Response to *Anonymous Reviewer 1*:

Many technical jargons were used without being clearly defined first. For examples, effective population size, selective value, founder effect.
Ln41-42; 55;58: added clarifications for the technical terms

Ln57-59: this is exactly what happens as infections spread from one cell to the next. We now know that if a very small number of viral genome copies (say under 10) invade a cell, the infection is unlikely to be successful. Thus, for most viruses productive cellular infection requires cooperation among large numbers of viral genome copies co-entering the same cell. Nevertheless, regardless of the number of genome copies a cell internalizes, only very few of them have the chance to replicate to yield progeny genome copies (intracellular bottlenecking). The same intracellular bottlenecking process repeats itself when these progeny genome copies enter the next cell, so that again only very few of them yield progeny. This has been well documented – e.g. Grubaugh et al Cell Host-Microbe 2016.
Ln59-60: We added the reference Grubaugh *et al.* 2016 as suggested.
"Repeated sampling of a small number of founders for example in intracellular bottlenecking process during cell-to-cell movement (Grubaugh et al. 2016)"

"It is true Muller's ratchet has the potential to cause some isolated subpopulations (cell lineages in an infected individual) to accumulate deleterious mutations and potentially go extinct, but the same process could also allow other subpopulations (intra-host cell lineages) to become more fit."
We totally agree with these comments as we also observe this in our simulations. In addition, the reference 8 (Cases-González *et al*. 2008) also indicates the potential beneficial effect of bottlenecks.

Ln60-63: As discussed above, I cannot agree that one virus particle is enough to initiate a successful infection in an intact plant. I challenge the authors to demonstrate that. Earlier studies came to such conclusion by measuring the progeny genome copies that replicated to detectable levels. However, just because very few viruses end up producing detectable progeny does not mean very few entered a cell/tissue/plant.
We have modified the text to make relax the affirmation (L64-65) :" The mean number of founders of infection can under some circumstances can be reduced to a very small quantity for plant viruses".

Ln207, define "100% infectivity".
Ln219, we have clarified the term 100% infectivity. "Results in nearly 100% infection of inoculated plant"

Ln142-145, unclear. Were the up to 10 lesions mixed before grounding?
For the 'intermediate N_e' treatment the 10 lesions were grounded in a same mortar.
Ln136-137: we have rewritten as "pooled and grounded together" to be more precise in this step of the preparation of inoculum.

How did you ensure all of the 10-microliter-extract, or all virions in it, invaded tobacco cells?
Obviously, we cannot be sure that all virions in a 10 uL extract enter into a cell. The only thing we are sure, and is absolutely common sense, is that the more virions in an extract, the higher the probability of infecting cells. Our model indicates a low number of virions to form a lesion. We did not expect that all virions contained in an extract will enter in the inoculated plant. The relevant fact here is to have 20-fold more virions in a case ('intermediate N_e') than in the other ('smallest N_e').

The issue here may indirectly prove that you need a high number of virions to enter each cell for successful infection to occur (the content of a single lesion may become so diluted or wasted in the preparation and inoculation processes so that not enough viral genomes can invade a single cell together.

All the lesions were treated in the same way, of course some degradation may occur, but this random effect is unbiased among treatments. The question of dilution was taken into account and we used the minimum quantity of buffer in order to have enough concentrated material to inoculate our plants.

Ln207-208, Did all lesions invariably lead to poor infectivity? Do you sense something wrong here?

In the text:

Ln221-222: "After a single passage of TEV in *C. quinoa,* infectivity in *N. tabacum* was reduced to 0.261 ±0.035."

Here lesions from the first inoculation of infected tobacco were tested for they capacity to give symptoms to tobacco. Some have 100% infectivity, but was not the majority as the average infectivity for all tested lesion (1st passage) was nearly 26%.

Ln210, 212, unclear. "serial passages" – all in quinoa?

Ln 224-225: "Serial passages of single lesions (small N_e treatment) produced in *C. quinoa* resulted…"

Accordingly, to the Figure 1a, the smallest N_e treatment is done only on *C. quinoa*.

Response to *Ana Morales-Arce:*

The introduction is clear about the theory and objectives behind the experiment. It aims to investigate the survival of viruses infecting plants despite the action of Muller's ratchet and transmission bottlenecks. The references cited are sufficient. I also highlight the study's main contribution in that it regards a whole organism (in this case, the quinoa and tobacco plants) rather than culture cells, traditionally used to explore the same questions. The research questions are straightforward, though hypotheses or predictions still need to be included. In general, there isn't a clear answer to the title question; perhaps it could be more explicit that they are conducting an experimental approach in the title.

We very much appreciate this suggestion, but after careful consideration, we have decided that the title reflects quite well the content of the manuscript. Therefore, we have opted to maintain it as written.

The methods are clear enough to allow for experiment replication, though it took me a while to understand them. Figure 1 could be further improved to make understanding the passages and differences between the Ne expected sizes easier. For instance, it could be helpful to include the approximate—number of generations. In general, quinoa plants are infected to create lineages, a reduced number of individuals, and allow for the accumulation of mutations. Then viruses are transmitted to a natural host of tobacco plants that will show a systemic infection. This approach is consistent with the question. Further, the fitness measure would be the infectivity measure in the tobacco plants, and this specifically would make many assumptions. I would expect that the plants measured are all clones and not influenced by external environmental factors during the 9dpi, which I doubt was the case.

Unfortunately, estimating the exact number of generations taking place during each infection is not straightforward. Keep in mind the replication process within single cells is mostly linear (Luria's stamping machine; Martínez *et al*. 2011 *Genetics* 188, 637-46), but the cell-to-cell expansion results in an exponential growth. In addition, new growing leaves add a stop-and-burst component to virus accumulation. Therefore, for a case of overlapping random number of generations, the best estimate we can generate, so far, is the number of population doubling from inoculation to sampling.

Notice that the key parameter for our study is the bottleneck size, N_e, which is experimentally easy to manipulate and would result in the a range of values independent on the number of generations that took place between inoculation and sampling .

Further, the authors tested their experimental results by comparing them with a model. The model is explained in terms of the parameter values, and the authors discuss them quite well. However, it could also be helpful for the reader to have a background in the choices of these ranges, i.e., why the replication (r)= [1,50] and why they chose 200 replicates. The size of the bottleneck or the initial infection number of individuals is better covered but less for the rest.

We done different assay of number of run (50, 100, 150, 200, 300, and 500) for the 100 first combinations (with the lowest values) and the last ones (with the highest values). Results were consistent from 150 runs. We then decided to run each combination 200 times.

We initially conducted an analysis using all variables within the range of [1, 20]. Upon eliminating results that did not correspond to the experiment, we identified an overestimation of the initial number of individuals, which was subsequently refined to a range of [1-8]. Furthermore, we observed an underestimation of the replication, particularly evident in the values of r_{max} for parameters A and D. As these values consistently fell within the ascending segment of the curve, we extended our exploration of this parameter to a broader range, encompassing $r \in [1, 50]$.

Additionally, the readers might benefit from having more information about the virus biology (what is in the literature), i.e., the replication time (generations), the mutation rates, genome recombination, and epistasis.

Ln83-85 we added a text to included information about the virus biology, particularly focusing on aspects such as the replication time (generations), mutation rates, genome recombination, and epistasis

The model scripts run and produce consistent values with the reported in the paper. I am not surprised the author disagrees with their model and the experiment. The model cannot reflect all the complexity involved, as they recognized in the discussion (for example, lines 292, 359). The model could recognize the limitations more explicitly and still show how valuable it is to try to model the transmission as it is done. Perhaps because the model is missing the reality of the number of infective particles in every passage, I understand the difficulties of measuring this before a passage, if it is possible in-vitro in this virus. The discussion also needs some specifics. For instance, when they mention the genetic properties of viral particles in lines 274-275, what exactly do they refer to?
Haplotype load? Population load? Other?

Ln268: "This finding suggests that there was a reduction in <u>genetic diversity</u> within those lineages over time as they were serially passaged."
The text mentions the genetic diversity and not genetic proprieties.

And finally, in line 359, the authors mention cooperation. How could this happen? Do they mean epistasis? or host-pathogen interactions?

Viruses can cooperate through sharing "public goods", for example a virion that is deleterious in its own replicase can benefit from the viral protein of another virus in order to multiply.

What about the replication system of the virus? Are they exempt from progeny skew? Especially if the founder population is small.

In small founder populations, especially within a single host plant, there is a possibility of progeny skew, as genetic drift and random variations can have a more significant impact when the population size is limited. In our case we simulate bottleneck differences in order to isolate different genotypes in lesions or to let the population expand in its natural host.

I also would be careful to claim mutation rate changes (line 411) as these are not measured here. A good question remaining is the role of mutation rate and mutation rate changes in these experiments. If it happened, what is the cause? how do you measure it experimentally?

We do not speak about mutation rate change. It is about the probability to have a beneficial mutation in the *C. quinoa* host. In other words, in changes in the distribution of mutational fitness effects associated to host switches.

As mentioned in the text:

1- **"Indeed, the virus population is contained in few cells and cannot expand." This means that the probability of mutations is considerably reduced compare to an expansion in the natural host.**
2- **"Given the small population size low generation number, the distribution of mutational (Carrasco *et al*. 2007; Lalić *et al*. 2011) and recombination effects (Vuillaume *et al*. 2011), the probability that any fortuitous genetic changes be beneficial and not neutral or deleterious is very low."**

This sentence is to remember to the reader that beneficial mutations (in TEV) are very rare.

Is the experimental plan consistent with the questions?

We believe so. Why this question? Could you be a bit more precise on what aspect of our experimental design is generating doubts?

"I am curious if there is work describing whether this occurs in the N. tabacum system when there are strict bottlenecks between passages. If not, why not?"

Actually, this question is answered in the manuscript where the results indicates that the expansion of the population within *N. tabacum* is a solution to the accumulation of deleterious mutations. Meanwhile one can ask the question of adaptability and evolution in fluctuating environment. We added a text in the introduction as suggested to clarify this point (Ln 96-102).

Response to Brent Allman:

Are the statistical analyses appropriate?
I really appreciated the analysis of lesions against the infectivity of N. tabacum.
This was a helpful point to make, and the authors may consider moving this figure from the supplement to the main text if they desire. I think it could fit nicely in Figure 2 or as its own figure but discussed earlier on in the results.
Thanks for this suggestion. We have moved the analysis of lesions against the infectivity of N. tabacum from the supplement to the main text as Figure 4.

I) Genetic Variance
The authors discuss "significant" changes in variance in the section beginning on line 233, Variance intra- and inter-lineages. However, this was not accompanied by any statistical test or approximate justification. Additionally, the use of these variances was omitted in the Methods section. Finally, I am having a difficult time interpreting the Figure 3 plots. Their description of Figure 3B seems to ignore a particular datapoint where the intermediate population appears to have a decrease in variance during one of the passages but the authors claim "the intermediate and large Ne lineages did not display any significant changes in intra-lineage variation throughout the passages."
We added statistical analysis of the inter (Wilcoxon and Friedman 's test; Ln:257-258 ; 261-262) and intra-lineages (linear regression model; ln:270-271) variance.

II) Simulation Model
I enjoy the inclusion of the simulations in the manuscript. I think the setup makes intuitive sense as someone familiar with these types of models. They appear to adequately search parameter space for reasonable parameters. However, to make their quantitative methods more rigorous, I would have liked to see the authors fit their model to their data. They claim that the model trends (Figure 5) match the data well (Figures 2 and 3). However, the authors could make a more convincing claim if they fit their model to data so the actual values of the model and data match – they don't match in 5A and 2B. My observation that they don't match by eye might be evidence that a better-fit parameterization is available in parameter space. I suggest the authors look into particle MCMC as a potential method for fitting this stochastic model to their data. With this method they will be able to estimate model parameters from their data, potentially showing where the small and intermediate Ne experiments disagree. A good place to learn about this method is this Endo et al paper, https://doi.org/10.1016/j.epidem.2019.100363. A couple of examples of its use in the literature can be found in Zhu et al, https://doi.org/10.3390/v13071216, and Rasmussen et al, doi:10.1371/journal.pcbi.1002136.
We fit our results to the model by selecting combinations of parameters and restricting them to a space of values that is biologically relevant. In our article, we discussed discrepancies between the model and the simulation, which can have several explanations. To summarize, there are two major points. First, the simulation misses some other variables that are inside the system. As Reviewer 1 pointed out, 'the same process could also allow other subpopulations (intra-host cell lineages) to become more fit,' which is what we observe in the simulation (Fig. 5A) for a few lineages.
To enhance the simulation model, incorporating PMCMC for a more precise parameter evaluation is a valuable suggestion. While we have thoroughly explored the biologically relevant parameter space and our results effectively elucidate the studied process, we currently lack the resources to conduct new analyses, including PMCMC.

Line 38: The authors have several citations for estimates of viral mutation rates. However, I would advocate for the inclusion of a slightly more contemporary citation among them since methods for

estimating mutation rates have been updated. Perhaps 10.1128/JVI.01031-17 or 10.1007/s00018-016-2299-6 could be a good fit.
The suggested references have been added.

On lines 83 to 85: The authors claim TEV does not migrate within the plant. However, the presence of lesions suggests local spread, and their aphid hosts may allow for migration or multiple transmission events in nature. Perhaps a point to address in the discussion.
On lines 88 to 89: "Because the virus is not moving systemically within the plant,…"
In plant virology, it's important to distinguish between two types of viral movement: cell-to-cell movement, which occurs during the early stages of infection and coincides with lesion formation, and systemic movement, where the virus spreads throughout the entire plant. In the case of TEV/C. quinoa, it's worth noting that the virus is incapable of invading the entire plant, indicating a limitation in its ability to establish systemic infection.

Figure 6 in the discussion is labeled Figure 5.
We fixed the wrong label.

Be aware that the hyperlink on line 162 of the PDF pointing to the simulation model on Zenodo goes to a broken page.
We checked and in our pdf version the link is working.

Is the manuscript well written?
While there is incorrect grammar in some sentences of the article and README file, the clarity of the message is not hindered by these small errors. Example in the README file: "A few number of particle initiate a lesion" could be changed to "A small number of particles initiate a lesion" or "A few particles initiate a lesion." It seems most errors are made when using the plural and the future tense. To be ready for publication, I suggest passing the next version of the manuscript through software like Grammarly or ChatGPT to help address some of these small grammatical issues.
The manuscript and the README file underwent grammatical software checks to address any grammatical or orthographical concerns.