Preferential acquisition and inoculation of \( \text{PVY}^{\text{NTN}} \) over \( \text{PVY}^{\text{O}} \) in potato by the green peach aphid \( \text{Myzus persicae} \) (Sulzer)

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Potato virus Y (PVY) is currently the most economically important disease affecting seed potato production in the US and is the primary reason seed lots fail recertification (Frost et al., 2013). Further exacerbating the situation is the emergence of recombinant PVY strains (e.g. PVYNNTN) that induce tuber necrosis and attenuated foliar symptoms in many popular North American potato cultivars (Gray et al., 2010). These plants often escape visual detection during field inspections, whereas most isolates of the ordinary strain (PVYO) cause distinctive foliar mosaic, but do not induce tuber necrosis. In the past ten years, PVYNNTN has become widespread and incidence is increasing in US seed potato production areas, though it still only accounts for about 20% of the total PVY incidence (Gray et al., 2010 and unpublished). In contrast, PVYNNTN now predominates in many seed potato production regions in Europe (Rolland et al., 2008) and is common in most potato production areas worldwide (Ali et al., 2013, 2010; Bouhachem et al., 2008; Ramirez-Rodriguez et al., 2009; Wang et al., 2012).

While less definitive foliar symptoms induced by PVYNNTN have contributed to its emergence in US seed stocks, other factors may be involved. Multiple studies report that PVYNNTN is more efficiently transmitted by aphid vectors than other strains (Cervantes & Alvarez, 2011; Srinivasan et al., 2012), although contrasting findings have also been published (Davis et al., 2005; Verbeek et al., 2010). In mixed strain infections, PVYNNTN seems to outcompete other PVY strains in potato, which may contribute to enhanced transmission efficiency (Cervantes & Alvarez, 2011; Syller & Grupa, 2014). During surveys of US seed potatoes (Gray et al., 2010), PVYNNTN was often found in the same field and occasionally in the same plant with PVYO. The experiments reported here investigated the acquisition and inoculation efficiencies of PVYNNTN and PVYO when they were (1) acquired sequentially by the same aphid and then inoculated to a healthy recipient plant or (2) acquired individually by separate aphids and then sequentially inoculated to the same healthy recipient potato plant.

All potato (\( S. \, \text{tuberosum} \, \text{L.} \)) plants were grown in Cornell Mix (Boodley & Sheldrake, 1977) and maintained in a greenhouse screened to exclude aphids. Healthy recipient potato plants were grown from true seed of the cultivar 'Red Maria'. Seedlings were used at the 6–8 leaf stage. Plants of the cultivars, 'Pike' and 'Superior', were mechanically inoculated with PVYO or PVYNNTN, maintained via shoot cuttings rooted in vermiculite and used as the sources of virus for the subsequent aphid transmission experiments. The PVYO isolate Oz was described previously (Karasev et al., 2011); the PVYNNTN isolate ME-4-2006 was obtained during a national survey of late generation seed potato lots (Gray et al., 2010).
The green peach aphid, *Myzus persicae* (Sulzer), clone used in transmission tests had been maintained on caged *Physalis floridana* (Rydb.) plants in a plant growth room at our laboratory for approximately 15 years (Gray, 2008). Aphids, starved for at least 1 h, were placed on detached PVYO- or PVYNTN-infected source leaves and observed for probing behaviour during a 1–3 min acquisition access period (AAP). Individual aphids were transferred to the youngest fully developed leaf on a healthy Red Maria seedling and the plant caged for an inoculation access period (IAP) of at least 2 h. The sequential acquisition experiments, designated O/NTN and NTN/O, used a single *M. persicae* given an AAP on a leaf infected with one strain and then moved immediately to a leaf infected with the other strain for the second AAP before being placed on the recipient plant (Fig. 1). The sequential inoculation experiments, designated O + NTN and NTN + O, used two aphids per plant each given an AAP on a single strain. There was an approximately 20–30 min lag between placing the first and second aphids on the recipient plant (Fig. 1). Control treatments consisted of uninoculated plants and plants inoculated with single aphids that had been given an AAP on PVYO- or PVYNTN-infected leaves. The experiment was repeated six times, twice using Pike source tissue and four times using Superior source tissue. In each experiment 20 to 22 recipient plants were used per treatment. Following the IAP, the plants were treated with a mixture of pymetrozine, Endeavour 50WG, at 1.6 g (93 m²⁻¹) [0.058 oz (1000 ft²⁻¹)] and bifenthin, Talstar P, at 15 ml (93 m²⁻¹) [0.5 fl oz (1000 ft²⁻¹)] to kill all aphids before being moved to the greenhouse.

Inoculated plants were observed for symptom development and tested at 35 to 42 days post-inoculation (days p.i.) by TAS-ELISA (Agdia) using a monoclonal antibody (4C3) that recognizes all strains of PVY or monoclonal antibodies specific for PVYO/C serotypes (Mab2) or for PVYN serotypes (1F5) (Baldauf et al., 2006). Source leaves and a subset of plants infected with PVYO and/or PVYNTN were also tested by immunocapture multiplex reverse transcription (RT)-PCR (Lorenzen et al., 2006).
Statistical analyses of experiments were conducted using effect likelihood ratios and odds ratios determined by fitting data to a nominal logistic model, contingency analysis, distribution probability analysis and chi-square tests. Experiment was not significant and experimental results were combined for analyses shown. All analyses were done with JMP Pro version 10.0.2 (SAS Institute) or QuickCalcs (GraphPad Software).

When using infected Pike leaves as the source of virus, the transmission efficiency of PVYNNTN was similar across all treatments described in Fig. 1 (\( \chi^2 = 1.36, P = 0.8507 \)). The number of plants infected with PVYNNTN was similar whether the AAP was on PVYNNTN-infected tissue (18.6\%, \( n=43 \)), on PVYNNTN and then PVYO (16.7\%, \( n=42 \)), on PVYO and then on PVYNNTN (19.5\%, \( n=41 \)) or when two aphids, each given an AAP on one virus, were placed on the recipient plant in either order (PVYNNTN first, 14.0\%, \( n=43 \); PVYO first, 23.3\%, \( n=43 \)).

In contrast to the mild mosaic symptoms observed on Pike plants infected with PVYNNTN, infection with PVYO led to vein necrosis and chlorotic/necrotic spots on leaves. PVYO was not transmitted from Pike source tissue in any of the experiments. The variety ‘Pike’ may possess one or more of the poorly characterized Ny resistance genes that confer incomplete resistance to PVYO, manifested as a hypersensitive response and restriction in virus movement (Rowley et al., 2015). This could have prevented PVYO acquisition and transmission. Ny resistance is often strain specific, temperature sensitive and ineffective against the recombinant strains of PVY, e.g. PVYNNTN. Ny-like resistance to PVYO is common in North American cultivars, and reduced spread of PVYO by aphids and through tubers from those cultivars could be contributing to the shift in PVY strain predominance from PVYO to PVYNNTN and other recombinant PVY strains.

The Superior plants infected with PVYO or PVYNNTN expressed typical mosaic symptoms without vein or leaf necrosis. When leaves from PVY-infected Superior plants were used as the virus source, the number of PVY-infected recipient plants was significantly different across treatments \((\chi^2 = 13.82, P = 0.0168)\) (Table 1). PVYNNTN transmission efficiency was higher, 28.1\%, than PVYO, 17.5\%. When a single aphid was fed sequentially on PVYO then PVYNNTN, or the reverse order, the transmission efficiencies were 19.5\% (O/NTN) and 22.2\% (NTN/O), respectively. The highest transmission efficiencies, 35.4\% and 36.6\%, were in the sequential inoculation treatments, O+NTN and NTN+O, respectively. These higher PVY transmission efficiencies were perhaps expected, since these plants were inoculated by two potentially viruliferous aphids. The transmission efficiencies for PVYO in treatments O+NTN (15.9\%) and NTN+O (19.5\%) were not significantly different \((\chi^2 = 0.38, P = 0.8274)\) from the PVYO control (17.5\%) (Table 1). Similarly, PVYNNTN transmission efficiency was 24.4\% in both sequential inoculation treatments and not significantly different \((\chi^2 = 0.38, P = 0.8268)\) from the PVYNNTN control (28.1\%) (Table 1). These results suggest that while multiple viruliferous aphids landing on the plant will increase the probability of infection, the probability of either PVYO or PVYNNTN being inoculated does not change, i.e. overall inoculation efficiency by multiple aphids carrying different virus strains is additive relative to single aphids inoculating the plant.

Aphids sequentially acquiring both virus strains in treatments (O/NTN or NTN/O) actually inoculated a successful mixed infection in only one out of 163 plants (0.6\%) (Table 1). Although rare, these results show a single aphid is capable of probing sequentially on two plants, each infected with a different strain, and transmitting both strains to a single plant. Therefore, an aphid need not acquire both strains from one source plant in order to transmit multiple viruses. In fact, acquisition and subsequent transmission of both virus strains from a mixed-infected plant is not generally efficient (Srinivasan et al., 2012; Syller & Grupa, 2014) and mixed-infected plants are not abundant in the field (Gray et al., 2010). Our results suggest that mixed-infected plants would more likely be generated in the field via inoculation by multiple aphids that have fed on plants infected with different strains than by single aphids probing multiple plants singly.

### Table 1. Number of Red Maria seedlings infected with PVY, PVYO, PVYNNTN or mixed-infected (Mix) in the four experiments, using Superior as source tissue

Mixed-infected plants are counted also as both O-infected and NTN-infected. Letters next to the numbers indicate significant differences in the odds ratios for uninfected plants across treatments within each Total column.

<table>
<thead>
<tr>
<th>Sequential treatment</th>
<th>Plants (n)</th>
<th>Total PVY</th>
<th>% PVY</th>
<th>Total PVYO</th>
<th>% PVYO</th>
<th>Total PVYNNTN</th>
<th>% PVYNNTN</th>
<th>Total Mix</th>
<th>% Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>O control</td>
<td>80</td>
<td>14 a</td>
<td>17.5</td>
<td>14 b</td>
<td>17.5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>O/NTN</td>
<td>82</td>
<td>16 a</td>
<td>19.5</td>
<td>3 a</td>
<td>3.7</td>
<td>14 a</td>
<td>17.1</td>
<td>1 ab</td>
<td>1.2</td>
</tr>
<tr>
<td>NTN/O</td>
<td>81</td>
<td>18 ab</td>
<td>22.2</td>
<td>4 a</td>
<td>4.9</td>
<td>14 a</td>
<td>17.3</td>
<td>0 a</td>
<td>0.0</td>
</tr>
<tr>
<td>NTN control</td>
<td>82</td>
<td>23 abc</td>
<td>28.1</td>
<td>–</td>
<td>–</td>
<td>23 a</td>
<td>28.1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>O + NTN</td>
<td>82</td>
<td>29 bc</td>
<td>35.4</td>
<td>13 b</td>
<td>15.9</td>
<td>20 a</td>
<td>24.4</td>
<td>4 bc</td>
<td>4.9</td>
</tr>
<tr>
<td>NTN + O</td>
<td>82</td>
<td>30 c</td>
<td>36.6</td>
<td>16 b</td>
<td>19.5</td>
<td>20 a</td>
<td>24.4</td>
<td>6 c</td>
<td>7.3</td>
</tr>
</tbody>
</table>

http://jgv.microbiologyresearch.org
infected with different strains. In the sequential inoculation treatments, four of 29 and six of 30 plants became infected with both viruses in the O + NTN and NTN + O treatments, respectively (Table 1).

When single aphids were given sequential AAPs on both strains of the virus there were significant differences in the numbers of PVYO-, PVY NTN- or mixed-infected plants in each of the two treatments (O/NTN, \( \chi^2 = 15.89, P = 0.0004 \); NTN/O, \( \chi^2 = 17.33, P = 0.0002 \)) (Fig. 2a). PVYNTN was transmitted 3–4 times more efficiently than PVYO and only one plant became infected with both viruses (Fig. 2a). The order in which the strain was acquired by the same aphid had no significant effect on the numbers of plants infected with either PVYNTN (\( \chi^2 = 0.001, P = 0.9715 \)) or PVYO (\( \chi^2 = 0.162, P = 0.6865 \)), suggesting that PVYNTN can displace PVYO when PVYO is acquired first, but the reverse does not happen. Alternatively, both virus strains may bind the aphid stylets equally, but PVYNTN is preferentially released when the aphid salivates. Fasting prior to the first AAP and not the second AAP may also have impacted virus strain acquisition or transmission by affecting aphid behaviour or altering the complement of inhibitors or helper components in the stylet. However, Syller & Grupa (2014) also reported that the acquisition order of PVYO and PVYNTN did not affect transmission efficiency, but in their experiments PVYNTN and PVYO were transmitted with similar efficiencies, suggesting fasting is not a likely factor.

PVYNTN infected more recipient plants in the sequential inoculation treatments (O + NTN, NTN + O) (Table 1, Fig. 2b), although differences in the number of plants infected with each strain or strain mixture were only significant in the O + NTN treatment (\( \chi^2 = 7.71, P = 0.0055 \)). The mechanism for this outcome is unknown, but it is unlikely both aphids would inoculate the same or closely associated cells. Therefore, any synergistic or competitive effects of both strains would occur after many rounds of replication and cell-to-cell or systemic movement. The order of inoculation and perhaps the time interval between inoculations likely influence the outcome. Perhaps PVYNTN has a stronger silencing suppressor (Kasschau & Carrington, 1998) than PVYO, which could facilitate superinfection of PVYO in a PVYNTN-infected plant with suppressed plant defences, although PVYNTN appears to win over PVYO more of the time. Interestingly, in the 11 mixed-infected plants from all treatments (Table 1), PVYNTN always out-competed PVYO. At 35 to 42 days p.i., the average ELISA absorbance values for PVYNTN were similar in mixed-infected (1.952, \( n = 11 \)) and singly infected (2.304, \( n = 28 \)) plants, while the average ELISA absorbance values for PVYO were 0.230 (\( n = 11 \)) and 2.469 (\( n = 17 \)) in mixed-infected and singly infected plants, respectively. At 98 to 140 days p.i., nine surviving mixed-infected plants were retested with TAS-ELISA, multiplex RT-PCR and by two bioassays: back-inoculation to a systemic host, Nicotiana tabacum, and to a local lesion host, Chenopodium amaranticolor, Coste & A. Reyn. Seven of the nine plants failed to test positive for PVYO; the other two plants gave slightly above background levels for PVYO with TAS-ELISA and RT-PCR, but PVYO was not detected by the plant bioassays. Aphids were allowed an AAP on leaves from these two plants and 20 aphids from each source leaf were transferred to a single recipient plant. PVYO was transmitted from one of the two mixed infected plants. Finally, tubers collected from the mixed-infected plants were sprouted and the sprouts tested for PVY by TAS-ELISA and RT-PCR; only PVYNTN was detected in the developing sprouts. Syller & Grupa (2014) reported that PVYNTN reached higher titres than PVYO in mixed-infected potato plants.

Fig. 2. The distribution of Red Maria seedlings infected with PVYO, PVYNTN or both strains (Mixture) within the sequential transmission treatments using Superior as source tissue. Letters above the bars indicate significant differences in the distribution of infected plants. (a) Sequential acquisition in which one aphid given an AAP sequentially on each strain, the first listed strain first, was placed on a recipient plant. (b) Sequential inoculation in which two aphids each given an AAP on a different strain, were placed on a recipient plant, the first strain listed first.
and that PVYO was often undetected by ELISA by 35 days p.i., but detected by back inoculation to tobacco and by aphid transmission. Again, PVYNTN may have a stronger silencing suppressor giving it a competitive edge or perhaps PVYO is more efficiently targeted by the silencing machinery. Alternatively, the RNA of PVYO might be transenveloped by the PVYNTN coat protein, directly affecting the ELISA, immunocapture multiplex RT-PCR results and the transmission by aphids, or the PVYNTN RNA may outcompete the PVYO RNA for both coat proteins leaving the PVYO RNA more vulnerable to cellular degradation. Clearly, PVYO survives in some plants at low levels, but PVYNTN has a distinct advantage of being acquired and transmitted from these mixed-infected plants.

PVYNTN transmission efficiency by M. persicae as measured by counting infected plants 35 to 42 days p.i. is higher than PVYO transmission from mixed infections (Srinivasan et al., 2012; Syller & Grupa, 2014) despite the use of different clones of M. persicae, different isolates of the virus strains and different host plant species (both as source and recipient). Also, PVYNTN titre in mixed-infected recipient plants is usually higher than PVYO titre (Srinivasan et al., 2012; Syller & Grupa, 2014 and this study). Our results here suggest PVYNTN will also have a competitive advantage over PVYO as a result of sequential acquisition or inoculation. It is likely that these are consistent phenomena across most isolates within these strains. Whether this is true for other aphid vector species remains to be determined. However, what is unknown is the contribution of each step in this process, i.e. do both strains differ in acquisition and inoculation efficiency or is the differential transmission primarily a result of virus–plant interactions that allow PVYNTN to outcompete PVYO in a greater number of instances? Perhaps aphids actually play a minor or no role in differential transmission, since we do not know if both strains are equally acquired (bound) on the aphid stylet or equally inoculated (released) into the recipient plant with equal efficiency. Our results suggest that aphids do play a role and that PVYNTN is either acquired or released more efficiently.

Nonetheless, because transmission efficiency was measured as the number of plants that become infected 35 to 42 days p.i., it may be that both viruses are equally inoculated into plants by the aphid and the resulting final number of plants infected at 35 to 42 days p.i. is strictly a function of plant–virus interactions and competitions. A careful dissection of mechanisms will require additional carefully planned experiments. Regardless of the mechanisms, it is apparent that the end result is a higher likelihood of PVYNTN-infected plants over PVYO-infected plants via aphid transmission, and perhaps tuber transmission. This may, in part, help explain the observed shift in PVY strain distribution and incidence in the US seed potato crop over the past decade.

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