth regulators commonly used in plant tissue culture (e.g. Murashige & Skoog 1962) . Serial transfer of aphids to the infected leaf material and then to the virus-free test plants following the initial starvation period is designed to reflect transmission of non-persistent viruses by transient alate aphids. This three step procedure is laborious, however, and does not lend itself to studies where large numbers of aphids have to be physically transferred. The leaf overlay method was found to result in comparable transmission rates to the manual serial transfer method (Table 2) and the reduced handling affords less opportunity to damage the aphid stylets or disrupt feeding. A large degree of variability can occur between experiments that is likely attributable to stages in aphid development, behavioral factors, plant

leaf age and morphology, environmental factors (Smith et al. 1994), and the technical agility of personnel doing the aphid transfers. Several studies have used 100 or more aphids per leaf to ensure consistent transmission (Quiot et al. 1995; Damsteegt et al. 2001). Other studies, using the 'free roaming method', placed infected plants containing indeterminate numbers of aphids among healthy seedlings allowing aphids to move to the seedlings at their volition (Damsteegt et al. 2001; 2004). Generally, most researchers have found that 10 – 30 aphids per leaf or plant gave consistent virus transmission (Marénaud & Massonie 1977; Dosba et al. 1987; Labonne et al. 1994; Kamenova et al. 1998; Gildow et al. 2003). Our studies generally demonstrated acceptable transmission efficiencies with between 25 and 50 aphids (Table 3), although fewer than 25 aphids were not tested. Unless otherwise stated, all of our subsequent research has standardized on 25 aphids/leaf to reduce transfer times, allowing for more replications, while still maintaining sufficient inoculum pressure for consistent virus transmission. Virus levels in leaves inoculated with 50 or 25 aphids were moderately high, with PCR ct values averaging 25 compared to 18 in leaves from symptomatic seedlings grown in containment chambers that we used as controls. Although not permitted under containment guidelines at this facility, the most efficacious approach may be to rear aphids on infected plum seedlings and to apply excised leaf disks containing 25 or more aphids directly onto the target leaves. This would alleviate damage to the aphid resulting from the physical transfer process. The detached leaf method outlined here is flexible and allows for transfer of infected leaf pieces infested with aphids that would more closely simulate transmission of PPV from peach to peach by colonizing aphids.

Susceptibility of plants to virus infection is affected by environmental conditions such as temperature, relative humidity and light. Temperature has a significant effect on plant susceptibility to virus infection and virus multiplication rate as well as plant response to infection

and disease symptoms (Kassanis 1957; Swenson 1963; Syller 1991). In the current study, peach leaves receiving different combinations of incubation temperatures for short storage times pre- and post-inoculation, as indicated in table 4, did not show any significant differences in virus transmission rates. Similar observations have been recorded from other studies; pre and post - inoculation treatments did not alter the susceptibility of host plants to *Potato virus Y* and *Potato leafroll virus* (Singh et al. 1988), *Cucumber mosaic virus* (Stimmann & Swenson 1967), or *Bean yellow mosaic virus* (BYMV) (Swenson 1968). According to Szittya et al., (2003) in cold conditions, plants tend to become more susceptible to virus infection. Susceptibility of bean and pea plants to BYMV inoculated by aphids has been reported to increase when plants were kept pre-inoculation at 18^{o}C and 15^{o}C respectively, while plant kept post inoculation at 30^{o}C resulted in more infected plants (Swenson & Sohi 1961). Our pre- and post-conditioning studies did not show any effect of temperature on susceptibility of peach leaves to PPV over the short period of 48 h, showing that leaves can be harvested from the field and kept chilled prior to use.

The detached leaf assay system outlined in this study produced PPV infection rates for leaves inoculated by viruliferous *M. persicae* that were equivalent to those using intact peach seedlings. Utilization of this technique will allow for the rapid evaluation of changes in host suitability for trees growing under field conditions and could be used for other studies that were previously difficult to perform with whole trees. Previous research we conducted on the host range of PPV, for example, required culture of inoculated woody plants for many months, often with an intervening period of cold, before the plants tested positive using DqRT-PCR (data not shown). Based on our results with leaves collected from peach trees in the field, the detached leaf technique could provide reliable results over a period of 2-3 weeks using a minimal amount of space.

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Table 1. Percentage infection of peach seedlings and detached peach leaves following manual serial aphid transmission of PPV from infected plum leaves.

		Seedlings		Detached leaves	
Trial	Host	n=	% infection	n=	% infection
1	Peach	20	55	10	20
2	Peach	22	45	10	20
3	Peach	34	36	12	25
4	Peach	21	24	25	17
5	Peach	30	25	7	29
6	Peach	47	15	12	25
		x ± sd:	33.3± 14.9		21.5± 3.4

Six replicated trials inoculating either peach seedlings or detached peach leaves. Fifty aphids were used for each inoculation. Means and standard deviations were determined using the means of the six trials. The two means are not significantly different based on Welch's test (P= 0.1529 at $\alpha = 0.05$, n=6).

Table 2. Comparison of PPV infection rates of detached leaves by the manual serial aphid transfer method and the leaf overlay method.

Manual Transfer Method				Leaf Overlay Method			
Trial	Host	n=	% Infection	Trial	Host	n=	% Infection
1	Peach	10	20	1	Peach	16	25
2	Peach	34	15	2	Peach	20	15
3	Peach	34	20	3	Peach	5	25
4	Peach	56	11	4	Peach	27	37
5	Peach	10	28	5	Peach	30	23
6	Peach	15	15	6	Peach	42	22
		x ± sd:	18.2± 5.9				24.5± 7.1

Six replicated trials comparing efficacy of the inoculation methods. Twenty five aphids were used for each inoculation. Means and standard deviations were determined using the means of the six trials. The two means are not significantly different based on Welch's test (P= 0.1288 at $\alpha = 0.05$, n=6).

Table 3. Percentage infection of detached peach leaves influenced by the number of aphids applied to infected plum leaf segments overlaid on the peach leaves.

			% Infection	
Trial	Host	n=	50 aphids	25 aphids
1	Peach	27	37	8
2	Peach	12	41	17
3	Peach	30	23	23
4	Peach	10	20	30
5	Peach	10	40	30
6	Peach	10	10	10
		x ± sd:	28.5± 12.7	19.7± 9.6

Mean and standard deviation was determined using the means of the six trials. The two means are not significantly different based on Welch's test (P= 0.2071 at $\alpha = 0.05$, n=6).

Fig. 1. Addition of aphids to PPV infected plum leaves positioned over detached peach leaves on a gel bed. Peach leaves were incubated 2 weeks in a containment room before being tested by DRT-PCR for PPV infection.

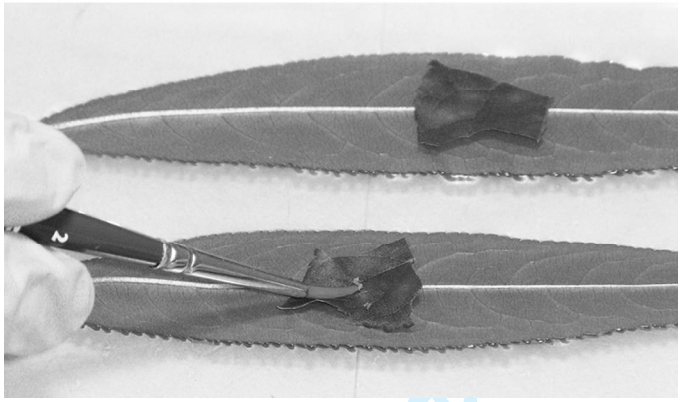

Table 4. Effect of pre- and post-incubation temperatures on PPV multiplication in peach leaves collected from the field during the growing season.

Temperature	Pre %Transmission*	Post %Transmission*
Control	9.86 ± 0.98 a	9.10 ± 1.27 a
4 °C	10.86 ± 1.22 a	9.69 ± 0.44 a
10 °C	10.86 ± 1.22 a	10.47± 0.67 a
20 °C	7.20 ± 3.11 a	7.80 ± 0.28 a
	**Chi-square= 13.2473 (3 df); P= 0.0041; Contingency Coefficient= 0.0934	**Chi-square= 9.0982 (3 df); P= 0.0280; Contingency Coefficient= 0.0776

* Transmission (%) followed by different letters within each assay indicates significant differences ($P \leq 0.05$) according to Chi-square test and Fisher exact test when the expected values were lower than 5.
** Contingency Coefficient measure the association between the two variables, values closer to 1 indicate higher degree of association between the variables