*Scientific paper*

# The Titre of the Virus in the Inoculum Affects the Titre of the Viral RNA in the Host Plant and the Occurrence of the Disease Symptoms

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***Dedicated to the memory of Prof. Dr. Igor Kregar***

## Abstract

*Potato virus Y* (PVY) is the most economically important potato virus, therefore extensive research is focusing on elucidation of its interaction with the host. To obtain repeatable results, strict standardization of research methods is crucial. Mechanical inoculation by rubbing sap from a PVY infected plant onto the leaf surface together with a fine abrasive powder is the most convenient way of experimental transmission of PVY to host plants. However, factors determining reproducibility of this process need to be determined. In the present study, it was shown that higher titre of the virus in the inoculum resulted in faster increase of $PVY^{NTN}$ RNA titre in the inoculated leaves, as well as in faster translocation of $PVY^{NTN}$ from inoculated leaves into upper non-inoculated leaves. The final titre of $PVY^{NTN}$ RNA in upper non-inoculated leaves was independent of the virus titre in the inoculum. In addition, the occurrence of the disease symptoms was followed and the dependence to the titre of the virus in the inoculum was observed.

**Keywords:** *Potato virus Y*; PVY; potato; mechanical inoculation; inoculum; virus titre; symptoms

## 1. Introduction

*Potato virus Y* (PVY) is the most economically important potato virus affecting potato production worldwide[1] and it was classified as one of ten most important plant viruses overall.[2] In sensitive potato cultivars, it can cause potato tuber necrosis ring spot disease.[3] On the green part of a plant, local lesions, chlorosis, mosaic, crinkling, systemic necrosis, leaf drop and plant death can appear.[4] The outcome of the interaction depends on genotypes of both, potato host plant and PVY pathogen. In addition, the response of a potato towards PVY infection can vary with various environmental conditions and various developmental and physiological growth stages of a plant.[5]

PVY infected potato plants can exhibit either a compatible or an incompatible response.[6] Plants exhibiting a compatible interaction are susceptible and the virus can replicate and invade the plants.[7] Susceptible potato plants can be either sensitive or tolerant to PVY infection. Sensitive potato plants develop disease symptoms, while tolerant plants develop no or very mild symptoms, although they can accumulate high titre of the virus.[8]

In an incompatible interaction, plants resist the virus by restricting cell invasion, virus replication and/or virus spread. Plants can respond to virus infection with an extreme resistance (ER) or a hypersensitive response (HR). In the case of ER, potato plants show no symptoms or very limited necrosis in the form of pinpoint lesions.[9] Virus titres remain extremely low, below the limit of detection also in the initially infected leaf.[9,10,11,12] In the case of HR, virus translocation from the initially infected leaf to other parts of the plant is prevented, although the virus replication and initial cell-to-cell movement are not blocked. Later, most of the infected cells die, which result in a localised necrotic lesion at the site of infection.[12,13]

In the natural environment, PVY is transmitted by sap-feeding aphid vector, or vegetatively through propagated potato tubers. Experimentally, PVY can be transmitted by mechanical means such as grafting or rubbing of a sap from a PVY infected plant onto the leaf surface together with a fine abrasive powder, e.g. carborundum.[14] The latter method is a convenient mean for experimental transmission of the PVY to host plants, although strict standardization of the method is crucial for repeatable re-

sults. The aim of the present study was to investigate the relation between the titre of the virus in the inoculum and the titre of the viral RNA in the host plant, and additionally to determine whether there is the correlation between PVY titre in the inoculum and induction of disease symptom development in inoculated plants.

## 2. Experimental

96 virus free potato plants (*Solanum tuberosum* L.) of cultivar Désireé and 105 virus free plants of cv. Igor from stem node tissue culture (cultivated for 2 weeks) were planted in soil and kept at 21 ± 2 °C in growth chambers, with illumination at 70 $\mu Mm^{-2}s^{-1}$ (Osram L36W/77 lamp), a photoperiod of 16 h and relative humidity 70%. After 4 weeks, three bottom leaves of each plant were mechanically inoculated by rubbing the inoculum onto the leaf surface together with a fine abrasive powder of carborundum (0.037 mm). Inoculum was prepared by grinding $PVY^{NTN}$ (NIB-NTN isolate, AJ585342, referred also as NTN-Slo Isolate[15]) infected plants of cv. Pentland Squire from node tissue culture in the grinding buffer (0.02 M phosphate buffer, 0.01 M DETC, pH 7.6) in the ratios (w/v) 1:5, 1:10, 1:50 and 1:100. For control mock inoculation, healthy plants of cv. Pentland Squire from node tissue culture were ground in the grinding buffer in the ratio (w/v) 1:5. After 10 min incubation, the inoculum was washed from the leaf surface by tap water.

On potato plants of cv. Igor disease symptom development was monitored at 0, 3, 4, 5, 6, 7, 10 and 14 days post inoculation (dpi). At the same time points, samples from three plants per treatment were collected for the quantification of PVY RNA. For that purpose, the right distal quarter of the second inoculated leaf and the right distal quarter of the second upper non-inoculated leaf were collected and further analysed separately from each plant of cv. Désireé.. Collected leaf tissues were frozen immediately in liquid nitrogen and stored at –80 °C for further analysis.

Plant tissue was homogenised by Tissue Lyser (Qiagen, Hilden, Germany). RNA was isolated by MagMAX™ Plant RNA Isolation Kit (Applied Biosystems, Foster City, CA, USA) including DNAse treatment. RNA concentration, quality, and purity were assessed using agarose gel electrophoresis and NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) . RT-qPCR reactions were performed on 2 µl of extracted RNA in a total volume of 10 µl using the AgPath-IDTM One-Step RT-PCR Kit

(Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Primer and probe concentrations, together with PCR amplification conditions, were as in Kogovšek *et al.*[16] Two dilutions of RNA were analysed, each in duplicate. Reactions were carried out using the ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). RT-qPCR conditions included a reverse transcription step (10 min at 48 °C) followed by 40 amplification cycles (1 min at 60 °C, 15 s at 95 °C). Data were analysed using the SDS v 2.3 software (ABI, Foster City, CA, USA). PVY RNA amount was quantified using a relative standard curve method by normalization to the endogenous control cox mRNA by quantGenius.[17] The significance of differences ($p \leq 0.05$) between mean values was determined by the Student's t-test.

## 3. Results and Discussion

The titre of $PVY^{NTN}$ RNA was studied in potato host plants of tolerant cv. Désireé after inoculation with the inocula obtained by grinding $PVY^{NTN}$ infected plant tissue in grinding buffer in different ratios (w/v), 1:5, 1:10 and 1:100, and therefore containing different titres of $PVY^{NTN}$. The amount of $PVY^{NTN}$ RNA detected immediately after inoculation (0 dpi) correlated with the titre of $PVY^{NTN}$ in the inoculum, indicating that the remnants of $PVY^{NTN}$ RNA on the leaf surface after washing the inoculum was detected.

From 0 dpi to 4 dpi the detected amount of $PVY^{NTN}$ RNA decreased (Figure 1) due to degradation of $PVY^{NTN}$ RNA on the leaf surface. It was already demonstrated in previous studies[18] that it is possible to detect the traces of the inoculum on the leaf surface of extremely resistant cv. Santé (in which PVY cannot multiply) even at 14 dpi; and to detect viral RNA in dry inoculum 12 days after its preparation. Double-stranded RNA products shown to arise during viral replication of *Potyviridae*[19,20] can explain the stability of viral RNA.

It is hypothesised that PVY enters the initial potato cells for further multiplication through broken leaf hairs

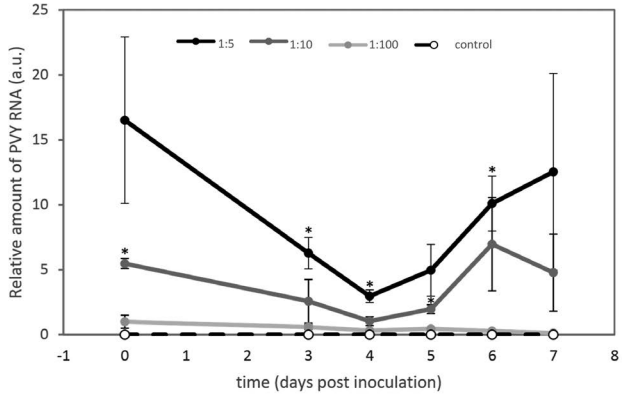


**Figure 1.** The relative amount of $PVY^{NTN}$ RNA measured by RT-qPCR in the inoculated leaves of cv. Désireé from 0 to 7 dpi. Error bars represent the standard error (n = 3). Statistical comparison was made between plants inoculated with the inoculum in which $PVY^{NTN}$ infected potato sap was diluted in buffer in the ratio 1:100 and plants inoculated with the inocula in which $PVY^{NTN}$ infected potato sap was diluted in buffer in the ratios 1:5 or 1:10. Statistically significant differences ($p \leq 0.05$) are indicated with.*

(broken by rubbing at mechanical inoculation). At 5 dpi the amount of newly multiplied $PVY^{NTN}$ RNA exceeded the amount of $PVY^{NTN}$ RNA in the remnants of the inoculum on the leaf surface of the plants inoculated with the inocula in which $PVY^{NTN}$ infected potato sap was diluted in buffer in the ratios 1:5 or 1:10 (Figure 1). From 5 to 7 dpi, higher $PVY^{NTN}$ RNA titres were observed in plants inoculated with more concentrated inoculum (Figure 1). In leaves, inoculated with the inoculum in which $PVY^{NTN}$ infected potato sap was diluted in buffer in the ratio 1:100, the titre of $PVY^{NTN}$ probably increased later than 7 dpi, as also the translocation of $PVY^{NTN}$ from inoculated into upper noninoculated leaves occurred later (Figure 2).

In inoculated leaves of potato the concentrical distribution of PVY around the point of infection was shown during HR.[12] In inoculated leaves of tobacco (*Nicotiana tabacum* L.) cv. Xanthi movement of PVY from initially inoculated cells to neighbour cells in concentrical circles, loading of PVY into the veins and translocation of PVY into upper non-inoculated leaves was shown in tolerant interaction.[21] Our results in tolerant potato-PVY interaction showed that the translocation of $PVY^{NTN}$ from inoculated into upper non-inoculated leaves of potato cv. Désireé occurred regardless of the virus titre in the inoculum, while the speed of the process was dependent on the virus titre in the inoculum (Figure 2). Although the multiplication of $PVY^{NTN}$ in inoculated leaves and the translocation of $PVY^{NTN}$ from inoculated into upper non-inoculated leaves was slower in the case of lower viral titre in the inoculum, the final titre of $PVY^{NTN}$ RNA in upper non-inoculated leaves 14 dpi was independent of the virus titre in the inoculum (Figure 2). Even much longer times (a few months) are required for the spread of $PVY^{NTN}$ from nutrient solution containing extremely low titre of $PVY^{NTN}$, through the roots, to the green parts of the plants as shown in experiments using a hydroponic system.[22]

Additionally, the disease symptom development after inoculation with different $PVY^{NTN}$ titre was monitored on potato plants of cv. Igor, which is one of the most susceptible and sensitive potato varieties to $PVY^{NTN}$. A few days after infection, local lesions appeared on inoculated leaves, followed by leaf chlorosis (yellowing of the leaves) and leaf drop of inoculated leaves. In general, the severity of the disease symptoms correlated with the titre of $PVY^{NTN}$ in the inoculum. The appearance and the development of disease symptoms was slower in the plants inoculated with lower titre of $PVY^{NTN}$ in the inoculum (Figure 3), what is shown also by the correlation between the titre of $PVY^{NTN}$ in the inoculum and the leaf drop (Figure 4). By 14 dpi, all 3 inoculated leaves dropped in plants inoculated with the inocula diluted in the ratios 1:5 or 1:10. At the same time, in plants inoculated with the inocula diluted in the ratios 1:50 or 1:100 only the 1st and the 2nd inoculated leaf dropped, while the 3rd inoculated leaf showed more severe symptoms in plants inoculated with the inoculum diluted in the ratio 1:50 as compared to the plants inoculated with the inoculum diluted in the ratio 1:100 (Figure 3). No symptoms appeared on control potato plants, where also no leaf drop was observed at 14 dpi (Figure 3, right panel).

Besides the high titre of $PVY^{NTN}$ in the inoculum, also too much carborudum or too excessive rubbing at mechanical inoculation promotes the leaf drop on inoculated plants (data not shown). Early leaf drop of inoculated leaves can prevent the spread of the virus to upper non-inoculated leaves and the development of symptoms in upper non-inoculated leaves. In $PVY^{NTN}$ – cv. Igor interaction, the absence of symptoms in upper non-inoculated leaves indicates with a high probability the absence of the virus in upper non-inoculated leaves.

Although the plants are kept in the same growth chamber controlled environment with the same tempera-

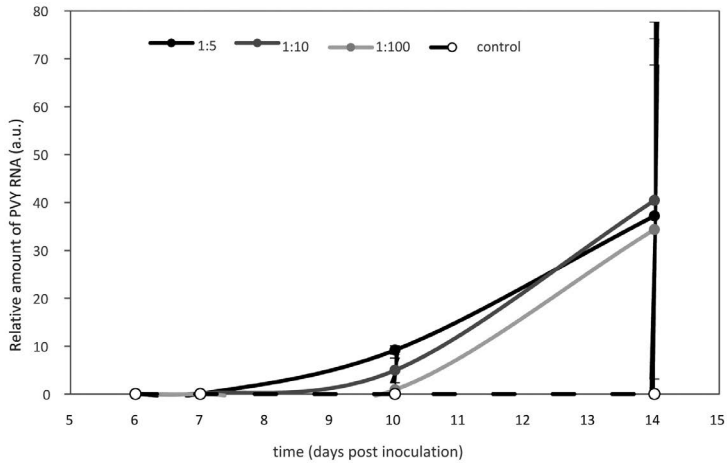


**Figure 2.** The relative amount of $PVY^{NTN}$ RNA measured by RT-qPCR in upper non-inoculated leaves of cv. Désireé from 6 to 14 dpi. Error bars represent the standard error (n = 3). Statistical comparison was made between plants inoculated with the inoculum in which $PVY^{NTN}$ infected potato sap was diluted in buffer in the ratio 1:100 and plants inoculated with the inocula in which $PVY^{NTN}$ infected potato sap was diluted in buffer in the ratios 1:5 or 1:10. None of the differences was statistically significant ($p \leq 0.05$).

**Figure 3.** Disease symptoms on potato plants of cv. Igor at 14 days after inoculation with the inocula in which $PVY^{NTN}$ infected potato sap was diluted in buffer in the ratios 1:5 (left), 1:10 (second from the left), 1:50 (middle) or 1:100 (second from the right); and control potato plants (right).

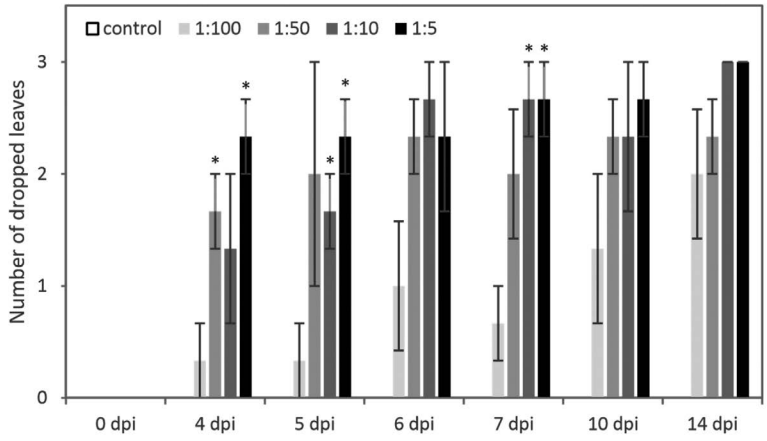


**Figure 4.** The average number of dropped inoculated leaves per plant in cv. Igor after infection with $PVY^{NTN}$ from 0 dpi to 14 dpi. Error bars represent the standard error (n = 3). Statistical comparison was made between plants inoculated with the inoculum in which $PVY^{NTN}$ infected potato sap was diluted in buffer in the ratio 1:100 and plants inoculated with the inocula in which $PVY^{NTN}$ infected potato sap was diluted in buffer in the ratios 1:50, 1:10 or 1:5. Statistically significant differences ($p \leq 0.05$) are indicated with.*

ture, relative humidity, illumination and photoperiod, the plant's response to viral infection (including the development of symptoms) can vary between experiments (biological repetitions) due to yet unknown parameters (e.g. physiological state of the plant, season…).

## 4. Conclusions

We can conclude that higher titre of $PVY^{NTN}$ in the inoculum resulted in faster increase of $PVY^{NTN}$ RNA titre in the inoculated leaves and in faster translocation of $PVY^{NTN}$ from inoculated leaves into upper non-inoculated leaves. The final amount of $PVY^{NTN}$ RNA in upper noninoculated leaves was independent of $PVY^{NTN}$ titre in the inoculum. In addition, the occurrence of the disease symptoms was slower in the plants inoculated with lower titre of $PVY^{NTN}$ in the inoculum as compared to the plants inoculated with higher titre of $PVY^{NTN}$ in the inoculum. Thus to obtain reproducible results of any biochemical, physiological or molecular study of potato – PVY interaction one should use standardised titre of the virus in the inoculum.

## 5. Acknowledgment

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## Povzetek

Virus krompirja Y (*Potato virus Y*, PVY) je ekonomsko najpomembnejši virus krompirja, zato se številne raziskave osredotočajo na študij interakcije PVY z gostiteljsko rastlino. Za zagotavljanje ponovljivosti rezultatov je pomembna standardizacija raziskovalnih metod. Najpogostejši način prenosa PVY na gostiteljsko rastlino v eksperimentalnih pogojih je mehanska inokulacija, pri kateri na površino lista nanesemo abrazivno sredstvo in sok okužene rastline, pri čemer pa je potrebno določiti dejavnike, ki so ključni za ponovljivost tega postopka. V raziskavi smo pokazali, da se je višji titer virusa v inokulumu odražal v hitrejšem dvigu titra RNA $PVY^{NTN}$ v inokuliranih listih ter v hitrejšem širjenju $PVY^{NTN}$ iz inukuliranih listov v zgornje neinokulirane liste. Končni titer RNA $PVY^{NTN}$ v zgornjih neinokuliranih listih pa ni bil odvisen od titra virusa v inokulumu. Poleg tega smo opazili tudi, da je bila hitrost pojava bolezenskih znamenj odvisna od titra virusa v inokulumu.