Effects of *Potyvirus* Effective Population Size in Inoculated Leaves on Viral Accumulation and the Onset of Symptoms

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Abstract

Effective population size ($N_e$) is a key parameter for understanding evolutionary processes, but it is generally not considered in epidemiological studies or when studying the infection of individual hosts. Whether $N_e$ has an effect on the onset of symptoms and viral accumulation for *Tobacco etch virus* (TEV) infection of *Nicotiana tabacum* plants is considered here. Using mixtures of TEV variants carrying fluorescent markers, dose-dependence of $N_e$ was confirmed and the inoculation procedure was found to be the main source of variation in these experiments. Whereas the onset of symptoms was independent of $N_e$, accumulation at 6 days post-inoculation was lower and more variable for small $N_e$ values ($N_e < 5$). The observed variation in accumulation was not heritable, however, suggesting that this variation was not due to the fixation of deleterious mutations in the small founder populations. On the other hand, virus-induced fluorescence and accumulation in the inoculated leaf were strongly $N_e$-dependent. Systemic accumulation was independent of $N_e$, although removal of the inoculated leaf led to a small reduction in systemic accumulation for small $N_e$ values. For whole plants $N_e$-dependent effects on accumulation were no longer observed nine days post-inoculation. Therefore, the effects of $N_e$ on accumulation are mainly due to limited expansion in the inoculated leaf and are transient. In this system $N_e$-dependent effects will be strongest at low doses and early in infection. We conclude that $N_e$ can have implications for epidemiology and infection at the individual-host level beyond determining the rate of mixed-genotype infection.
Introduction

Effective population size (Ne) is a concept introduced by Wright (1931) to better understand how the number of individuals within a population affects the outcome of evolution (38). Ne gives an approximation of the number of breeding individuals in an idealized population, or in the case of viruses, the number of transmitting individuals (8,25). This number indicates the strength with which genetic drift will act on the virus population (38), as well as the frequency at which mixed-genotype infections will occur (40). Mixed-type infections are relevant for understanding evolution, since they are a prerequisite for recombination between different viral strains (10).

Census size (N) on the other hand, is the observed number of individuals in the population at a given moment. Whereas N values tend to be huge for viruses, Ne values can be very small under certain conditions (i.e., low pathogen doses or low host susceptibility) (15).

Many studies have shown that Ne can be very small for plant viruses, when considering the transition from initial to systemic infection (9,14,22,29) or vector-borne transmission (1,5,14,24). It was recently shown that small Ne values also occur during infection following mechanical inoculation, and that dose response, the number and distribution of primary infection foci and the frequency of mixed-genotype infections conform to independent action hypothesis (IAH) model predictions (39). The IAH model assumes that each virion has a non-zero probability of infection, and that virions infect the host such that the action of each virion is independent of that of other virions (2). Alternative models of infection can incorporate dependent action (DA; i.e., dose-dependent effects on the probability of infection for each virion) or heterogeneous host susceptibility (HHS; i.e., differences in infection probability per virion over hosts) (28). Although there is support for the IAH model for some mono-partite viruses (39,40), the model can be rejected a priori for multi-partite viruses that encapsidate the different genome segments separately. In these cases, there will be complementation between the different virion types, and hence a dependent action model would be more appropriate. It must also be stressed that the models of infection considered here describe conspecific virus populations, and that the terms ‘genotypes’ and ‘mixed-genotype infection’ refer to virus variants that are identical except for a neutral marker (40). Therefore, both the models and the experiments have no bearing on the occurrence of a variety of interactions between distinct genotypes. In the field, interactions between virus genotypes can be complex and highly relevant, e.g. (12,27). IAH leads to a dose-
dependent $N_e$; $N_e$ is then simply the product of dose and the probability of infection (39).

Although $N_e$ has been quantified for a number of plant viruses, the relationship between $N_e$ and $N$ has, to our knowledge, not been quantitatively described for viral infection of a complex multicellular host.

The concept of $N_e$ is rooted in evolutionary biology, and indeed has been shown to be extremely important for understanding evolutionary processes (25,38). On the other hand, $N_e$ is usually not considered an important parameter on relatively short time scales, such as those often studied in epidemiology. For modeling the epidemiology of micro-parasites, host organisms are usually considered within the SIR (susceptible, infectious and removed) framework, or variations thereof including the SEIR (susceptible, exposed, infectious and removed) model with a latent period of infection. This widely used and immensely successful approach (18) therefore focuses on how many host organisms are in a particular state, and not on attributes of the virus population (19).

For plant pathogens, classic epidemiological models have also considered the amount of uninfected (i.e. susceptible), latently infected (i.e. exposed) and infectious tissue (17,35), again focusing on host states. Recently it has been suggested that integration of the between-host and within-host levels, and the approaches used to study them, will increase our understanding of infectious disease dynamics (13,23). Whether the inclusion of the concept of $N_e$ could lead to a better understanding of the epidemiology of plant viruses is therefore considered here.

A similar question could be asked concerning even shorter time scales: is $N_e$ a useful and necessary concept for describing the infection process in individual plants? In experiments comparing different plant genotypes, viral dose (3) or mRNA inoculum (21) is often held constant. Even though dose can be used to predict $N_e$ under IAH (39), the two are not equivalent. If it is assumed dose is fixed, $N_e$ will not be the same in all hosts, even when there is no experimental variation (i.e. no variation in dose and a homogeneous host population). When the IAH model with a fixed probability of infection over hosts is supported, $N_e$ follows a Poisson distribution (39,40). For example, for a Poisson-distributed $N_e$ with a mean of 5 it would be expected that ~25% of the hosts to be infected with ≤ 2 and ≥ 8 individuals.

There is one important case in which $N_e$ is highly relevant for processes both at the epidemiological and individual-plant scales: the occurrence of mixed-genotype infections. $N_e$ is...
an important determinant of whether mixed-genotype infections will occur, together with the frequencies and infection probabilities of different viral genotypes in the virus population. Mixed-genotype infections can, in turn, have important implications for infectious disease dynamics on different spatiotemporal scales (12,27,30,31,33). However, besides this obvious case, the question whether \( N_e \) merits attention on relatively short temporal scales remains.

Here the relationship between \( N_e \) and the infection process in individual plants is investigated, to explore whether \( N_e \) has further implications. It was previously reported that \( N_e \) is predicted by IAH and therefore dose-dependent for Tobacco etch virus (TEV, genus Potyvirus, family Potyviridae) in tobacco plants (Nicotiana tabacum L.), allowing for simple estimates of \( N_e \) by considering the number of primary infection foci (39). This approach was exploited to look for effects on the onset of symptoms and viral accumulation. Virus-induced symptoms may play a role in attracting vectors (16,32), and can therefore influence horizontal transmission of viruses; viral accumulation will affect the probability of a vector becoming viruliferous (26).

**Materials and Methods**

**Inoculation experiments with mixtures of TEV-GFP and TEV-mCherry**

To generate virion stocks, RNA was transcribed *in vitro* for pMTEV-GFP and pMTEV-mCherry (39) as described previously (6). *N. tabacum* cv. Xanthi was used for all experiments. Plants were infected with 5 μg RNA in the third true leaf, and the symptomatic systemically infected tissue was collected 9 days post-inoculation (dpi) and used for purification of virions (6). Virions were resuspended in 100 μL 0.05 M borate buffer (pH 8.0, 5 mM EDTA) with 20% glycerol. Virion concentration was determined by RT-qPCR using *in vitro* synthesized Turnip mosaic virus (TuMV) RNA as an internal control (39).

One-to-one mixtures of TEV-GFP and TEV-mCherry virions were then made, and 5 μL of a serial dilution of these mixtures were used to rub inoculate 25-day-old *N. tabacum* plants (39). Plants were kept in a greenhouse until inoculation, and then transferred to a growth chamber and kept at 24°C. All plants used for experiments were selected stringently for having the same size and synchronous development. GFP and mCherry fluorescence were observed using a Leica MZ16F stereomicroscope, using a 0.5× objective, and GFP2 or DSR filters (Leica), respectively.
Foci of primary infection in the inoculated leaf were counted 3 dpi, and systemic infection was determined 6 dpi. Those plants with virus-induced fluorescence in systemic tissues at 6 dpi are considered to be “infected plants”, unless otherwise noted. Whether TEV symptoms were present was also determined at 6 dpi, unless otherwise noted. For the time course experiment, the occurrence of TEV symptoms was recorded at all times of tissue collection (3, 6, 9 and 12 dpi). For the experiments to determine the median time until onset of symptoms (\(ST_{50}\)), symptoms were recorded daily from the moment of inoculation until all plants had developed symptoms.

Two replicates of the main experiment were performed. The total number of plants challenged with each virion dilution was: 20 (1:10, 1:30 and 1:90), 25 (1:270), 35 (1:810), 75 (1:2430) and 30 (non-virus control). A larger number of plants were inoculated with higher virion dilutions in order to have sufficient infected plants for subsequent analysis. For the time course experiments, 30 plants were infected with a 1:10 virion dilution, 75 plants were infected with a 1:270 virion dilution, and 15 non-virus controls were taken. For the \(ST_{50}\) experiments, 30 plants were infected with a 1:10 virion dilution, 50 plants were infected with a 1:270 virion dilution and 10 plants were taken as mock-inoculated controls.

**RT-qPCR for viral accumulation**

Plants were collected 6 dpi by removing all the aerial tissue, although multiple time points were used for the time course assay (3, 6, 9, and 12 dpi). Moreover, for the experiments on accumulation in the inoculated leaf, the inoculated leaf and the remaining aerial tissue were collected and stored separately. The collected tissue was weighed then stored at \(-80^\circ C\). Total RNA was extracted from 100 mg of tissue using the Invitrap Spin Plant RNA Mini Kit (Stratec Molecular). One-step RT-qPCR using specific primers for the coat protein (forward primer: 5’-TTGGTCTTGTGCAACGTG-3’ and reverse primer 5’-TGTGCGTTCTAGTGTCTTCTC-3’) was performed using Primerscript RT-PCR Kit II (Takara), according to the manufacturer’s instructions using a PRISM Sequence Analyser 7500 (Applied Biosystems). Data were then analyzed with 7500 Software version 2.0.4 (Applied Biosystems).
Statistical analysis

All statistical analysis was performed in R version 2.10.1 (The R Foundation; Austria, Vienna) unless otherwise noted. The dose-foci relationship was analyzed as previously reported (39). Briefly, foci data ($\lambda$) were transformed as $y = \ln(\lambda + 1)$, and the equation $y = \ln(\rho d + 1)$ was then fitted with non-linear regression (SPSS 16.0; SPSS Inc., Chicago, Illinois, USA), where $d$ is the dose and $\rho$ is the average infection probability. The $\ln(\lambda + 1)$ transformation was used as some leaves were uninfected.

The DA (dependent action) model of dose-response relationships (28) is:

$$I = 1 - e^{-\phi d}$$

where $I$ is the fraction of systemically infected plants, $d$ is the virion dose and $\phi$ determines what the effect of dose on the rate of infection is. The IAH model of infection is similar to the DA model, but $\phi$ is fixed to 1 so that there are no dose-dependent effects on the actual infection probability per virion (28). When $\phi > 1$, there is synergism between virions (dose response is steeper than predicted by IAH), whilst when $\phi < 1$, there is antagonism (dose response is shallower than predicted by IAH). The dose-response model incorporating HHS (heterogeneous host susceptibility) (4) is:

$$I = \left(\frac{1}{1 + d\rho v}\right)^\frac{1}{2}$$

Where $v$ determines the variability in host susceptibility, and is a nonnegative real number. Increases in $v$ represent increasing HHS, with zero indicating no variation in host susceptibility.

These three models (IAH, DA and HHS) were fit to the data using a maximum likelihood approach, were likelihoods were calculated using binomial probabilities. Binomial probabilities could be used as the data were analyzed as infected/non-infected and symptomatic/non-symptomatic. Grid searches were performed first over a large parameter space, followed by searches over consecutively smaller spaces. This approach ensured that the solution found was both precise and global.
A geometric model was fitted to the \( N_e \)-accumulation relationship. As \( N_e \) increases by one, viral accumulation \((A)\) becomes:

\[
A_{N_e+1} = A_{N_e} + ck^{N_e}
\]

Where \( c \) is a constant scaling the rate of change and determining whether \( N_e \) increases (positive values) or decreases (negative values). Here \( k \) is a constant that determines whether the increase or decrease remains the same \((k = 1)\), becomes larger \((k > 1)\) or smaller \((k < 1)\) as \( N_e \) increases. The solution for \( A_{N_e} \) is:

\[
A_{N_e} = \begin{cases} 
A_0 + \frac{c(1-k^{N_e})}{1-k} & \text{for } k \neq 1 \\
A_0 + cN_e & \text{for } k = 1
\end{cases}
\]

This model was fit to the data using non-linear regression (SPSS 16.0).

For the accumulation data from the time course experiments, the logistic model was fitted:

\[
n_t = \frac{n_0\kappa}{n_0 + (\kappa - n_0)\kappa^{-r_0}}
\]

where \( n_t \) is viral accumulation at time \( t \), \( n_0 \) is viral accumulation at time zero, \( \kappa \) is the carrying capacity and \( r_0 \) is the initial growth rate. Note that according to the definitions used here, \( n_0 \) is equivalent to \( N_e \). The different symbols are used, however, to distinguish between estimates made by counting foci of primary infection \((N_e)\) and estimates from fitting the logistic model to RT-qPCR data from the time course \((n_0)\).

**Results and Discussion**

**Infection process conforms to IAH model predictions**

The IAH model is supported for TEV infection of *N. tabacum* (39). Here, *N. tabacum* plants were inoculated with different doses of a 1:1 mixture of viruses tagged with a fluorescent markers, TEV-GFP and TEV-mCherry, as previously described (39). The number of foci of
primary infection was quantified using a stereomicroscope 3 dpi, and whether systemic infection had occurred was determined 6 dpi. All infection parameters conformed to IAH model predictions: (i) the number of primary infection foci (Figure 1A), (ii) the distribution of primary infection foci (Table 1), (iii) dose infection (Figure 1B, Figure 2, Table 2), and (iv) the rate of mixed-genotype systemic infection (Figure 1B, Table 3). Model selection was used to determine the most suitable dose infection model, and the IAH model was compared to more complex models incorporating a dose-dependent probability of infection (DA) or differences in host susceptibility to the virus (HHS) (see Materials and Methods). Both the DA and HHS models predicted dose-infection relationships slightly shallower than the data. The DA model predicted antagonistic interactions between virions ($\phi < 1$), and the HHS model predicted the occurrences of differences in host susceptibility ($v > 0$). There were, however, no appreciable differences in support for the different models (Table 2) and hence the IAH model is not rejected. Furthermore, in only one case was a plant observed in which exclusion of one genotype (TEV-mCherry) from systemic infection by the other (TEV-GFP) occurred. In total, only one out of 291 infected plants showed this phenomenon, a statistically insignificant difference ($P = 0.576$) compared to previous observations (3/275, (39)). The data are therefore in agreement with IAH predictions, as in a previous study (39), allowing inferences to be made on $N_e$ and how it changes with dose.

**Sources of variation in the infection process**

Infection parameters were not significantly different from IAH model predictions. Nevertheless, the distribution of primary infection foci tended towards a higher variance than predicted at high doses (Table 1), and dose response appears to be slightly shallower, as also indicated by estimated parameter values for dose response ($\phi < 1$ or $v > 0$; Table 2). Both trends have been observed before in this experimental system (39), as well as for other plant viruses (11,20). Moreover, these trends appear to be quite general since they also appear in other experimental systems (4,28,36,40), where it must be noted that a higher variance in the distribution of founders is equivalent to an increase in mixed-genotype infection frequency (36). Thus, although deviations from the model were not in themselves sufficient to reject IAH or support an alternative model of infection, the generality of these subtle discrepancies gives good grounds for investigating the underlying mechanisms. Heterogeneity in host susceptibility has been
implicated in departures from the IAH model; this model can reasonably account for dose response and mixed-genotype infection data if the probability of infection is allowed to vary over hosts (4,36).

To test what a possible source of minor variation in these experiments could be, both halves of leaves of *N. tabacum* plants were inoculated with TEV-GFP and the number of primary infection foci was scored three days later. If the variation is at the between-plants level, one would expect to see a correlation between the numbers of foci on half leaves of the same plant. On the other hand, if variation occurs at the within-plant level – which would strongly implicate the inoculation procedure as the source of variation – one would expect to see no correlation between the number of foci on half leaves. No correlation between half leaves was found (Table 4), suggesting the main source of the extra variation in the number of foci is the inoculation procedure itself.

**No effects of dose and *N*<sub>e</sub> on the occurrence or onset of TEV symptoms**

Whether TEV symptoms were present in challenged plants was determined on day six. Plants were classified as being symptomatic or non-symptomatic. The frequency of symptoms clearly increases with dose (Figure 2). On other hand, a Jonckheere-Terpstra (JT) test for trend shows that – when only infected plants were considered – the frequency of symptoms did not increase with dose (JT test: *P* = 0.820). The number of foci of primary infection is a good approximation of *N*<sub>e</sub> (39). Whether there is a relationship between *N*<sub>e</sub> and the frequency of symptoms in infected plants could therefore be tested, which also gave a non-significant result (JT-test: *P* = 0.782). Therefore, if a plant is infected then the probability that it will develop symptoms by 6 dpi appears to be independent of dose or *N*<sub>e</sub>. Different dose-response models were then fitted to the dose-symptomatology data (see Materials and Methods). Although the IAH model is not well supported in this case, support for the alternative models – DA and HHS – is almost equal, with only marginally more support for the HHS model (Table 2). Hence, the dose-response models do not help discriminate between these two mechanisms that could underlie the development of symptoms.
Viral accumulation was measured 6 dpi by RT-qPCR for the capsid protein (CP gene) in 10 randomly selected samples for each dose. It was found that viral accumulation was significantly higher in symptomatic plants than in non-symptomatic plants (1-tailed Mann-Whitney test: \( P = 0.033 \)). It therefore appears as if infected plants have a probability of developing symptoms by day six which is independent of virion dose and \( N_e \), but which is affected by viral accumulation. This result is relevant from an epidemiological perspective because symptoms can affect vector-borne transmission (16,32), and it therefore supports the exclusion of \( N_e \) in epidemiological models.

In the initial experiment, symptomatology was recorded only at 6 dpi. No dose or \( N_e \)-dependent effects were observed, and it was therefore decided to measure the median time until onset of symptoms (\( ST_{50} \)) for high (1:10 virion dilution) and low (1:270 virion dilution) doses to rule out dose-dependent effects. The first symptom observed in most infected plants was vein clearing in the 5th true leaf, and all plants developed symptoms by 8 dpi. The Kaplan-Meier estimate for the mean time until onset of symptoms ±1 SEM was 5.47±0.15 for the high dose, and 5.66±0.13 for low dose, and the difference is non-significant (Log rank test: \( \chi^2 = 0.924 \), 1 d.f., \( P = 0.336 \)). We therefore conclude dose and \( N_e \) have no effect on the onset of symptoms in this experimental setup, and that all infected plants eventually develop symptoms.

Effects of dose and \( N_e \) on viral accumulation

Next it was considered whether there is an effect of dose on viral accumulation, as measured by the RT-qPCR assay. Viral accumulation increases significantly with dose (Figure 3; JT test: \( P < 0.001 \)). A significant relationship between \( N_e \) and viral accumulation was also found (Figure 4; JT test: \( P < 0.001 \)). However, viral accumulation was negatively affected only for small values of \( N_e \) (i.e., \( N_e < 5 \)), and accumulation appears to reach a plateau for larger \( N_e \) values. To test if this is the case, a geometric model of viral accumulation was fitted to the data (see Materials and Methods). Estimated model parameters show that for every unit increase in \( N_e \), the increase in accumulation changes by a factor \( k = 0.564±0.095 \) (estimate ±1 SEM), which is significantly less than 1 (one-sample \( t \)-test: \( t_{59} = 4.590 \), \( P < 0.001 \); Table 5). Hence, estimated model parameters also support the idea that accumulation is dependent on \( N_e \) and that the dependence is
significantly below the linear expectation. This test result further reinforces the idea that this effect is manifest only when \( N_c \) is small.

**\( N_c \)-dependent effects on accumulation are not a heritable trait of the virus population**

There are a number of plausible reasons why accumulation might be lower for small \( N_c \) values. TEV has a reasonably high mutation rate (34) and the fitness effect of many TEV mutations is deleterious (7). Therefore, if only a small number of TEV genomes start infection, there may be a reasonably high probability that all the infecting genomes carry deleterious mutations and that these mutations result in lower accumulation. For those plants infected with \( N_c = 1 \), genetic variation in the population must be generated *de novo*. Therefore, lower accumulation due to deleterious mutations in the viral genome should be heritable to a large extent. New plants were therefore infected with sap of equal virion concentrations, calculated as genome equivalents by RT-qPCR, from the first replicate of the original experiment. Virus populations from those plants that gave the highest and lowest accumulation were used. There were significant differences in accumulation when the three populations with the highest and lowest accumulation were considered (nested ANOVA; Table 6 ‘Experiment 0’). We hypothesize that if accumulation is a heritable trait, the difference between these two populations will persist among their descendants. Although compensatory mutations or reversions could occur, they would not be expected in all replicates on such short time scales (i.e., two passages since the bottleneck where \( N_c = 1 \)).

Two independent experiments were performed. In experiment 1, sap from each of the three plants which gave the highest and lowest accumulation was used to infect three new plants, and accumulation was then determined by RT-qPCR. In experiment 2, sap from each of the two plants which gave the highest and lowest accumulation was used infect five new plants. There was no evidence for heritable differences in accumulation in both experiments (nested ANOVA; Table 6). We can therefore conclude that the effect of small \( N_c \) values on viral accumulation is not necessarily explained by the presence of deleterious mutations in the inoculum. This conclusion is not at odds with previous results on the distribution of mutational fitness effects, as these experiments considered within-host competitive fitness instead of accumulation (7). Virus genotypes can have identical accumulation while having marked differences in competitive
within-host fitness (M.P. Zwart, J.A. Daròs and S.F. Elena, unpublished data), suggesting that within-host fitness is more sensitive to mutational effects than viral accumulation.

**N_e-dependent effects on accumulation are due to infection dynamics in the inoculated leaf**

A second possible explanation for \( N_e \)-dependent differences in accumulation is the infection process in the inoculated leaf. The infection process in the inoculated leaf is different from that in the systemically infected tissue. In the inoculated leaf, the foci of primary infection will typically only expand locally and along vascular tissue while exiting the leaf (Figure 5). It is therefore likely that there is dose-dependent accumulation in the inoculated leaf at low doses when the inoculated leaf does not become saturated with primary infection foci. Moreover, as viral accumulation was measured early in infection when the inoculated leaf (third true leaf) is the largest leaf, the contribution of accumulation of the inoculated leaf to accumulation in the entire plant may be considerable.

To test this possibility, plants were infected with either a high or low virion dose. Plants with \( N_e = 1 \), as determined by microscopy at 3 dpi, were selected from the low dose treatment for further analysis, and plants with \( N_e > 10 \) were selected from the high dose treatment. At 6 dpi, fluorescence in the inoculated leaf and the systemically infected tissue was observed, and the inoculated leaf and the remaining aerial tissue were then collected separately. Viral accumulation was then determined separately for the inoculated leaf and the rest of the plant. Whereas viral accumulation in the systemically infected tissue was not dependent on \( N_e \) (Tamhane’s test: \( P = 0.119 \)), viral accumulation in the inoculated leaf was clearly dependent on \( N_e \) (Tamhane’s test: \( P < 0.001 \)). There was a more than 5-fold \( N_e \)-dependent difference in accumulation in the inoculated leaf (mean accumulation \pm standard deviation; \( N_e = 1 \): \((3.59 \pm 1.52) \times 10^6 \), \( N_e > 10 \): \((2.05 \pm 0.58) \times 10^7 \)). Thus our hypothesis that differences in viral accumulation are largely due to the inoculated leaf was confirmed.

**N_e-dependent differences in viral accumulation when the inoculated leaf is removed**

As a further test of how \( N_e \) in the inoculated leaf affects accumulation in systemic tissue, plants were infected with a high and low virion dose and subsequently foci numbers were confirmed at 3 dpi. As soon as virus-induced fluorescence was visible in the systemically infected tissue of all
plants in the experiment (5 dpi), the inoculated leaf was removed. The remaining aerial plant
tissue was collected 6 dpi and viral accumulation was determined (Figure 6). A significant effect
of Ne on accumulation was found (t-test on log10-transformed accumulation: \( t_{14} = 2.802, P =
0.014 \)). Nevertheless, there was only a 1.5-fold difference in accumulation (mean accumulation
\( Ne = 1 \): \( 1.14 \pm 0.40 \times 10^7 \), \( Ne > 10 \): \( 1.73 \pm 0.29 \times 10^7 \)). The differences in
accumulation for large and small \( Ne \) values were therefore much smaller than the differences
observed in the inoculated leaf at 6 dpi.

Why does the removal of the inoculated leaf 5 dpi results in lower viral accumulation in plants
with a small \( Ne \), whereas there were no \( Ne \)-dependent differences when the inoculated leaf was
not removed? We think this effect occurs because the flux of virions egressing the inoculated
leaf at any time point depends on the number of primary infection foci, whilst on the other hand
the systemic tissue of the plant can eventually be largely saturated by the virion production of
even a single focus. Note that this explanation does not imply a high multiplicity of infection
(MOI) in systemically infected tissue, as mechanisms regulating MOI can act at the cellular level
(e.g., co-infection exclusion). This idea would result in a small time window in which there are
\( Ne \)-dependent effects on systemic accumulation, which has all but vanished by 6 dpi. Moreover,
the time at which individual primary infection foci start contributing to the flux of virions is
likely to be subject to stochastic effects. These effects might come about due to the proximity of
the initial infected epidermal cell to vascular bundles, and in particular the number of mesophyll
cells that must be traversed by means of cell-to-cell movement before phloem can be accessed.
When \( Ne \) is large, there will not only be more foci, but some of them are more likely to be
fortuitously situated with respect to phloem in the inoculated leaf than when \( Ne \) is small. This
mechanism may also explain why there is greater variation in viral accumulation at 6 dpi when
\( Ne \) is small (Figures 4 and 6). In summary, although systemic accumulation at 6 dpi appears to
be \( Ne \)-independent, these results show that at earlier time points there are \( Ne \)-dependent effects.
We have found that \( Ne \)-dependent effects on systemic infection may be stronger if the inoculated
leaf is removed earlier during infection in a related experimental study (N. Tromas, G.
Lafforgue, S.F. Elena & M.P. Zwart, unpublished manuscript).
**N_e-dependent effects on accumulation are transient**

N_e-dependent effects on accumulation at 6 dpi are due to the level of infection in the inoculated leaf. At earlier time points in infection, these data suggest that there may also be N_e-dependent accumulation in systemic tissue (Figure 6). We therefore hypothesized that the effect on accumulation in the whole plant should be transient. As the plant grows and infection expands, the inoculated leaf should contribute less to viral accumulation in all tissues, and its contribution eventually becomes negligible. We speculate, moreover, that given enough time the number of virions egressing the inoculated leaf will be sufficient, in combination with secondary infections, to saturate the systemic tissues irrespective of N_e. A time course experiment was therefore performed, with a setup identical to the first experiment described in this study. However, plants were only infected with a high (1:10 virion dilution) or low (1:270 virion dilution) dose. After the number of foci of primary infection had been quantified at 3 dpi, plants were randomly assigned to be collected and stored after 3, 6, 9 and 12 dpi. Fluorescence and symptomatology were recorded at all times of collection for all available plants. In this experiment, there was also not an effect of dose on the onset of symptoms (Log rank test: $\chi^2 = 0.228$, 1 d.f., $P = 0.633$), confirming previous observations. Accumulation was measured for whole plants collected at different time points by RT-qPCR (Figure 7), and a logistic model was fit to the data (see Materials and Methods). There were significant differences only at days three (Mann-Whitney U-test: $P = 0.008$) and six ($P = 0.008$), but not later on (day nine: $P = 0.175$; day 12: $P = 0.841$). Fitting of the logistic model rendered similar estimates of $\kappa$, the carrying capacity, for both treatments (Table 7). These similar estimates further confirm that N_e-dependent effects on accumulation are transitory, because virus accumulation saturates at the same level regardless of N_e.

**Concluding remarks**

These results demonstrate a manner in which N_e can affect the outcome of the infection process, in particular virus accumulation. Although this effect was demonstrated by means of mechanical inoculation, a similar effect could conceivably occur during infections initiated by vector-borne transmission (37). The number of feeding or probing events by viruliferous vectors, or alternatively the density of viruliferous vectors, could lead to differences in accumulation due to
differences in the number of foci of primary infection, thereby contributing significantly to overall accumulation. On the other hand, the effect of $N_e$ on accumulation was transitory, being observed only before 9 dpi. Moreover, $N_e$ had no effect on accumulation at 6 dpi for most observed values ($N_e \sim 5-50$), and the onset of symptoms was independent of $N_e$. As a first approximation, ignoring $N_e$ in models of infection or epidemiology that do not consider mixed-genotype infections therefore does not seem unreasonable for this model system. However, at low doses $N_e$ can have transient effects on viral accumulation and incorporating this mechanism in models may lead to better insight into the infection and epidemiology of plant viruses.

A practical consideration also arises from the work presented here. For experiments comparing the accumulation of different genotypes in this model system, dose only affects accumulation if $N_e$ values in individual plants drop below $\sim 5$, and this effect is due mainly to infection dynamics in the inoculated leaf. To avoid $N_e$-induced effects in experiments comparing accumulation of different viral genotypes, there are three possibilities: (i) measuring accumulation only in the systemically infected tissue, a common laboratory practice, (ii) use of high doses, or (iii) measuring accumulation late in infection (i.e., at least 9 dpi for this system) when the contribution to the inoculated leaf to total inoculation will be negligible and the systemic tissue has probably been saturated with virions from the inoculated leaf and secondary infections in systemic tissue. The exact threshold value for $N_e$-induced effects ($\sim 5$) will probably depend on the exact experimental conditions used (e.g., size of the inoculated leaf, temperature), and should therefore not be seen as an absolute value.

**Acknowledgments**

We thank Francisca de la Iglesia for technical assistance and three anonymous reviewers for their constructive comments.

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Figure Legends

Figure 1: IAH model predictions and data compared
A comparison of IAH model predictions and data for the dose foci relationship (panel A), and the rate of infection and the rate of mixed-genotype infection (panel B). In panel A, ln-transformed dose is plotted on the abscissa, and ln-transformed number of foci plus one is on the ordinate. In Panel B, log mean foci is on the abscissa, whilst the frequency of infection (squares and solid line) or mixed-genotype infections (diamonds and dotted line) is on the ordinate. For both panels, error bars represent 95% confidence intervals. None of the data points in panel B are significantly different from model predictions (Table 3).

Figure 2: Dose symptomatology relationship at 6 dpi
On the abscissa is log virion dose, and on the ordinate frequency of symptomatology or infection at 6 dpi. The symptomatology data are given by the triangles (mean ± 95% confidence interval), whereas the squares represent infection data for comparison. The HHS model is plotted for dose symptomatology (dotted line), as this model gave the lowest Akaike Weight (see Table 1). However, the HHS model does not have appreciably more support than the DA model. For dose infection, the IAH model is plotted as this is the simplest model and neither of the other models has appreciably more support (see Table 2). Note that dose symptomatology is much shallower than dose response, and that the frequency of symptomatic plants is considerably lower than the frequency of infected plants.
Figure 3: Effects of dose on viral accumulation at 6 dpi

On the abscissa is virion dose, and on the ordinate is viral accumulation at 6 dpi, the number of genome copies per 100 ng of total RNA extracted from plants. Note that both scales are logarithmic. The mean accumulation ± standard deviation is given, and appears to increase with dose. A Jonckheere-Terpstra test confirmed that there is a significant increase in accumulation with dose.

Figure 4: The effects of $N_e$ on accumulation at 6 dpi

On the abscissa is $N_e$ and on the ordinate is viral accumulation at 6 dpi, the number of genome copies per 100 ng of total RNA extracted from plants. Circles represent measurements for individual plants ± standard deviation. A Jonckheere-Terpstra test demonstrates that there is a significant increase of accumulation with $N_e$. The line depicts the fitted geometric model, which confirms that the increase of accumulation with $N_e$ only occurs for small $N_e$ values.

Figure 5: Fluorescence in the inoculated leaf at high and low doses.

GFP and mCherry fluorescence seen in the inoculated N. tabacum leaf at six dpi. For panels A-C a high virion dose was given ($1.44 \times 10^7$), whereas for panels D-F a low virion dose was given ($5.34 \times 10^5$) and plants with a single focus of primary infection at day three were selected. RT-qPCR also demonstrated significantly lower accumulation in the inoculated leaf of plants infected by only a single focus. Note the variation in the amount of fluorescence at low doses (panels D-F). In some cases, only the focus of primary infection and a faint trail along the vascular tissue are present (panel D), whereas in other cases the virus does achieve limited expansion into the inoculated leaf (panel F).
Figure 6: Effects of $N_e$ on accumulation when the inoculated leaf is removed

Viral accumulation 6 dpi is given for plants infected with a large or small $N_e$. The inoculated leaf was removed 5 dpi, when all infected plants in the experiment had shown inoculated systemic fluorescence. Errors bars represent the standard deviation.

Figure 7: Time course experiment on the effects of $N_e$ on accumulation

On the abscissa time of collection is given, and on the ordinate is viral accumulation – the number of genome copies per 100 ng of total RNA extracted from plants – is given. Circles represent measurements for individual plants ($\pm$ standard deviation) from the low dose treatment (1:270 virion dilution; mean $N_e = 2.05 \pm 1.28$), whereas squares represent plants from the high dose treatment (1:10 virion dilution; $N_e = 23.30 \pm 10.57$). Lines represent a logistic model fitted to the high dose (solid line) and low dose (dotted line) data. There were effects of $N_e$ on accumulation only at low doses, whereas at high doses similar levels of accumulation were measured.
Table 1. Variance in the mean number of foci of primary infection

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Virion dose</th>
<th>Mean foci</th>
<th>Variance</th>
<th>z</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10</td>
<td>1.44 × 10^7</td>
<td>26.25</td>
<td>113.67</td>
<td>1.217</td>
<td>0.103</td>
</tr>
<tr>
<td>1:30</td>
<td>4.81 × 10^6</td>
<td>13.45</td>
<td>21.00</td>
<td>0.719</td>
<td>0.680</td>
</tr>
<tr>
<td>1:90</td>
<td>1.60 × 10^6</td>
<td>5.60</td>
<td>10.88</td>
<td>0.936</td>
<td>0.345</td>
</tr>
<tr>
<td>1:270</td>
<td>5.34 × 10^5</td>
<td>0.88</td>
<td>1.11</td>
<td>0.326</td>
<td>1</td>
</tr>
<tr>
<td>1:810</td>
<td>1.78 × 10^5</td>
<td>0.46</td>
<td>0.43</td>
<td>0.067</td>
<td>1</td>
</tr>
<tr>
<td>1:2430</td>
<td>5.94 × 10^4</td>
<td>0.21</td>
<td>0.17</td>
<td>0.184</td>
<td>1</td>
</tr>
</tbody>
</table>

The dilution of purified virions used to inoculate plants, and the estimated virion dose, estimated by the number of RNA molecules measured by RT-qPCR, are given. Mean foci is the mean number of foci of primary infection, of both TEV-GFP and TEV-mCherry, in an inoculated leaf. Variance is the calculated variance of the mean number of foci of primary infection. For a Poisson distribution, the mean is equal to the variance. Whether this holds for the data was tested by means of a one-sample Kolmogorov-Smirnov (KS) test, where \( z \) is the KS test statistic and \( P \) its significance. No test results were significantly different from model predictions, although the variance deviates more strongly from predictions at higher doses.
Table 2. Fitting of three dose response models to dose infection and dose symptomatology data

<table>
<thead>
<tr>
<th>Data</th>
<th>Model</th>
<th>Param.</th>
<th>NLL</th>
<th>AIC</th>
<th>ΔAIC</th>
<th>AW</th>
<th>ρ</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection</td>
<td>DA</td>
<td>2</td>
<td>9.10</td>
<td>22.20</td>
<td>0.523</td>
<td>3.69×10^-4</td>
<td>φ = 0.78</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IAH</td>
<td>1</td>
<td>10.55</td>
<td>23.10</td>
<td>0.90</td>
<td>0.333</td>
<td>2.33×10^-6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HHS</td>
<td>2</td>
<td>10.39</td>
<td>24.78</td>
<td>2.58</td>
<td>0.144</td>
<td>2.71×10^-6</td>
<td>v = 0.24</td>
</tr>
<tr>
<td>Symptomatology</td>
<td>HHS</td>
<td>2</td>
<td>12.19</td>
<td>28.39</td>
<td>0.794</td>
<td>2.95×10^-6</td>
<td>v = 4.91</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DA</td>
<td>2</td>
<td>13.55</td>
<td>31.09</td>
<td>2.70</td>
<td>0.206</td>
<td>2.71×10^-3</td>
<td>ϕ = 0.37</td>
</tr>
<tr>
<td></td>
<td>IAH</td>
<td>1</td>
<td>48.43</td>
<td>98.87</td>
<td>70.48</td>
<td>0.000</td>
<td>2.33×10^-7</td>
<td></td>
</tr>
</tbody>
</table>

Models fitted to the data are the Independent Action Hypothesis (IAH), Dependent Action (DA), and Heterogeneous Host Susceptibility (HHS). Param. indicates the number of model parameters, while NLL is the negative log likelihood. AIC is the Akaike Information Criterion, while ΔAIC gives the difference in AIC compared to the best-supported model, which is given on top for both infection and symptomatology. The infection probability (ρ) is given for all models, and fitted parameters φ and v are given where applicable. The difference between the DA and IAH models are only marginal for the infection data (ΔAIC < 2), whilst the HHS model gives somewhat better fit than the DA model for symptomatology data (ΔAIC > 2). For the DA model, in both cases φ < 1 which is representative of a shallow dose response and antagonistic interactions between virions.
Table 3. Comparison of data and model predictions for the rate of infection and mixed-genotype infection.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Virion dose</th>
<th>Mean foci</th>
<th>Dose foci relationship</th>
<th>Mixed-genotype infections</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>( I_{\text{obs}} )</td>
<td>( I_{\text{pred}} )</td>
</tr>
<tr>
<td>1:10</td>
<td>( 1.44 \times 10^7 )</td>
<td>26.25</td>
<td>1 (20/20)</td>
<td>1</td>
</tr>
<tr>
<td>1:30</td>
<td>( 4.81 \times 10^6 )</td>
<td>13.45</td>
<td>1 (20/20)</td>
<td>1</td>
</tr>
<tr>
<td>1:90</td>
<td>( 1.60 \times 10^6 )</td>
<td>5.60</td>
<td>1 (20/20)</td>
<td>0.996</td>
</tr>
<tr>
<td>1:270</td>
<td>( 5.34 \times 10^5 )</td>
<td>0.88</td>
<td>0.520 (13/25)</td>
<td>0.585</td>
</tr>
<tr>
<td>1:810</td>
<td>( 1.78 \times 10^5 )</td>
<td>0.46</td>
<td>0.343 (12/35)</td>
<td>0.369</td>
</tr>
<tr>
<td>1:2430</td>
<td>( 5.94 \times 10^4 )</td>
<td>0.21</td>
<td>0.213 (16/75)</td>
<td>0.189</td>
</tr>
</tbody>
</table>

Dilution is the dilution of the virus stock used to inoculate plants, and virion dose is the number of virions per inoculated plant estimated by RT-qPCR. The observed rate of infection (\( I_{\text{obs}} \)) and mixed-genotype infection (\( P_{\text{mod}}(R \cap G) \)) are compared to model predictions (\( I_{\text{pred}} \) and \( P_{\text{pred}}(R \cap G) \), respectively). \( P \) is the significance value of binomial test comparing data and model predictions. None of the observed infection of mixed-genotype infection rates are significantly different from model predictions.

Table 4. Correlation of the number of foci on separately inoculated half-leaves.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Virion dose</th>
<th>Mean foci per half leaf</th>
<th>Pearson correlation</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Left</td>
<td>Right</td>
<td>correlation</td>
</tr>
<tr>
<td>1:10</td>
<td>( 2.98 \times 10^7 )</td>
<td>11.47 ± 6.30</td>
<td>15.27 ± 8.46</td>
<td>0.314</td>
</tr>
<tr>
<td>1:30</td>
<td>( 9.92 \times 10^6 )</td>
<td>9.47 ± 3.20</td>
<td>9.67 ± 3.18</td>
<td>0.157</td>
</tr>
<tr>
<td>1:90</td>
<td>( 3.31 \times 10^6 )</td>
<td>4.47 ± 2.72</td>
<td>3.53 ± 2.70</td>
<td>0.119</td>
</tr>
<tr>
<td>1:270</td>
<td>( 1.10 \times 10^6 )</td>
<td>2.00 ± 1.31</td>
<td>1.20 ± 1.21</td>
<td>-0.090</td>
</tr>
<tr>
<td>All data</td>
<td></td>
<td>6.85 ± 5.35</td>
<td>7.42 ± 7.21</td>
<td>0.411</td>
</tr>
</tbody>
</table>

Half leaves were separately inoculated with TEV-GFP, and the number of foci per half leaf quantified. The mean number of foci per right of left half, and its standard deviation, are given. For no one dose was a significant correlation between half leaves observed. When the data from different doses were pooled (“All data”) the partial correlation, with dose taken as a factor, was significant.
Table 5. Estimated parameters for the geometric model of $N_e$-viral accumulation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>SE</th>
<th>Lower 95% CI</th>
<th>Upper 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_0$</td>
<td>$5.816 \times 10^5$</td>
<td>$1.229 \times 10^6$</td>
<td>$-1.877 \times 10^6$</td>
<td>$3.040 \times 10^6$</td>
</tr>
<tr>
<td>$C$</td>
<td>$3.848 \times 10^6$</td>
<td>$9.045 \times 10^5$</td>
<td>$2.038 \times 10^6$</td>
<td>$5.657 \times 10^6$</td>
</tr>
<tr>
<td>$K$</td>
<td>0.564</td>
<td>0.095</td>
<td>0.373</td>
<td>0.755</td>
</tr>
</tbody>
</table>

Estimated model parameters for the fitting of a geometric model to $N_e$-viral accumulation data (see Materials and Methods). Key model parameters are $c$, which is positive indicating that there is an increase of accumulation with $N_e$, and $k$, which is significantly less than one, indicating that with each increment in $N_e$, the increase in viral accumulation becomes smaller.
Table 6. Nested ANOVAs for analysis of viral accumulation in 3 experiments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Source of variation</th>
<th>SS</th>
<th>d.f.</th>
<th>MS</th>
<th>F</th>
<th>P</th>
<th>Variance expl(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Class (High or Low)</td>
<td>2.809</td>
<td>1</td>
<td>2.809</td>
<td>35.52</td>
<td>0.004</td>
<td>84.35±13.77</td>
</tr>
<tr>
<td></td>
<td>Lineage within class</td>
<td>0.316</td>
<td>4</td>
<td>0.079</td>
<td>57.93</td>
<td>&lt; 0.001</td>
<td>14.86±0.17</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>0.016</td>
<td>12</td>
<td>0.001</td>
<td>0.78</td>
<td>&lt; 0.001</td>
<td>0.78±0.00</td>
</tr>
<tr>
<td>1</td>
<td>Class (High or Low)</td>
<td>0.094</td>
<td>1</td>
<td>0.094</td>
<td>4.56</td>
<td>0.100</td>
<td>0.98±0.00</td>
</tr>
<tr>
<td></td>
<td>Lineage within class</td>
<td>0.082</td>
<td>4</td>
<td>0.021</td>
<td>0.76</td>
<td>0.571</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td>Replicate within lineage within class</td>
<td>0.325</td>
<td>12</td>
<td>0.027</td>
<td>20.25</td>
<td>&lt; 0.001</td>
<td>98.8±0.11</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>0.048</td>
<td>36</td>
<td>0.001</td>
<td>0.1</td>
<td>&lt; 0.001</td>
<td>0.1±0.00</td>
</tr>
<tr>
<td>2</td>
<td>Class (High or Low)</td>
<td>0.166</td>
<td>1</td>
<td>0.166</td>
<td>5.88</td>
<td>0.136</td>
<td>2.58±0.04</td>
</tr>
<tr>
<td></td>
<td>Lineage within class</td>
<td>0.057</td>
<td>2</td>
<td>0.028</td>
<td>0.40</td>
<td>0.678</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td>Replicate within lineage within class</td>
<td>1.137</td>
<td>16</td>
<td>0.071</td>
<td>16.10</td>
<td>&lt; 0.001</td>
<td>95.38±0.23</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>0.177</td>
<td>40</td>
<td>0.004</td>
<td>2.04</td>
<td>&lt; 0.001</td>
<td>2.04±0.00</td>
</tr>
</tbody>
</table>

Viral accumulation from three separate experiments was analyzed with nested ANOVAs. Experiment 0 is the analysis of the 3 Nc = 1 plants with highest or lowest accumulation in the original accumulation experiment (Figure 4). Experiment 1 and 2 are the first and second heritability of accumulation experiments, respectively. SS is the sum of squares, and MS is the mean square. Variance expl. is the percentage of variance explained by the model, which was estimated by a maximum likelihood-based variance components analysis in SPSS, and the asymptotic covariance is given as an indication of estimate error.
Table 7. Estimated parameters for the logistic model of viral accumulation over time

<table>
<thead>
<tr>
<th>Dose</th>
<th>Ne Parameter</th>
<th>Estimate</th>
<th>SE</th>
<th>Lower 95% CI</th>
<th>Upper 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>n₀</td>
<td>13.11</td>
<td>9.82</td>
<td>-7.60</td>
<td>33.82</td>
</tr>
<tr>
<td></td>
<td>r₀</td>
<td>2.303</td>
<td>0.174</td>
<td>1.936</td>
<td>2.669</td>
</tr>
<tr>
<td></td>
<td>κ</td>
<td>3.988×10⁷</td>
<td>8.768×10⁵</td>
<td>2.138×10⁷</td>
<td>5.838×10⁷</td>
</tr>
<tr>
<td>High</td>
<td>n₀</td>
<td>9928</td>
<td>3857</td>
<td>1792</td>
<td>18,066</td>
</tr>
<tr>
<td></td>
<td>r₀</td>
<td>1.344</td>
<td>0.098</td>
<td>1.138</td>
<td>1.550</td>
</tr>
<tr>
<td></td>
<td>κ</td>
<td>4.905×10⁷</td>
<td>5.226×10⁵</td>
<td>3.802×10⁷</td>
<td>6.008×10⁷</td>
</tr>
</tbody>
</table>

Estimated model parameters for the fitting of a logistic model to accumulation data over time for a high (1:10 virion dilution) and low (1:270 dilution) doses. Nₑ is the estimate ± standard deviation obtained by counting the number of foci of primary infection on day three. Estimates of κ, the carrying capacity, are similar and 95% confidence intervals (CIs) overlap. Estimates of n₀ and r₀ also differ, as attested by non-overlapping 95% CIs. For n₀ this difference (~738 fold) could be expected, and the low dose gives a lower parameter estimate. On the other hand, from a quantitative perspective the difference in n₀ estimates does not match the difference in Nₑ (~ 10.9 fold) or dose (27 fold). Different estimates of r₀ occur because at high doses the systemic population experiences less growth relative to the larger population in the inoculated leaf, and probably do not reflect differences in the basic rate of viral replication.