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Impact of the potato inoculation date on potato virus Y load and viral distribution in daughter tubers at harvest

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Running head: PVY translocation in daughter tubers

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ABSTRACT

Aged plants are more difficult to infect than young plantlets. This modification of susceptibility is described as mature plant resistance (MPR). For potato virus Y (PVY), MPR is known to lead to i) low infection rates of plants inoculated at post-flowering stage and ii) decrease of the number of infected daughter tubers. However, the impact of inoculation date on the capacity of PVY to accumulate in daughter tubers has not been studied so far. Field and greenhouse experiments were carried out to better understand PVY epidemiology and to help potato growers to evaluate consequences of early/late infections on the quality of their crops. In field trials, potato plants (cv. Bintje) were covered by insect proof nets from planting to harvest except for a 14-day period to expose plants to natural PVY infections. Under controlled conditions, potato plants were mechanically inoculated with PVY at different dates from pre-flowering stages (early inoculations) to post-flowering stage (late inoculations). At harvest, daughter tubers were individually collected and analysed to define proportions and viral load of infected tubers according to the time between virus inoculation and harvest. Our results showed that although the age of plants at the time of inoculation can modify their susceptibility to PVY infection, in return, early and late PVY inoculations lead to similar i) rates of infected tubers at the plant scale and ii) equivalent viral accumulation in infected tubers. All together, these data revealed that both early/late infections are high risks for the sanitary quality of potato tubers.

INTRODUCTION

Potato virus Y (PVY) (Shukla *et al.*, 1994) is the type-member of the genus *Potyvirus* (Brunt, 1992). PVY infects a wide range of host plants from the family Solanaceae and is transmitted in a non-persistent manner by more than 65 aphid species, including the green peach aphid (*Myzus persicae*) which is the most efficient PVY vector (Lacomme *et al.*, 2017). PVY is the most economically damaging virus on potato. Indeed, in the absence of protection against aphids (e.g. sprays of mineral oil), yield losses due to PVY infection can reach up to 80.0% (Valkonen, 2007). In the 1960s, PVY isolates were first divided into two main strain groups, PVY^N and PVY^O, based on their ability to induce (PVY^N) or not (PVY^O) tobacco vein necrosis on *Nicotiana tabacum* cv Xanthi and on their pathogenicity on potato (*Solanum tuberosum*) (Singh *et al.*, 2008). Then, since PVY variants with particular properties have been reported between late 1980s and early 2000s, PVY species are now classified into seven main groups: PVY^O, PVY^N, PVY^C, PVY^{N-Wi}, PVY^{NTN}, PVY^Z and PVY^E (Kehoe & Jones, 2016). Members of PVY^{NTN}, PVY^Z and PVY^E strain groups have been described as the most damaging PVY isolates on potato crops because of their ability to induce necrotic symptoms on tubers of susceptible cultivars (Beczner *et al.*, 1984; Piche *et al.*, 2004; Galvino-Costa *et al.*, 2012).

When a viruliferous aphid inoculates PVY to a host plant, the infection starts at the inoculated site. After a viral multiplication/accumulation phase in the first infected cells, the virus moves locally from cell-to-cell, crosses different cell layers of the inoculated organ and reaches the phloem tissues (Rupar *et al.*, 2015). Afterwards, viruses spread into the whole plant leading to a systemic infection of the host (Otulak & Garbaczewska, 2010). This process that drives the within-host spatio-temporal dynamics of PVY is generic. However, it has been demonstrated that its magnitude depends on several biotic (e.g. host genotype and viral strain) and environmental (e.g. temperature) factors. Indeed, it has been shown that the proportions of

infected organs (leaves, stems and tubers) were higher with PVY^N than with PVY^O (Basky & Almasi, 2005) and that the genetic background of potato cultivars significantly impacts the dynamics of systemic infections. According to the presence of *R* (ER, extreme resistance) or *Ny* (HR, hypersensitive resistance) genes, the movement of virus within the infected tissues is blocked (ER) or restricted (HR) to cells near the inoculation site (Szajko *et al.*, 2008). Finally, the age of the plant at the time of inoculation also influences within-host virus development. Indeed, it has been shown that old plants are more difficult to infect than young plantlets. This change in the susceptibility of the host with its age is common for infections with plant pathogens and has been described as ontogenic resistance, age-related resistance, developmental resistance, or as in our specific case study, mature plant resistance (MPR). MPR has been described for several viral pathosystems (e.g. wheat dwarf virus/winter wheat (Lindblad & Sigvald, 2004); potato virus S (PVS)/potato and potato virus X (PVX)/potato (Venekamp & Beemster, 1980)) but remains poorly understood. Previous studies suggest that MPR does not result from the expression of any resistance gene, but is rather a consequence of i) a drastic decrease of ribosome and RNA contents (Venekamp & Beemster, 1980) and ii) an increase of callose deposition at plasmodesmata (Kumar *et al.*, 2017). MPR has already been reported in several studies for the PVY/potato pathosystem (Beemster, 1972; Gibson, 1991; Shrestha *et al.*, 2014; Dupuis *et al.*, 2017). These studies suggest that the main observed developmental resistance is associated with the flowering stage when foliage has stopped to grow and when tubers has started to grow, i.e. about 70 days after planting (DAP). In addition, Dupuis (2017) demonstrated that part of the MPR phenomenon can be due to the decay of the mother tuber which impedes the movement of PVY from one stem to the other. MPR in potato plant, associated with a decrease in the number of infected tubers on the total tuber harvested, is known to depend on both host genotype and virus isolate. Shrestha *et al.* (2014) used a semi-quantitative ELISA approach to show that PVY load in potato leaf was similar within plants

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inoculated before and after the flowering stage. However, no data are available on translocation efficiency of PVY within infected tubers at the scale of the plant submitted to early or late PVY inoculations. In this study, we addressed the effects of plant age at infection for the within-host dynamics of PVY in infected plants. We used both field and greenhouse experiments to investigate PVY accumulation in plants and the translocation of virus to daughter tubers using several PVY isolates naturally and mechanically inoculated to potato plants. The main aim of this work is to provide new insights into the translocation of PVY to daughter tubers, and therefore into the epidemiology of the disease. This knowledge is particularly important for growers to evaluate the risk on the quality of potato tubers according to the timing of infections (early/late) along the cropping season.

MATERIALS AND METHODS

Field trials

Field trials were carried out in an experimental potato field located in the North of France. PVY-susceptible cv. Bintje potato seeds were used. For each year of experimentation, tubers came from a single certified super-elite class (less than 1.0% of virus-infected tubers) seed lot supplied by the French seed potato growers (FN3PT, Paris, France). The layout of the trial consisted of a 50-meters long and 5-meters wide plot with a single row of 120 potato seeds (plot A) and seven extra plots (plots B to H) of 5-meters long and 1-meter wide (Figure 1a). These extra plots were planted with 2 rows of 10 potato seeds. Aphid populations present in the trials were monitored twice a week from planting to harvest using yellow water pan traps.

In plot A, potato plants were exposed to aphids during the whole experiment. In plots B to H, an insect proof net (800 μ x1000 μ , Biotex, Saint Wandrille Ranc on, France) was used to protect plants from planting to harvest, except for a 14 day period during which the plants were exposed

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to natural aphid populations potentially presents at the trial site. This window of net removal (WNR) was different for each plot and corresponded to the 28th-42nd (i.e. D_{28/42}) day after planting (DAP) for plot B. To allow aphid exposure at the different potato physiological stages (Supplementary Figure 1), the net-free periods were scheduled at D_{42/56}, D_{56/70}, D_{70/84}, D_{84/98}, D_{98/112} and D_{112/126} for plots C, D, E, F, G and H, respectively (Figure 1a). To determine the sanitary status of planted material, plants from plots B to H were individually sampled when removing the insect-proof net and leaves were analysed using ELISA as described below. Likewise, plants in plot A were sampled at 28 DAP. PVY-infected plants were maintained in the experiment, but data associated with these plants and their tubers were removed from the analysis. At the end of each WNR period, plants were treated by an insecticide (Mandarin, Pro-active agent pyrethrinoid, 0.25L/ha, Philagro, Saint Didier au Mont d'Or, France) to prevent any further infections. To determine the sanitary status of plants after each WNR period, at 133 DAP, 20 plants evenly distributed in plot A and all plants from plots B to H were manually and individually harvested. Then, 15 daughter tubers were randomly collected for each plant, immediately transferred to the laboratory. Dormant tubers were directly analysed by real-time RT-PCR, and growing-on ELISA were applied on plantlets from replanted daughter tubers. The status (PVY-infected or healthy) and the serotype of PVY isolate(s) infecting mother plants of the different plots were defined using cumulated ELISA results from each of the 15 collected daughter tubers per plant. A mother plant was assigned to PVY-infected status when at least one of its 15 tested daughter tubers was infected. The infected mother plant was considered as having a mixed infection when either i) at least one of its daughter tubers was infected with PVY isolates belonging to different serotypes or ii) different PVY serotypes were individually detected in different daughter tubers.

This experimental procedure was repeated during two consecutive growing seasons between 3 May and 16 September 2016, and 20 April and 31 August 2017.

Greenhouse experiment

Greenhouse experiments based on mechanical inoculation of cv. Bintje were carried out in 2016 and in 2017. Potato seeds, initially stored at 4°C, were placed at 20°C in the dark for few weeks to break the tuber dormancy. As soon as sprouts emerged, 108 tubers were individually planted in an insect-proof greenhouse at 20°C ± 2°C with natural light. At 12 DAP, all except one emerging stem were removed from each plant. Sets of 12 plants were mechanically inoculated with the referent isolate PVY^N-605 (Jakab *et al.*, 1997). Each set of plants was inoculated according to Glais *et al.*, (2015). Briefly, crude sap produced from an PVY^N-605-infected source plant was rubbed on the apical 2-3 true youngest potato leaves of each plant of a set. Inoculation of a set was performed at 20, 47, 54, 61, 68, 75, 82, 89 and 100 DAP (Figure 1b) which corresponds to different developmental stages of potato (Supplementary Figure 2). Each potato plant was individually harvested at 140 DAP and the sanitary status of tubers were directly analysed by real-time RT-PCR, and an ELISA was applied on plantlets from replanted daughter tubers.

Plant sampling and PVY detection

Potato leaves collected from either one stem (in greenhouse experiments) or multiple stems of the same plant (in field trials) were placed in a plastic bag, afterwards transferred to the laboratory for ELISA analysis. Tubers were stored at 4°C for two months. Then, one eye-plug was sampled on the sprout rose-end on each tuber. Eye-plugs were placed into a gibberellic acid solution (4mg/L) for 1 h and dried overnight at room temperature before being planted in greenhouse at 20°C ± 2°C with natural light. Two months later, potato plantlets were sampled as mentioned above and tested by ELISA for the presence of PVY.

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Samples were tested by DAS-ELISA as described by Glais *et al.*, (2015) with a mixture of monoclonal and polyclonal antibodies raised against PVY (M. Guillet, FN3PT/INRAE, Rennes, France). Then, PVY-infected samples were tested with a PVY^N-monoclonal antibody (Bioreba, Reinach, Switzerland) and with a PVY^O-monoclonal antibody (15C10) provided by FN3PT/INRAE. Samples were considered infected when ELISA values (optical density at 405 nm) were higher or equal to three times ELISA values for healthy control samples.

In field trials, for each plot/year combination, a maximum of two samples infected by a PVY serotype (i.e. by either -O, -N or -U serotypes avoiding mixed infections) were selected for partial molecular characterization of viral genome. A total of 26 and 22 infected samples collected from potato plantlets grown from single infected daughter tubers were selected in 2016 and 2017, respectively (Supplementary Table 1). An immunocapture (IC)-RT-PCR procedure was applied according to Glais *et al.* (1998) for the fixation of PVY particles present in plant sap and the amplification of two regions of the PVY genome. The regions coding for HC-Pro/P3 proteins (nt 1672-2666, according to Jakab *et al.*, 1997) and coat protein (CP) (nt 8572-9373, according to Jakab *et al.*, 1997), where hot spots of recombination can be used to identify PVY strain group members, were amplified and sequenced through Genoscreen platform (France) by direct sequencing of amplification products in forward and reverse directions. The acronym and the sequence of primers used for the different steps (RT, PCR, sequencing) are described in Supplementary Table 2. Nucleotide sequences were edited by the BioEdit software (Hall, 2011) and compared with ClustalW (Thompson *et al.*, 1994) to three published PVY sequences: PVY^N605 (accession number X97895), PVY^O139 (accession number U09509) and PVY^{NTNH} (accession number M95491).

Potato tuber sampling, nucleic acid extraction and real-time RT-PCR diagnosis

A peel of approximately 20 mg was taken from the heel end of each washed tuber directly after the harvest. For potato plants from field trial, peels of 15 tubers per plant were pooled and ground with a Pollähne press (Meku, Wennigsen, Germany). Potato plants from the greenhouse experiments produced fewer and smaller daughter tubers. Thus, peels from 2 to 4 tubers per plants were pooled. The tuber sap was diluted (v/v) with 500 µl of Mortimer buffer (0.2% BSA, 0.5% polyvinyl pyrrolidone, 0.02 M PBS, 0.05% Tween 20). After centrifugation for 2 min at 10 000 rpm, the extraction of nucleic acids was performed on 200 µl of the supernatant using InviMag virus RNA Kit (Strattec Molecular, Berlin, Germany) following manufacturer's recommendations. This method associated with the KingFisher™ mL System (ThermoFisher Scientific, Waltham, USA) allows the automatic efficient handling of RNA on magnetic particles and by a series of three washes in different buffers to obtain purified nucleic acids. Briefly, a cell lysis was performed by adding 400 µl of Lysis buffer in the presence of 20 µl proteinase K and 20 µl carrier-RNA from the extraction kit to the 200 µl of the supernatant from tuber sap. This solution was incubated, while being agitated, at 65°C for 10 min. Then, the mixture was transferred into the KingFisher mL instrument, mixed with 260 µl of binding solution and 20 µl of solution containing the magnetic beads. Three automatic washing steps of the particles fixed to RNA were then performed and after 15 min, 100 µl of pure nucleic acids were eluted. An aliquot of 2 µl of viral RNA (± 70 ng/µl) was analysed by multiplex RT-qPCR in a total volume of 25 µl using the AgPath-ID™ One-Step RT-PCR kit (ABI, Foster, CA, USA) according to the manufacturer's instructions, except for the primers (Univ F, Univ R) and probe (Univ) used at concentrations according to Kogovsek *et al.* (2008), i.e. 300 nM and 200 nM, respectively. In addition, an endogenous control based on the amplification of the plant cytochrome oxidase gene (Cox) was used to validate the quality of each extraction. Concentrations of primers (COX F, COX RW) and probe (COXSOL 1511T) were used

according to Boonham *et al.* (2009). Real-time thermal cycling conditions were as follows: 10 min at 45°C for the reverse transcription (RT) of the viral RNA, then 15 min at 95°C for the RT inactivation and initial denaturation of nucleic acids, followed by 40 cycles of amplification with 45 sec at 60°C and 15 sec at 95°C. Data were analysed with the LightCycler® 480 software v.1.5.0 (Roche). Viral quantification was obtained by comparison with standard fractions containing the targeted PVY sequence at different concentration (from 10^8 to 10^2 copies per reaction) according to Balme-Sinibaldi *et al.* (2006). This standard corresponds to a plasmid (p3'end) in which PVY^N-605 nucleotide sequence from nt 4065 to 9801 has been introduced (Tribodet *et al.*, 2005). In our experimental conditions with tuber extracts, a sample is considered infected by PVY once the C_T value, defining the amplification cycle number at which the fluorescence exceeds the reporting threshold, is below 30. This C_T threshold corresponds to the RT-qPCR detection limit fixed under our experimental conditions at 10^3 copies of the targeted PVY RNA per sample. This C_T cut-off was validated in our laboratory by comparing qPCR assays carried out on tubers and ELISA tests carried out on plantlets after replanting the corresponding tubers. Based on this C_T threshold, more than 99.0% of correlation was found between both diagnostic techniques (data not shown).

Statistical analyses

Prevalence data were analysed using Generalised Linear Models with a Binomial distribution: $\text{ni-Binomial } [N, p]$, where n is the number of infected plants (or tubers) among a sample of N tested plants (or tubers) and p the probability of infection, with a logit link function. The effects of experimental year, the plant age at infection or exposure to vectors were tested with Wald tests. PVY load in daughter tubers was analysed by considering its natural log and a Generalised Linear Model with a Gaussian distribution to test the effects of experimental year and the plant age at infection with Wald tests. For each statistical model pairwise comparisons and Tukey

tests were used to compare and rank the levels of each tested variable. All the statistical analyses were performed with the R software (R core team, 2016).

RESULTS

Vegetative development of potato plants in trials

In the field trials, the physiological stages of the plant were noted at the beginning of each of the 7 WNR (Supplementary Figure 1). For both 2016 and 2017 experiments, at the WNR associated with plot B (28-42 DAP), potato plants reached 30-40 cm. At the following observations of growing stage, plants were in flower bud (plot C (42-56 DAP)), in full flowering (plot D (56-70 DAP)), flowers had fallen (plot E (70-84 DAP)), still well green (plot F (84-98 DAP)) and with a pronounced yellowing (plot G (98-112 DAP)). Then, potato plants were senescent with the drying of stems at the observation carried out on plot H (112-126 DAP).

In the greenhouse trials, the mechanical inoculation was performed at nine dates after planting. At 20 DAP, for both repetitions, potato plants were shorter than 30 cm, while they reached a height of about 80 cm and full flowering at 47 and 54 DAP (Supplementary Figure 2). At 61 DAP, plants were still green, but some flowers have started to fall. At 68 DAP, some leaves at different stages in plants became yellow, and only few flowers were left. At 75 and 82 DAP, yellowing was more pronounced on leaves and generalized with the fall of all flowers. At 89 DAP, the whole plants were yellow. Finally, at 100 DAP the yellowing of plants was noted with the drying and the fall of basal leaves.

Monitoring of aphid flights

In 2016, a total of 550 aphids were trapped during the monitored period (i.e. from the planting to the harvest of potato). Given the WNR periods, different aphid pressures were recorded (Figure 2a and Table 1). For WNR that corresponds to plot B, 54 aphids were collected. For the following WNR applied to plots C to H, 128, 105, 61, 33, 8 and 1 individuals were trapped. In 2017, a total of 1533 aphids were collected throughout the potato cultivation period. During the different WNR (corresponding to exposure of plots B to H) 93, 312, 767, 173, 37, 17 and 21 aphids were trapped, respectively. Whatever the date of the experiment, the higher number of trapped aphids was obtained in plot C (128 and 312 aphids for 2016 and 2017, respectively) and in plot D (105 aphids for 2016 and 767 for 2017), corresponding to 42-70 DAP. In return, in 2016 this number was much lower in plot G (8 aphids) and plot H (1 aphid), corresponding to 98-126 DAP. In both experiments, *Myzus persicae* constituted 48.0-80.0% of trapped aphid species according to the considered plot.

Monitoring of the percentage of infected plants

In 2016 field trial, only 3 out of the 258 tested plants (each of the plots D and E contained only 19 plants) were initially PVY-infected. These three plants belonged to the plot A. In 2017, 2 plants (1 from plot A, and 1 from plot F) were found infected at the beginning of the experiment. The infected status of these plants resulted from seed-borne PVY infections. Data from these PVY-infected plants were not considered in the analyses.

Plot A was exposed to natural infections during the whole potato growing season. In 2016, 19 of the 20 randomly selected plants of plot A were infected by PVY at the end of the experiment (Figure 2a). ELISA performed on plantlets from replanted daughter tubers revealed that potato

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plants from plots B to H displayed different infection rate according to the WNR period. Indeed, the percentage of infected plants was 35.0% (7/20) in plot B (WNR = 28-42 DAP) and increased steadily to 85.0% (17/20) and 100.0% (19/19) in plot C (WNR = 42-56 DAP) and plot D (WNR = 56-70 DAP), respectively. This rate remained high (90.0%-95.0%) for plots E (18/19; WNR = 70-84 DAP) and F (18/20; WNR = 84-98 DAP) but decreased to low values (10.0%-25.0%) for plots G (5/20; WNR = 98-112 DAP) and H (2/20; WNR = 112-126 DAP). Similar results were noted in 2017, although a low inter-annual variation of the percentage of infected plants was observed between these two biological repetitions (1.60% of the deviance, $p=1.11E-06$). Indeed, in 2017 the percentage of infected plants was 100.0%, 80.0%, 100.0%, 100.0%, 100.0%, 52.6%, 25.0% and 15.0% in plots A (20/20), B (16/20), C (20/20), D (20/20), E (20/20), F (10/19), G (5/20) and H (3/20), respectively. Under these experimental conditions, potato plants were i) highly infected by PVY from 42 to 98 DAP and ii) mostly uninfected when exposed to natural infections occurring from 98 DAP, when the number of trapped aphids was low. Statistical analyses highlighted that the period at which the plant was infected was significant and explained 73.4% of the observed infection rate ($p=2.2E-16$). In addition, a significant effect of the number of caught aphids according to the WNR period was also observed ($p=2.2E-16$) and explained 22.5% of the total deviance.

For PVY inoculations carried out under greenhouse conditions, similar infection pattern was observed in experiments in 2016 and 2017. Infection rates above 75.0% were obtained for inoculations carried out between the 20th and the 61st DAP while later inoculations were associated with infection rates below 60.0% (Figure 2b). The last two inoculations performed at 89 and 100 DAP were all unsuccessful.

Virus diversity within plots

ELISA characterization of PVY present in field infected plants showed that in 2016 plot A was infected by PVY of serotype-N (19/19) while some of these infected plants were also infected by PVY of serotype-O (4 Y^N/Y^O mixed infected plants). Plots B to H with different WNR periods had different patterns of PVY serotype. Indeed, plots B, G and H were infected by PVY of serotype-N only, whereas plants from plots C, D, E and F were infected either by PVY of serotype i) -N, ii) -N and O or iii) -N, -O and isolate(s) from the unconventional serotype-U (Lacroix *et al.*, 2010). These particular PVY^U isolates are characterized by a positive detection with the non-specific antibody raised against PVY, but a lack of detection with PVY^N and PVY^O monoclonal antibodies. Based on these serological properties, PVY^U isolates can only be identified in single infected daughter tubers but not in mixed infection. The proportions of each type of infection (i, ii and iii) were different between plots (Figure 3a).

Conversely to 2016, infections with PVY of serotype-O were more frequent in 2017. It was observed in plot A where 95.0% of potato plants (19/20) were in mixed infection with PVY isolates of serotype-O and -N. This trend was mainly observed in the first half of growing potato season (plots B to D) (Figure 3b). Indeed, we noted in plot B a high proportion (55.0%, 11/20) of plants infected only by PVY of serotype-O, and a high prevalence of mixed infections in plots C (65.0%, 13/20) and D (100.0%, 20/20). Plots E to H were mainly single-infected by PVY of serotype-N.

A partial molecular characterization of up to 2 isolates/serotype/plot was performed by sequencing fragments in the HC-Pro/P3 (994 bases from nt 1672 to 2666) and in the CP (801 bases from nt 8572 to 9373) genes (Supplementary Table 1). These two regions of the PVY genomes are known to be hot spots of recombination events between PVY^N and PVY^O sequences (Glais *et al.*, 2002). In 2016 experiment, 15 out of the 16 isolates of serotype-N

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displayed recombination junctions (RJ) in HC-Pro/P3 (at nts 2414-2418) and in CP (between nts 9163-9182) regions. These molecular features are characteristic of PVY^{NTN} isolates. The isolate without RJ displayed a N-type nucleotide sequence and should correspond to a PVY^N isolate or a non-recombinant PVY^{NTN} isolate (Glais et al., 2004). Eight serotype-O PVY isolates were sequenced. Three isolates showed no RJ and displayed an O-type nucleotide sequence (not shown) suggesting their assignment to PVY^O strain and five isolates displayed a RJ in HC-Pro/P3 region (at position 2392-2393 or 2395-2396) and a O-type nucleotide sequence in the CP region (not shown). According to these molecular characteristics, these five isolates belong to PVY^{N-Wi} strain. Two PVY^U isolates were included in the sequence analyses. One presents the two RJ described for PVY^{NTN} strain whereas the other PVY^U isolate has PVY^{N-Wi} molecular pattern (i.e. a RJ in the HC-Pro/P3 region). In 2017, the 13 sequenced serotype-N isolates displayed PVY^{NTN} type RJs in HC-Pro/P3 and CP regions. Eight out of nine sequenced serotype-O isolates corresponded to PVY^O isolates because they have no recombination junction and displayed an O-type nucleotide sequence. One serotype-O isolate showed a single RJ in the HC-Pro/P3 region located at position 2392-2393 leading to its assignment to the PVY^{N-Wi} strain.

Virus translocation in daughter tubers

Due to a low number of harvested tubers from experiments carried out under controlled conditions, translocation of PVY from inoculated leaves to daughter tubers was analyzed only under field conditions. In 2016, half of infected plants from plot A had between 25.0% and 92.8% infected daughter tubers (63.2% on average) (Figure 4a), with only two plants showing an infection of 100.0% of their progeny tubers (Table 2). In 2017, 50.0% of infected plants from plot A displayed between 92.3% and 100.0% of infected daughter tubers (97.2% on average) (Figure 4b), with 15 out of 20 infected plants showing an infection of 100.0% of their daughter

tubers (Table 2). Except this difference, the distribution of the percentage of infected daughter tubers per plants in plots B to H showed the same trend in both years, although a low inter-annual variation of this distribution was observed between 2016 and 2017 experiments (1.9% of the deviance, $p=2.26E-05$). In return, the effects of the WNR period (19.3% of the deviance, $p=1.17E-12$), and the WNR x year interaction (8.0% of the deviance, $p=1.4E-13$) were significant. Indeed, the pairwise comparisons of the percentage of infected tubers in plants between the different plots in 2016 highlighted that plots D and E (88.0% on average) showed a higher percentage of infected tubers than in plots B, C, F, G, H (65.0% on average), and this was not different from plot A (63.2% on average) (Table 2). In 2017, statistical analyses also highlighted a significant influence of the WNR period on virus translocation to tubers (40.1% of the deviance, $p=2.2e-16$). Plants infected in plots B, C, E, F, G, H had 58.0% on average percentage of infected tubers, whereas infected plants from plot D showed 96.3% of infected tubers, as in plot A (Table 2).

Viral accumulation in daughter tubers

PVY load in infected samples was estimated for each studied potato plant by bulking the peels of daughter tubers. In field conditions, the change of the distribution of PVY titer in daughter tubers with the date of infection plants showed similar pattern in both years, although significant effect, but low influential, of the year (6.3% of the deviance, $p=0.0010$) or of the year x WNR period (5.4% of the deviance, $p=0.023$) was found. In pooled data of 2016 and 2017, 12.6% of the deviance of the PVY titer in infected tubers was explained by the WNR period ($p=3.86E-06$). In 2016, tubers from half of infected plants in plot A had a PVY titer between $1.28E05$ ($\text{Log}=11.76$) and $1.28E06$ ($\text{Log}=14.06$) copies of viral RNA/2 μl of total RNA extract with an average virus load at $6.9E05$ PVY copies (Figure 5a). As plot A was continuously exposed to natural viral infection along the growing period, viral load in infected plants from this plot

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should correspond to the maximum of virus accumulation for this trial. Pairwise comparisons analyses highlighted that the PVY load was not different in tubers from infected plants of plots A, B, C, F, G and H ($3.94E5$ PVY copies on average) (Figure 5a). In return, a significantly higher PVY titer was observed in tubers from infected plants from plots D and E ($9.99E5$ PVY copies on average). In 2017 experiment, the period from which the plant was infected also explained 32.0% of the variability of PVY titer in progeny. Indeed, contrary to plants of plots B, E, F and H where no significant difference in PVY titer in infected tubers was noted ($4.1E05$ PVY copies on average), a higher PVY load was statistically observed in infected plants from plot D ($1.01E06$ copies on average) which is close to that of plants from plot A ($7.1E05$) (Figure 5b). Plants of plots C and G displayed an intermediate PVY load between these two groups.

To complement the analyses of viral load carried out at the plot scale, in 2016 experiment virus quantifications were carried out at plant scale in order to study PVY load in the daughter tubers from an infected mother plant. Two plants each from plots B, D, F, and H were randomly selected, and their daughter tubers were individually tested in real-time RT-PCR. Statistical analyses revealed that PVY load was not significantly different between infected daughter tubers from the same mother plant, nor between the two studied plants in each plot ($0.059 < p(Tuber) < 0.84$; $0.10 < p(Plant) < 0.82$) (Supplementary Figure 3).

For the greenhouse trials, a significant low inter-annual variation (7.27% of the deviance, $p=0.012$) in PVY load was observed. Indeed, an average PVY titer of $9.05E04$ copies of viral RNA/2 μ l of total RNA extract was obtained in 2016 experiment (Figure 5c), and $2.74E04$ in 2017 experiment (Figure 5d). Nevertheless, both trials highlighted the same trend, with no significant effect of the date of inoculation ($p > 0.05$).

DISCUSSION

The age of plants at the time of inoculation is known to be one of the factors that determines the susceptibility of potato to PVY (Beemster, 1972; Venekamp & Beemster, 1980; Gibson, 1991; Shrestha *et al.*, 2014; Chikh-Ali *et al.*, 2020). However, impact of mature plant resistance (MPR) on PVY accumulation in daughter tubers produced by infected potato has been poorly described in the literature. Such new insights into the PVY/potato pathosystem are important i) for researchers to better understand the epidemiology of this viral disease and ii) for growers to evaluate the consequences of early/late infections on the quality of their potato production. In the present study, field and greenhouse experiments were designed to assess how the dynamics of viral accumulation in tubers changes with the timing of inoculation during the growing season. Our study confirmed an ontogenetic change in plant resistance. This is characterised by an increase in host susceptibility followed by a strong decrease until near complete resistance, with the flowering stage as the benchmark of the appearance of MPR. This pattern of plant-virus interactions was observed in field and greenhouse conditions, and is consistent with published studies that pointed out a MPR switch about 60 DAP (i.e. 30 days after potato plant emergence) and the complete MPR about 90 DAP (i.e. 60 days after potato emergence) (Venekamp & Beemster, 1980; Gibson, 1991; Dupuis, 2017). In our study this evolution towards less susceptible plants was observed earlier in greenhouse conditions (61 DAP) than in field conditions (from 84 to 98 DAP). The difference in controlled conditions, and the time lag of almost 15 days of the potato flowering could explain this observed difference. In field trials, we observed a clear decrease of plant susceptibility which is statistically mainly due to the plant age at the infection, and to a lesser extent, due to the number of caught aphids at the different WNR periods. However, at the plant scale we noted that this phenomenon had no significant

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effect on viral translocation in daughter tubers. This means that if the infection occurred 28/42 (plot B) or 112/126 (plot H) days after planting the virus infected on average 58.0-65.0% of daughter tubers per infected plant. Some previous studies reported that the appearance of the MPR phenomenon occurs together with a decrease in the proportion of infected progeny tubers (Beemster, 1972; Venekamp & Beemster, 1980; Gibson, 1991; Dupuis, 2017; Chikh-Ali *et al.*, 2020). Moreover, Beemster (1972) stated that the longer the period is between inoculation of PVY^O and harvest, the more tubers are infected. However, all these studies compared the number of infected tubers to the total number of tuber tested in trials, and not to the total progeny tubers from infected plant, as in our study. These two levels of analysis could explain the discrepancy with our results. In return, for the two consecutive years a significantly higher percentage of infected daughter tubers was observed in plants infected from 56 to 84 days after planting (plots D/E-2016-88.0% on average; or D-2017-96.0% on average). In 1991, similar results were reported with the aphid inoculation of PVY^O at different weeks after the emergence of potato plants (Gibson, 1991). Indeed, this study showed that at 56 DAP, the PVY aphid inoculation of potato cultivar King Edward led to 62.0% of infected tubers from PVY-inoculated plants, whereas at 35, 42, 70 and 84 DAP, the percentages were 45.0%, 54.0%, 46.0% and 33.0%, respectively. To a lesser extent the same trend was also observed with the potato cv. Record.

For both field experiments, PVY load in daughter tuber lead to similar conclusions to those obtained for the percentage of infected tubers. Firstly, between early (plot B) or late infections (plot H) no significant differences were observed in PVY load in daughter tubers. This result is consistent with Shrestha *et al.* (2014) who revealed that mean PVY titer estimated by semi-quantitative ELISA in potato leaf sap was not statistically different between i) aphid-mediated or mechanical inoculations and ii) early and late inoculations. Our results highlight that if plant infection occurs in the period 7 to 21 or 91 to 105 days before harvesting, the virus accumulates

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at the same level in daughter tubers. Secondly, a higher PVY titer was also observed in progeny when mother plants were infected 56 to 84 DAP (plots D and E, 2016) or 56 to 70 DAP (plot D, 2017). The difference of PVY translocation and accumulation in tubers in these plots compared to all other plots could not be explained by the presence of particular PVY isolates within these WNR periods because similar PVY isolates (NTN, N, O, and W isolates) were also identified in other plots. These results suggest that a plant infected between 56 and 84 DAP may favour viral translocation and accumulation in daughter tubers rather than when infections occur earlier or later. In our experiments, this time window overlaps a period from full flowering to drop of flowers. The flowering physiological stage seems to be associated with the tuber growth with an influx of water and starch to the tubers (Ellissèche, 1996). This transfer of nutrients to tubers at this period could explain the promoted spread and load of PVY in daughter tubers.

In addition, our results pointed out the expected effect of the number of caught vectors in field that was significant to explain both prevalence and load of PVY in daughter tubers. Nevertheless, the ontogenetic change of plant susceptibility with age should be another factor interfering with virus infection because the same number of vectors has contrasted effects on the rate of infected daughter tubers and PVY load in infected hosts when the age of plant at infection changes. Results in plot A demonstrated this interaction, because a low aphid flight activity as in 2016 (550 individuals) led to a lower percentage of infected daughter tubers and PVY titer, than with a high aphid flight activity as in 2017 (1533 individuals). However, the number of aphids may not be the only factor affecting PVY translocation and accumulation in daughter tubers from infected plants. Indeed, even if in 2016 experiment plots B and E shared a similar number of trapped aphids (54 and 60, respectively), a similar number of trapped *M. persicae* (34 and 39, respectively), and similar PVY isolates in infected plants, a significant difference in PVY translocation and PVY titer in daughter tubers was observed.

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The most interesting point of this study is the fact that accumulation of PVY in infected daughter tubers had similar level even if mother plant was infected during its active growth or senescence phases. This result was obtained in field conditions (where potato PVY infections can result from multiple aphid-mediated viral inoculations) and in greenhouse conditions (where potato PVY infection resulted from a single mechanical inoculation). That means that similar PVY load in infected tubers was reached soon after (i.e. a few days) an inoculation (e.g. plots H, inoculated 7/21 days before harvest/diagnosis) and was maintained at this level even for long infection period (e.g. plot B, inoculated 91/105 days before harvest/diagnosis). Mehle *et al.* (2004), who studied the spread of PVY^{NTN} in various potato organs, demonstrated that i) the PVY^{NTN} isolate reaches the roots in 9 days (or less according to the potato cultivar) and ii) accumulates in infected plant at a level which does not increase between the ninth and the twentieth day after inoculation.

In our field study, PVY accumulated in infected tubers from cv. Bintje at a level above 10E05 copies/2 μ L of total RNA extracts. It would be worth investigating PVY translocation and accumulation for a range of cultivars with contrasted level of resistance to confirm or reject that PVY load in tubers remains above the threshold sensitivity of molecular detection tool. Early detection of PVY in dormant tubers through real-time RT-PCR method recently became a standard in testing procedure for several certified European laboratories (Schumpp *et al.*, 2021). It is therefore essential to guarantee the reliability of this molecular tool to detect PVY in dormant tubers of any potato cultivar that has been subjected to either early or late inoculation. Nowadays, seed potato growers still neglect the impact of late PVY infections, typically after flowering, on tuber production. However, our work demonstrates that late infections still represent a risk regarding vertical transmission of PVY in potato. Thus, growers may consider

that PVY remains a risk to their crop from planting to harvest, and in the case where tubers are produced for replanting, monitoring, control and protection of the crop is essential during the whole growing season.

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Figure 1: Schematic representation of field trials and description of procedures applied in field (a) and under greenhouse conditions (b) with potato plants of cv. Bintje. In field conditions, plot A corresponds to potato plants exposed to the natural viral infections during the whole experiment, i.e. 133 days. Plots B, C, D, E, F, G and H correspond to plants exposed to aphid flights during a period of 14 days without net protection at different periods (i.e. 28/42, 42/56, 56/70, 70/84, 84/98, 98/112 and 112/126 days after planting). Numbers 1 to 120 and 1 to 20 for plot A and plots B to H, respectively, correspond to the identification number of each plant. For the experiment carried out under greenhouse conditions, dates of PVY inoculation after planting and the number of days between inoculation and harvest are indicated.

Figure 2: Percentage of PVY-infected potatoes of cv. Bintje in field (a) and under greenhouse conditions (b) according to the exposure periods to natural viral infections after planting (field experiments) and to the date of the mechanical inoculation of the virus (greenhouse experiments). For field and greenhouse experiments, dark and light grey correspond to data obtained in 2016 and 2017, respectively. The dotted lines with black square and empty triangle indicate the number of trapped aphids in 2016 and 2017, respectively. Aphid data were determined using water trap located in field trials. Black square and empty triangle in plot A correspond to the total number of trapped aphids during the potato growing season in 2016 and 2017, respectively. The empty arrows indicate the peak flowering time of potato plants.

Figure 3: Proportion of the different PVY serotypes identified in infected potato plants from plots A to H in 2016 (a) and 2017 (b) field trials. Color code corresponds to plants infected by PVY isolates of serotype N (in black), O (in grey), N+O (mixed infections) (in white) and N and/or O + PVY^U isolate(s) which are not detected with Y^N and Y^O monoclonal antibodies

(mixed infections) (in spotted grey). The empty arrows indicate the peak flowering time of potato plants.

Figure 4: Boxplots of the distribution of the percentage of infected daughter tubers resulting from the infection of the mother plants obtained in the different periods of potato exposure to aphids after planting (plots A to H), and from 2016 (a) and 2017 (b) field trials. Boxplots labelled with “a, b, ab, bc, d, cd” are significantly different at $p < 0.05$. The cross in the boxplots corresponds to the average percentage of infected tuber in each plot. The circles correspond to outliers. The empty arrows indicate the peak flowering time of potato plants.

Figure 5: Boxplots of the distribution of PVY titer in pooled daughter tubers of all infected potato plants in plot A to H corresponding to i) the different exposure period of plants to natural viral infection in 2016 (a) and 2017 (b) field trials, and ii) inoculations under controlled conditions carried out in greenhouse in 2016 (c) and 2017 (d). Boxplots labelled with “a, b, ab” are significantly different at $p < 0.05$. The circles correspond to outliers. The empty arrows indicate the peak flowering time of potato plants.

Supplementary Figure 1: Physiological stages of potato plants of cv. Bintje in field conditions at 43 (plot C), 58 (plot D) and 118 (plot H) days after planting.

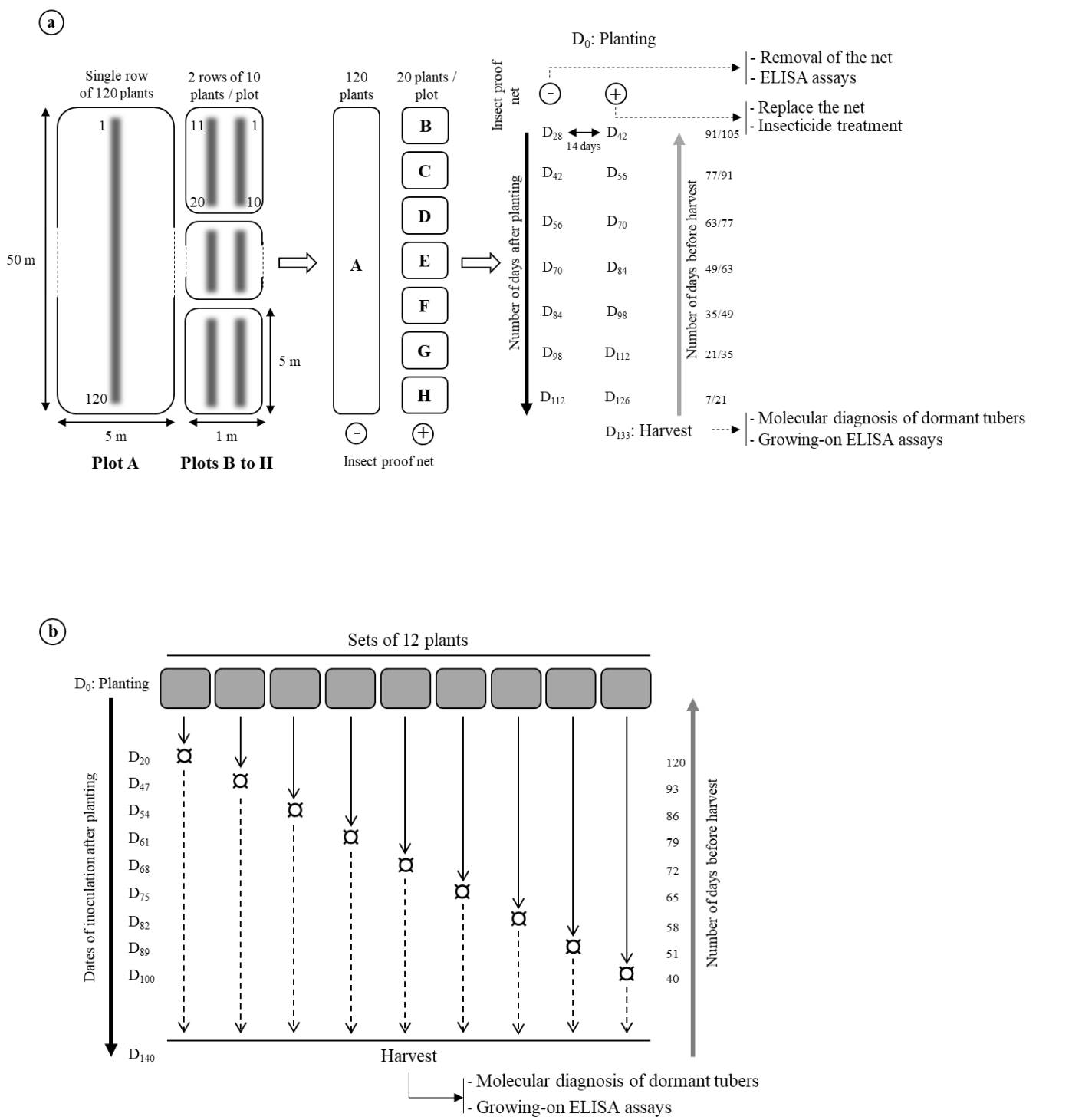
Supplementary Figure 2: Physiological stages of potato plants of cv. Bintje under greenhouse conditions at 20, 47, 54, 61, 68, 75, 82, 89 and 100 days after planting.

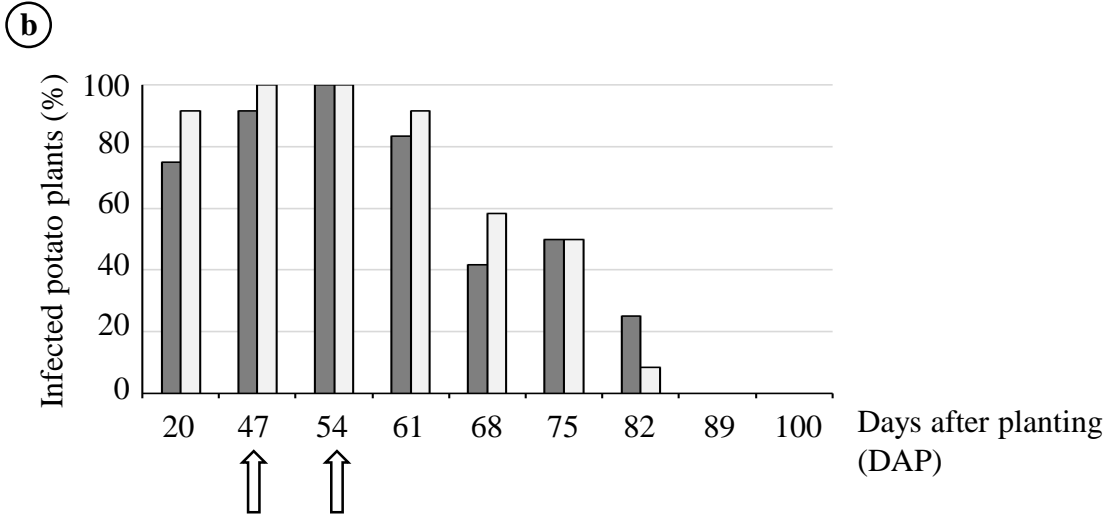
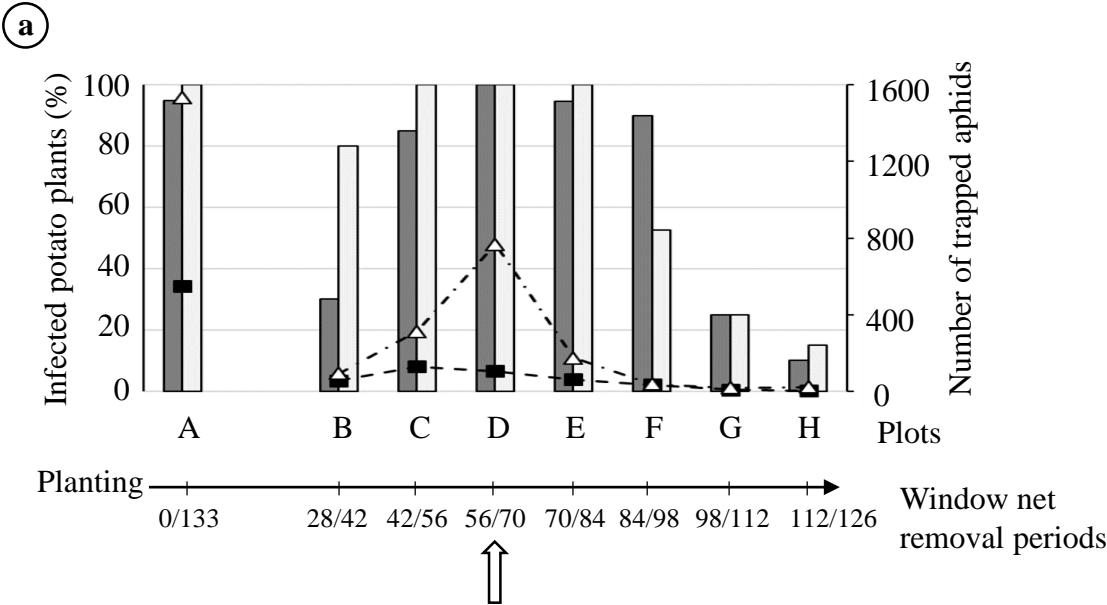
Supplementary Figure 3: Boxplots of the distribution of PVY load in individual infected daughter tubers of two infected potato plants each from plots B, D, F and H, (field trial, 2016). The circles correspond to outliers. For each plot, PVY load in individual daughter tubers was analysed by considering its natural log and a Generalised Linear Model with a Gaussian distribution to test the effects of the tuber and the plant with Wald tests.

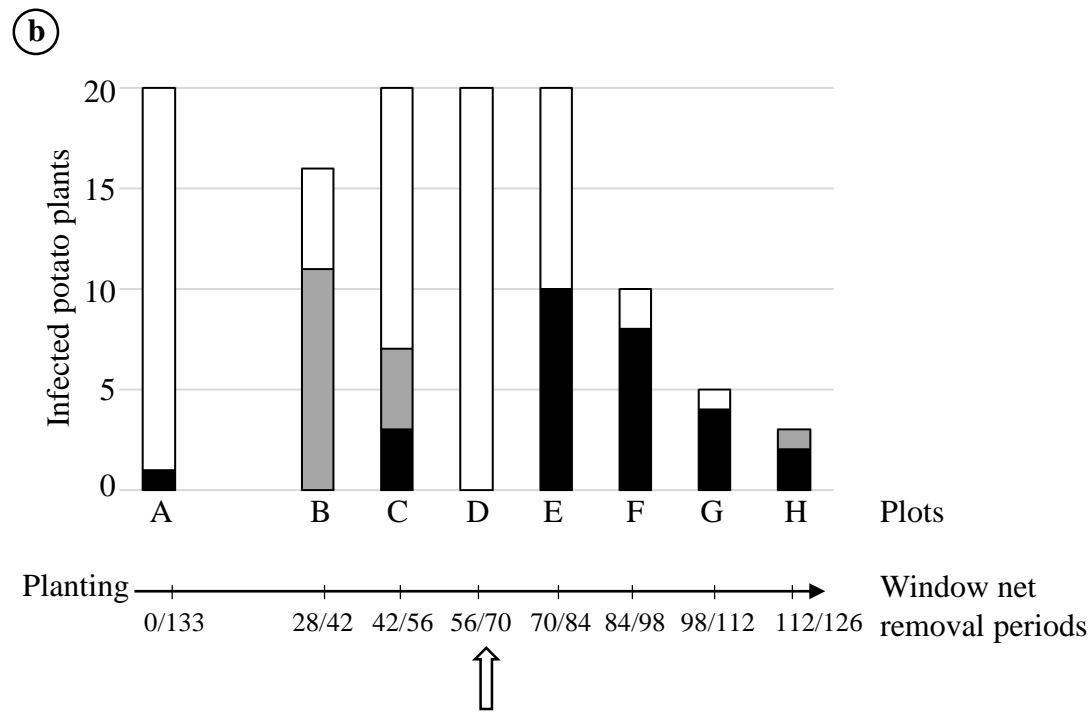
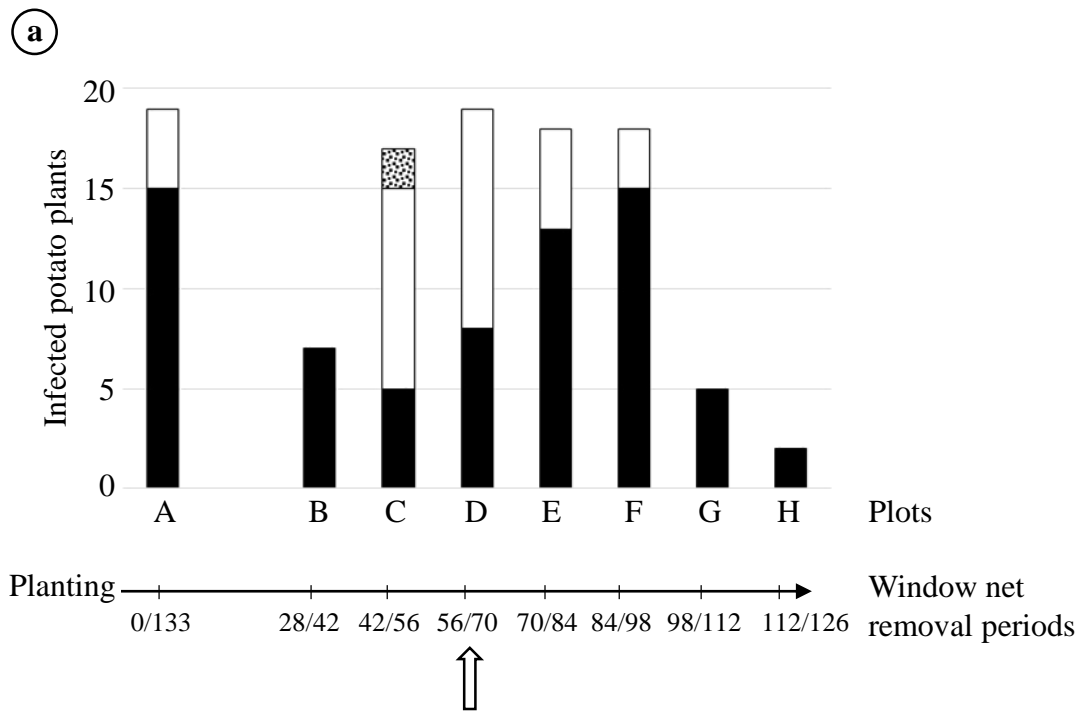
Supplementary Table 1: Serological and molecular characterization of PVY isolates in field trials (2016 and 2017).

Supplementary Table 2: Details of primers used for the reverse transcription (RT), nucleic acid amplification (PCR) and for nucleic acid sequencing steps.

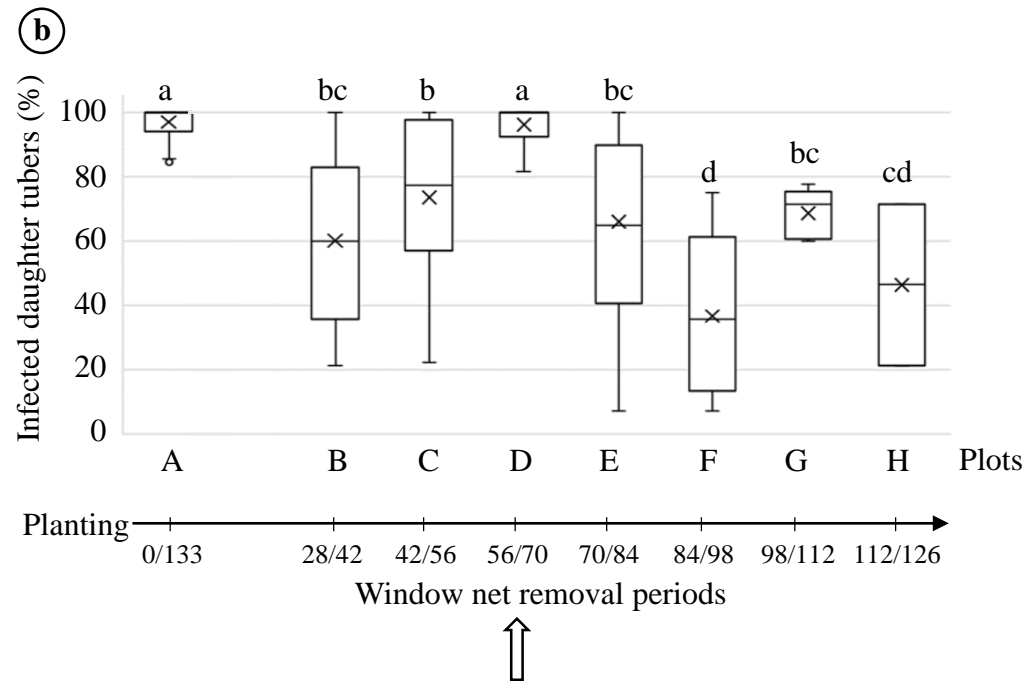
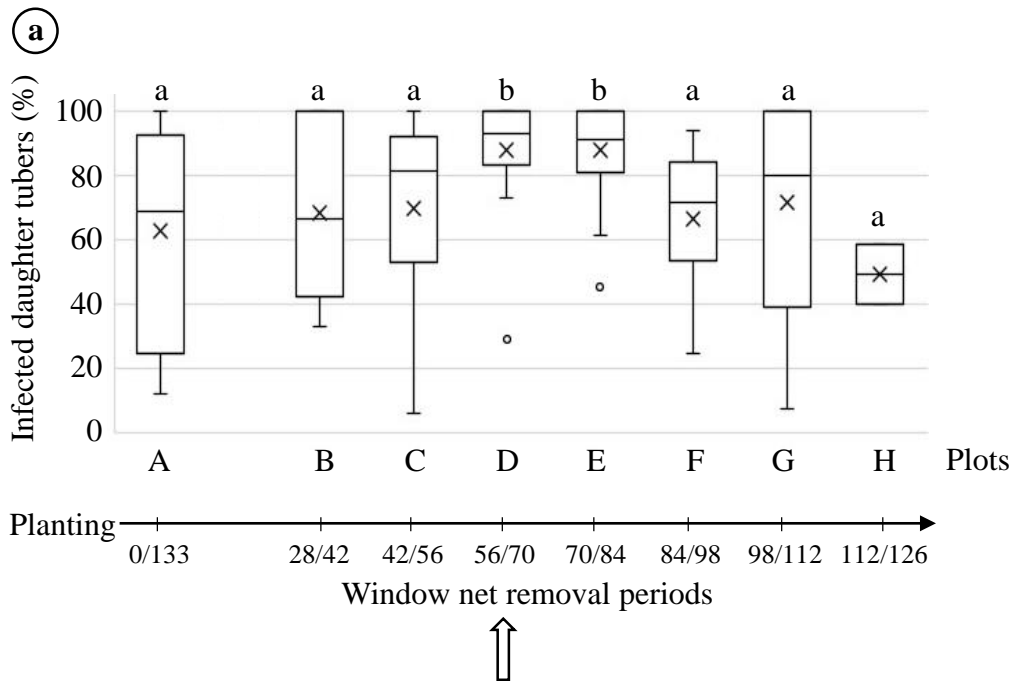
Boulard et al., **Figure 1**







Boulard et al., Figure 4



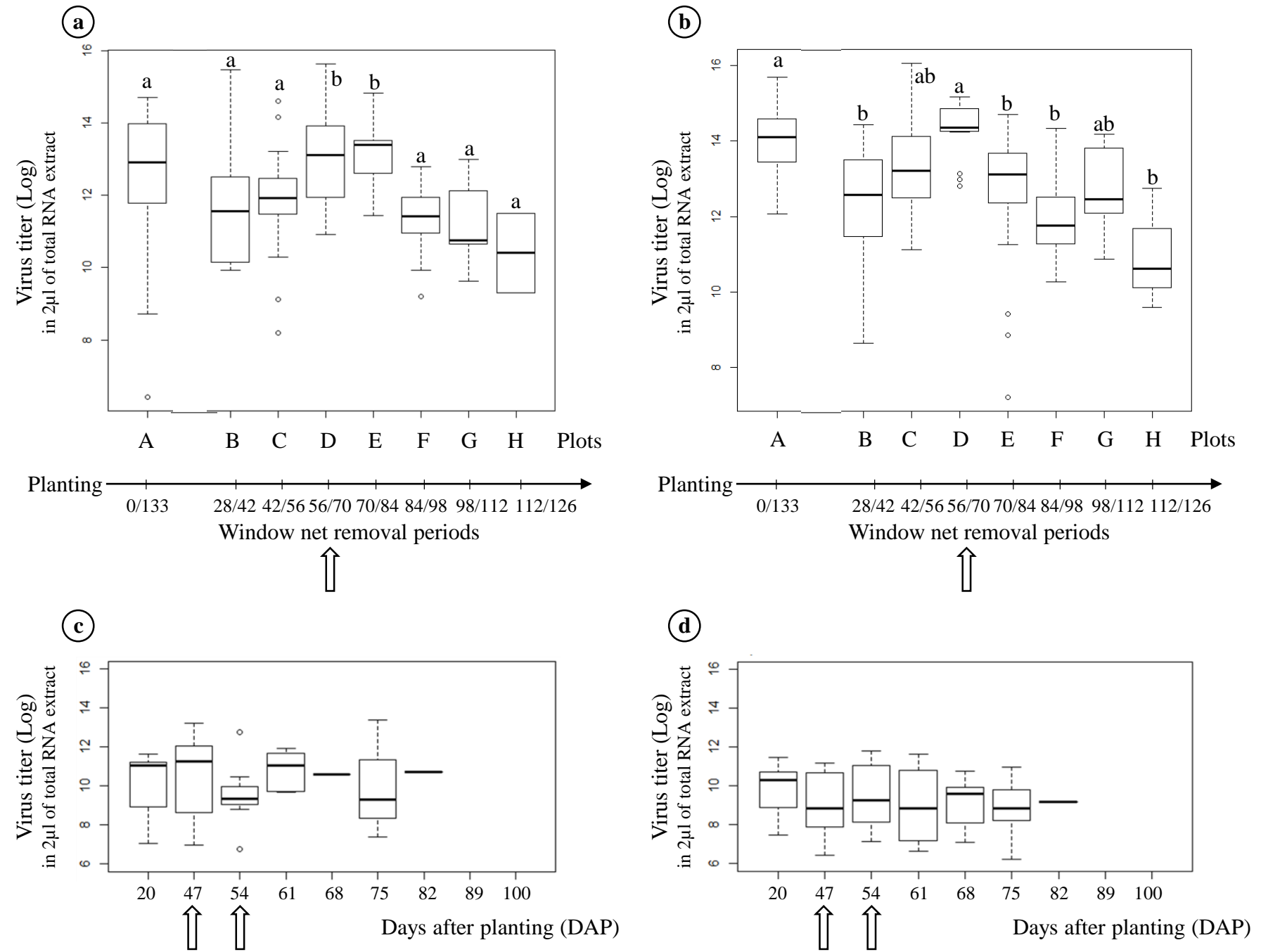


Table 1: Abundance of aphid species caught in water pan traps in field trial at the different window net removal periods in 2016 and 2017.

	Aphid species	2016							2017						
		B	C	D	E	F	G	H	B	C	D	E	F	G	H
reported PVY-transmitting aphids	<i>Acyrtosiphon pisum</i>	5	5	4	3	1	0	0	14	43	41	6	2	1	1
	<i>Aphis fabae</i>	2	1	3	3	2	0	0	8	6	18	3	0	0	0
	<i>Aphis spp</i>	0	1	0	3	0	0	0	3	4	10	2	1	0	1
	<i>Brevicoryne brassicae</i>	1	0	1	0	0	0	0	0	0	4	0	1	2	1
	<i>Capitophorus horni</i>	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	<i>Metopolophium dirhodum</i>	0	0	0	0	0	0	0	0	0	30	5	1	1	0
	<i>Myzus persicae</i>	34	98	62	39	19	0	0	51	222	610	125	19	9	10
	<i>Phorodon humuli</i>	0	0	0	0	0	0	0	0	0	1	0	0	0	0
	<i>Rhopalosiphum padi</i>	2	1	0	0	0	0	0	0	0	3	1	1	0	0
	<i>Sitobion avenae</i>	2	1	1	1	1	0	0	0	1	3	5	2	0	1
non reported PVY-transmitting aphids	<i>Anoecia corni</i>	0	1	4	0	1	0	0	0	0	0	1	2	0	0
	<i>Anuraphis farfarae</i>	0	0	0	1	0	0	0	0	0	0	0	0	0	0
	<i>Cavariella theobaldi</i>	0	0	0	0	0	0	0	0	0	0	1	0	0	0
	<i>Eriosoma ulmi</i>	0	1	0	0	0	0	0	0	0	1	0	0	0	0
	<i>Hyalopterus pruni</i>	0	1	0	0	0	0	0	0	0	3	1	0	0	0
	<i>Myzus sp</i>	0	0	3	0	0	0	0	0	0	3	0	0	0	0
	<i>Pterolcallis alni</i>	0	0	0	0	0	0	0	0	0	1	0	1	0	2
	<i>Rhopalosiphum minsertum</i>	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	Other	8	18	27	10	7	8	1	17	36	39	23	7	4	5
	Total number of trapped aphids	54	128	105	60	33	8	1	93	312	767	173	37	17	21

Table 2: Sanitary status of daughter tubers in field trials (2016 and 2017).

Trial	Plot	Window net removal period	Average percentage of infected daughter tubers	Percentage of mother plants with 100% infected daughter tubers
2016	A	0/133	63.2	10.5 (2/19)*
	B	28/42	68.6	43.0 (3/7)
	C	42/56	70.0	11.8 (2/17)
	D	56/70	88.3	42.1 (8/19)
	E	70/84	88.1	44.4 (8/18)
	F	84/98	67.0	0 (0/18)
	G	98/112	71.6	40.0 (2/5)
	H	112/126	49.4	0 (0/2)
2017	A	0/133	97.2	75.0 (15/20)
	B	28/42	60.3	18.7 (3/16)
	C	42/56	73.7	25.0 (5/20)
	D	56/70	96.3	60.0 (12/20)
	E	70/84	66.0	20.0 (4/20)
	F	84/98	36.8	0 (0/10)
	G	98/112	69.0	0 (0/5)
	H	112/126	45.0	0 (0/3)

* : number of plants with 100% infected daughter tubers / total number of PVY-infected plants