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# Frequency-dependent assistance as a way out of competitive exclusion between two strains of an emerging virus

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Biological invasions are the main causes of emerging viral diseases and they favour the co-occurrence of multiple species or strains in the same environment. Depending on the nature of the interaction, co-occurrence can lead to competitive exclusion or coexistence. The successive fortuitous introductions of two strains of *Tomato yellow leaf curl virus* (TYLCV-Mld and TYLCV-IL) in Réunion Island provided an ideal opportunity to study the invasion of, and competition between, these worldwide emerging pathogens. During a 7-year field survey, we observed a displacement of the resident TYLCV-Mld by the newcomer TYLCV-IL, with TYLCV-Mld remaining mostly in co-infected plants. To understand the factors associated with this partial displacement, biological traits related to fitness were measured. The better ecological aptitude of TYLCV-IL in single infections was demonstrated, which explains its rapid spread. However, we demonstrate that the relative fitness of virus strains can drastically change between single infections and co-infections. An epidemiological model parametrized with our experimental data predicts that the two strains will coexist in the long run through assistance by the fitter strain. This rare case of unilateral facilitation between two pathogens leads to frequency-dependent selection and maintenance of the less fit strain.

## 1. Introduction

Virus emergence threatens human, animal and plant health, with the associated economic impact. Because viruses evolve at high substitution [1] and recombination [2] rates that are essential for host switch, these processes are often invoked as causes of virus emergence. However, the vast majority of virus emergence events are in fact biological invasions (i.e. introductions of viruses in new areas without host switch) [3]. Since the early domestication of plants, human activity has greatly impacted viral evolution by promoting invasion and spread of viruses through the transport of plants away from their centres of diversification. In the past century, the whole process has been exacerbated by the increase in the world trade of plants and plant products [3,4] that favour virus and vector dissemination.

These dissemination events favour the co-occurrence of multiple viruses in the same environment. The competitive exclusion principle states that two species competing for the same limiting resources cannot stably coexist: only one species can survive in a static fitness landscape [5,6]. However, competition is not the only outcome of co-occurrence. Depending on the nature of the interactions between individuals (antagonism, neutrality or synergism), co-occurrence can lead to competitive exclusion but also to coexistence, and even to the emergence of new recombinant genomes [7–9]. Indeed, each population may modify the fitness landscape of the other populations, affecting them in a frequency-dependent manner [10,11]. Thus, properly parametrized epidemiological models incorporating interdependence between the protagonists are needed to predict the long-term outcome of co-occurrence.

For investigations on biological invasions of viral populations, it is particularly interesting to study the tomato yellow leaf curl virus complex (referred to as TYLCVs, genus *Begomovirus* of the Geminiviridae family [12]) which is responsible for the emergent and devastating tomato yellow leaf curl disease (TYLCD). These begomoviruses are transmitted in a circulative non-propagative and persistent manner by the whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae), considered the main driver of the emergence of TYLCV [4,13]. Since the first description of the disease in the late 1920s in the Jordan Valley [14], several strains and variants have been described, and at least two of them (TYLCV-Mld and TYLCV-IL) overlap in their worldwide distribution [12,15]. TYLCV-Mld was first reported in 1997 as the causal agent of outbreaks of TYLCD on tomato crops in Réunion Island [16], an island located 700 km off the coast of Madagascar. The spread and molecular evolution of TYLCV-Mld in Réunion Island is well documented from 1998 to 2004 [16,17], when more severe symptoms of TYLCD, never seen before, were observed. Molecular diagnosis revealed the introduction of the 'Israel' (also called 'severe') strain of TYLCV (TYLCV-IL) [18]. We took advantage of this second introduction to study the invasion and competition of the two main strains of one of the most emergent virus species in small, isolated Réunion Island, where no other viral disease affects tomato in open fields.

The fate of the two TYLCV strains depends on their competing abilities for host and vector, both in single and mixed infections. While the biological traits associated with TYLCD dynamics (host range, within-plant accumulation, transmission rate) have been studied, and local emergence and co-occurrence of multiple species/strains have been reported throughout the world [7,8,15,18,19], extensive studies of interactions between species/strains are rare. Here, we demonstrate that, over a 7-year period following its emergence in Réunion Island, the severe strain TYLCV-IL progressively displaced TYLCV-Mld, which mostly remained in mixed infections. We link this epidemiological pattern to key biological traits that may cause fitness differences between the two strains (within-plant virus dynamics, within-insect viral load and transmission rate). We estimate the relative fitness of the two strains both in the field and in the laboratory, and we use our experimental results to parametrize a model predicting the long-term outcome of competition between the two strains. As a valuable by-product of the transmission experiments, we demonstrate, for the first time, that the average number of effectively transmitted viral genomes (i.e. the number of viral genomes that are inoculated and then systemically infect the plant) can be as low as one or two even for a virus transmitted in a circulative manner (by a vector carrying several millions viral genomes).

## 2. Material and methods

### (a) Field survey

Nine locations in the main tomato-growing areas of Réunion Island (electronic supplementary material, figure S1) were surveyed twice a year (February–March and September–October). From 2003–2004 to 2007–2008, tomato plants (*Solanum lycopersicum*) exhibiting symptoms suggestive of TYLCD infections were sampled. In order to prevent biasing the sampling towards the more severe strain, we collected the widest range of symptoms resulting from TYLCV infection, including slight to severe stunting or dwarfing, leaf curling, leaf deformation and leaf chlorosis. In 2008–2009 and 2009–2010, blind random sampling was undertaken: 30 samples were collected randomly per sampling site, regardless of symptoms. All the samples were dehydrated using anhydrous calcium chloride and stored prior to DNA extraction.

### (b) DNA extraction and TYLCV typing

Total DNA was extracted using the DNeasy plant miniprep kit (Qiagen) according to the manufacturer's instructions. DNA was resuspended in 100 μl of ultrapure water and stored at −20°C until use. To differentiate between TYLCV-IL and TYLCV-Mld, we used multiplex polymerase chain reaction (PCR) as described elsewhere [20].

### (c) Test for the presence of recombinant variants

To the best of our knowledge, there is no report of natural recombinant between TYLCV-Mld and TYLCV-IL in epidemiological situations where these strains coexist. However, to account for such possible emergence in Réunion Island, the viral replication-associated protein open reading frame (Rep ORF, the genomic region that differentiates the two strains [21]) of co-infected field samples was amplified and sequenced (electronic supplementary material; all the nucleotide sequences are available on request). Seven and 10 sequences were obtained from samples collected in 2007–2008 and 2009–2010, respectively. In addition, eight full-length genomes from six samples detected in 2009–2010 as infected by TYLCV-IL (three samples) and co-infected by TYLCV-IL and TYLCV-Mld (three samples) were amplified, cloned and sequenced [22]. For both datasets (partial and full genomes), sequences were aligned with the TYLCV-IL and TYLCV-Mld reference sequences (EMBL–GenBank–DDBJ accession numbers AM409201 and AJ865337, respectively [18]) from Réunion Island using the CLUSTALW [23] subalignment tool available in MEGA 4 [24]. The presence of recombinant sequences was checked using RDP3 software [25].

### (d) Within-plant virus accumulation experiments

Within-plant virus accumulation of each TYLCV strain in tomato (cv. Farmer, Known-You Seed) was estimated by monitoring viral DNA accumulation in single and mixed infections with real-time PCR (electronic supplementary material; qPCR data are available on request). The plants were placed in an insect-proof greenhouse in a complete randomized block design to prevent any risk of block effects owing to differential environmental factors that might interfere with plant physiology, and hence with viral accumulation. Because a closely related begomovirus was shown to accumulate evenly among the three youngest (apical) leaves [26], the apex—including the last three leaves—of plants inoculated respectively by TYLCV-IL[RE4], TYLCV-Mld[RE] and both clones was randomly sampled between 2 and 35 days post-inoculation (electronic supplementary material, table S1). Samples were dehydrated and ground with TissueLyser (Qiagen); 20 mg were then collected for total DNA extraction as described above. Viral and plant DNA were quantified using the StepOnePlus PCR system (Applied Biosystems) and real-time duplex PCR as described elsewhere [27].

### (e) Insect transmission assays

The transmission rate of the two viral strains was estimated after acquisition of the virus by *B. tabaci* (biotype B population currently known as Middle East–Asia Minor 1 cryptic species) [28] from single- and mixed-infected plants (electronic supplementary material). To avoid biasing transmission rate estimates from mixed infections, viral load was estimated using real-time PCR as described above, and only plants with a ratio
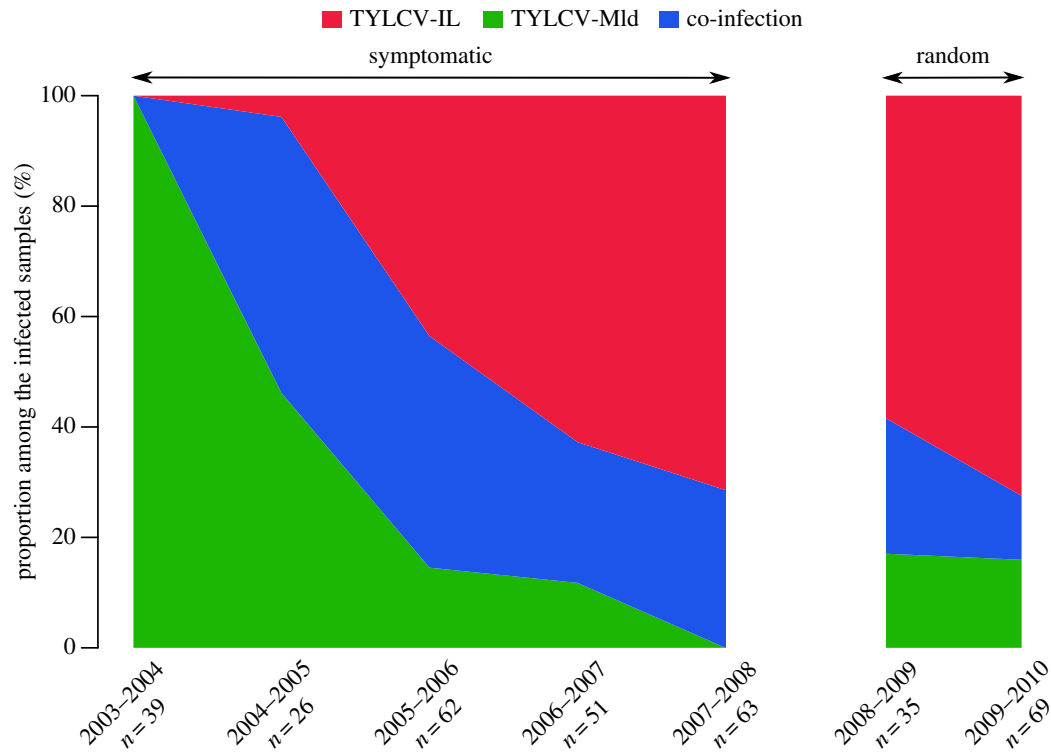
**Figure 1.** Relative dynamics of *Tomato yellow leaf curl virus* strain Mild (TYLCV-Mld) and strain Israel (TYLCV-IL) in Réunion Island from 2004 to 2010. Before the gap, only symptomatic plants were sampled; on the last two sampling seasons, blind sampling was conducted.

of the two TYLCV strains ranging from 0.95 to 1.05 were used. After a 72 h acquisition access period (AAP), adults were individually collected and deposited on healthy tomato plantlets (cv. Farmer, Known-You Seed) at the one-leaf growth stage. In order to discard insects with an unknown inoculation access period (IAP), only plantlets with living *B. tabaci* after the 72 h IAP were used to estimate transmission rates. The plantlets were then sprayed with insecticide and placed in an insect-proof greenhouse. After five weeks, the symptoms were scored, and plants were tested for the presence of TYLCV-IL and/or TYLCV-Mld using multiplex PCR as described above [20]. Two independent transmission experiments were performed for single infections, and three for mixed infections.

### (f) Within-insect viral quantification

Synchronous *B. tabaci* female adults were given a 72 h AAP in the cages to tomato plants (cv. Farmer, Known-You Seed) inoculated four weeks earlier with TYLCV-IL or TYLCV-Mld. Total DNA was extracted as described elsewhere [29] and stored at −20°C until use, and the within-insect viral load of each TYLCV strain was estimated with real-time PCR (electronic supplementary material; qPCR data are available on request).

### (g) Models and estimates of the relative success of the two viral strains

All the modelling and statistical analyses were performed using the R statistical software [30] (electronic supplementary material).

## 3. Results

### (a) Rapid displacement of TYLCV-Mld by the newcomer TYLCV-IL

The distribution of the two strains of TYLCV in Réunion Island was analysed based on 975 samples collected between the 2003–2004 and 2009–2010 growing seasons in the main tomato-producing areas (electronic supplementary material, table S2). The substantial proportion of uninfected plants observed among the sampled symptomatic plants probably results from abiotic stresses leading to leaf chlorosis. The blind random sampling performed in 2008–2009 and 2009–2010 provided an estimate of disease prevalence: 15.6% (2008–2009) and 16.4% (2009–2010) of the samples were infected with at least one strain of TYLCV. After the introduction of the IL strain of TYLCV, the change in the proportion of the two strains in the infected field samples indicated a displacement of TYLCV-Mld by TYLCV-IL (figure 1; electronic supplementary material, figure S1). Although TYLCV-Mld was dominant in 2003–2004, its relative frequency progressively decreased, whereas that of TYLCV-IL increased. If the symptom-based sampling strategy could have somehow biased the analysis with a preferential collection of plants infected by the IL strain either in single or mixed infection, then the extensive random sampling in 2008–2009 and 2009–2010 confirmed the displacement of the Mld strain, with 72.5% of the 2009–2010 TYLCV-positive samples infected with the IL strain alone, 15.9% with the Mld strain alone and 11.6% co-infected.

Although TYLCV-Mld was rapidly displaced by TYLCV-IL, it is interesting to note that it persisted, notably in mixed infections (figure 1; electronic supplementary material, table S2). Because mixed infections were frequent, and begomoviruses are known to be highly recombinogenic, it was important to determine whether a new recombinant between TYLCV-Mld and TYLCV-IL had appeared and reached a significant prevalence. As the Rep ORF is the portion of the genome that enables clear differentiation between these strains [21], we cloned and sequenced this ORF from seven and 10 samples collected in 2007–2008 and 2009–2010, respectively. None of these 17 sequenced samples presented any
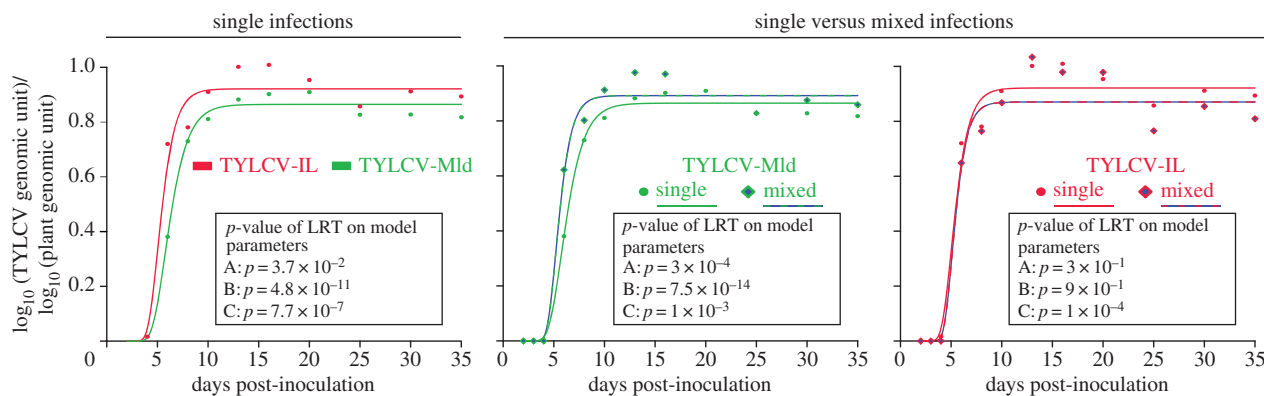
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**Figure 2.** Estimated (curve) and mean (dots) normalized viral load after agro-inoculation of the Réunion Island isolates of *Tomato yellow leaf curl virus* strain Mild (TYLCV-Mld) and/or strain Israel (TYLCV-IL) in tomato plants. LRT, likelihood ratio test.

**Table 1.** Estimated parameters [95% CIs] of the log−log growth models for within-plant accumulation of the Réunion Island isolates of *Tomato yellow leaf curl virus* strain Mild (TYLCV-Mld) and strain Israel (TYLCV-IL) in single and mixed infections.

| infection | virus | log−log model parameters | | |
| | | A[a] | B[b] | C[c] |
| --- | --- | --- | --- | --- |
| single | TYLCV-IL | 1.06 [0.88 − 1.24] | 5.38 [5.18 − 5.58] | 0.92 [0.9 − 0.94] |
| | TYLCV-Mld | 0.81 [0.7 − 0.92] | 6.21 [6.09 − 6.33] | 0.86 [0.85 − 0.87] |
| mixed | TYLCV-IL | 1.25 [0.96 − 1.54] | 5.4 [5.21 − 5.59] | 0.87 [0.85 − 0.89] |
| | TYLCV-Mld | 1.19 [0.98 − 1.4] | 5.52 [5.38 − 5.66] | 0.89 [0.88 − 0.9] |

[a]Slope during the exponential phase.
[b]Time to reach 50% of the viral load at the plateau.
[c]Viral load at the final plateau.

recombination pattern, all the sequences being of TYLCV-Mld or TYLCV-IL types. These analyses were repeated in 2009–2010 with full-length genomes, which confirmed the previous results as all sequences were shown to be of the TYLCV-IL type ($n = 5$) or of the TYLCV-Mld type ($n = 3$). This indicates that no new recombinant viral strain was prevalent on tomato crops in Réunion Island and that the Mld strain of TYLCV was displaced by the IL strain, and not by a recombinant strain.

### (b) TYLCV-IL is better at accumulating within the plant in single infection, but not in competition

Within-plant accumulation of the two TYLCV strains was evaluated in agro-inoculated tomato plants using real-time PCR assays. Preliminary studies revealed that the kinetics of viral accumulation fit a logistic growth model, characterized by an initial exponential increase in viral load and a final plateau. To accurately infer the model parameters, we decided to maximize the number of samples collected during the first few days post inoculation (dpi). After fitting log−log growth models to the experimental data and comparing the model parameters using likelihood ratio tests (figure 2 and table 1), it appeared that in single infections TYLCV-IL accumulates faster than TYLCV-Mld (reaching 50% of the plateau viral load 0.8 day before TYLCV-Mld; $p = 5 \times 10^{-11}$) and to a higher level (sixfold; $p = 8 \times 10^{-7}$).

In mixed infections (figure 2 and table 1), TYLCV-Mld accumulates faster than in the single infection (reaching 50% of the plateau viral load 0.7 day earlier; $p = 7 \times 10^{-14}$) and with a higher viral load (twofold; $p = 0.001$). By contrast,

TYLCV-IL accumulates less in the mixed infection (3.5-fold; $p = 10^{-4}$) than in the single infection (no difference was found in the speed of viral accumulation). Moreover, in the mixed infections, none of the three model parameters differed significantly between TYLCV-IL and TYLCV-Mld, reflecting similar within-plant accumulation.

### (c) TYLCV-IL is better transmitted than TYLCV-Mld in single infections but not in mixed infections

The rapid spread of TYLCV on tomato crops in Réunion Island was clearly associated with the presence of the invasive biotype B of *B. tabaci* [31]. Significant differences were found between the two TYLCV strains, with transmission rates of 59.0% and 65.8% for TYLCV-Mld, and 82.9% and 91.0% for TYLCV-IL in the two experiments ($p = 6 \times 10^{-4}$; figure 3). In line with these results, the mean numbers of efficiently transmitted genomes also differed significantly: 1.0 (CI: 0.71–1.4) for TYLCV-Mld and 2.0 (CI: 1.4–2.8) for TYLCV-IL (figure 3). As we revealed that TYLCV-IL accumulates more in tomato plants, it could be argued that the difference observed in the transmission rate is mainly due to a difference in the ability of *B. tabaci* to acquire the two TYLCV strains. However, after an AAP of 72 h, no significant difference was found in the viral load between the two strains in *B. tabaci* ($p = 0.87$; electronic supplementary material, figure S2), indicating that the higher transmission rate of the IL strain results from either a more efficient inoculation or initiation of infection.
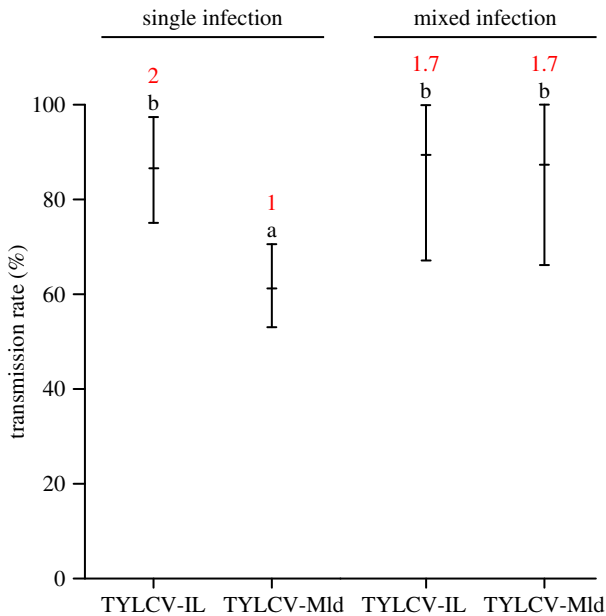
**Figure 3.** Results of transmission experiments conducted with *B. tabaci* biotype B adult females after acquisition on tomato plants single-infected by TYLCV-Mld or TYLCV-IL and mixed-infected by TYLCV-Mld and TYLCV-IL. Vertical bars around each point represent the 95% CI and the bars topped by the same letter are not significantly different at $p = 0.05$ (Tukey's paired comparison test). Each estimated mean number of efficiently transmitted viral genomes is indicated above the corresponding transmission rate.

Transmission from co-infected plants resulted in significantly ($p < 10^{-4}$ in each case) more co-infected plants (75.8%) than plants infected by either TYLCV-IL alone (12.3%) or TYLCV-Mld alone (11.9%). Moreover, no difference ($p = 0.996$) was found between the proportion of plants infected by TYLCV-IL alone and TYLCV-Mld alone (electronic supplementary material, table S3). As a result, the mean numbers of efficiently transmitted genomes were very similar in these experiments, being approximately 1.7 (CI: 1.3–2.1 for TYLCV-Mld and CI: 1.3–2.2 for TYLCV-IL).

It was also apparent that TYLCV-Mld is significantly better transmitted from mixed-infected plants than from plants with a single infection, with average transmission rates of 81% and 63%, respectively ($p = 6.2 \times 10^{-6}$). By contrast, the transmission rate of TYLCV-IL from mixed- or single-infected plants was similar (82% and 86% respectively, $p = 0.628$).

## (d) Epidemiological assistance to TYLCV-Mld by the fitter strain TYLCV-IL

How do the observed field dynamics and experimental accumulation and transmission differences translate into measures of relative fitness? What qualitative predictions can we make about the long-term dynamics of the two strains? The answers to these questions rely on modelling approaches. There are several ways of quantifying the relative success of two strains [32]. Using laboratory and field data, three such parameters were estimated: relative reproductive success, epidemiological prevalence and Malthusian fitness of TYLCV-IL and TYLCV-Mld. First, the relative reproductive success of the two strains during colonization of an empty niche was estimated experimentally based on their $R_0$ ratio (i.e. the relative number of secondary infections caused by a single infectious host introduced into a fully susceptible host population).

Because all the parameters are similar for the two strains except the transmission probabilities (electronic supplementary material), the $R_0$ ratio simplifies into a ratio of the transmission probabilities. Recalling that this ratio is a characteristic of the early phase of an epidemic (i.e. when co-infections are rare), it corresponds to the transmission ratio measured experimentally in the single infections. Thus, $R_{0,I}/R_{0,M} = (45/52)/(38/60) = 1.37$: the IL strain should be much more invasive than the Mld strain, with each infected plant producing 37% more new infections with the IL strain than with the Mld strain. From the epidemiological survey, we were able to estimate both the relative prevalence ($RR$) and the difference in fitness ($s$) between the two strains in the field. The key evolutionary parameter in the pathosystem is the selection coefficient $s$; the estimated value of $s = 0.0495$ month$^{-1}$ (figure 4, inset) translates into a doubling time of only 14 months for the relative prevalence $RR$. $RR$ increased steadily over time in favour of the IL strain (figure 4), and the rate of increase of $RR$ was similar before and after the introduction of the random sampling scheme (gap in figure 4). Because of its selective advantage, the IL strain became three times more prevalent than the Mld strain within 6 years of its introduction in Réunion Island.

All these measures of relative success indicate that the IL strain is much fitter than the Mld strain, except that both strains are equally transmissible from mixed-infected plants. This last feature leaves the door open for the possible persistence of the Mld strain in the agrosystem. Scaling up from experimental transmission rates with individual plants to disease dynamics in a plant population, our epidemiological model addressed this issue. For pathogens reaching the host carrying capacity, one would expect the overall transmission rate from mixed-infected plants to be the same as the transmission rate from plants infected by the IL strain only (maximum carrying capacity for transmission in a single infection). In this case, the Mld strain would have collapsed despite assistance from the IL strain (figure 5a). However, when, as observed in the experiment, assistance is associated with an increase in the overall transmission rate from mixed-infect plants, a stable epidemiological equilibrium is reached where both strains coexist (figure 5b); incidentally, the Mld strain is still present in Réunion Island in 2013 (data not shown). Note also that the qualitative results are stable throughout the range of biologically relevant parameter values (not shown): the two strains coexist because of mixed infections, co-occurrence favours the Mld strain more than it impairs the IL strain, and total TYLCV prevalence is higher than the prevalence of TYLCV-IL in the absence of TYLCV-Mld. The model also shows that the selection coefficient is affected by mixed-infected plants, $s$ being initially constant (and equal to the difference in Malthusian fitness between the two strains in the absence of the other strain) before progressively changing when the density and frequency of co-infections increase significantly (electronic supplementary material, figure S3). A salient feature of this dynamics is the resulting temporary increase in the Malthusian fitness of the Mld strain, which contributes to the sharp decrease in $s$ (electronic supplementary material, figure S3), although a more detailed analysis suggests that density dependence is also involved (electronic supplementary material, figure S4). In addition, the high frequency of co-infections modifies the epidemiological dynamics of each strain in comparison with the corresponding single-strain epidemics: TYLCV-Mld prevalence is greatly increased, whereas
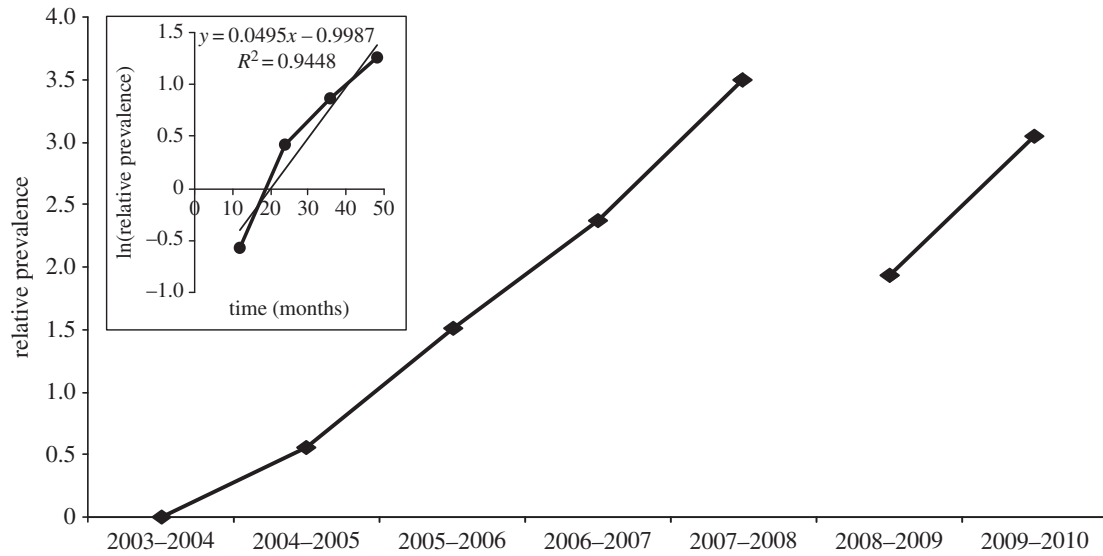
**Figure 4.** Relative success of *Tomato yellow leaf curl virus* strain Israel (TYLCV-IL) versus strain Mild (TYLCV-Mld). The relative prevalence *RR* of TYLCV-IL versus TYLCV-Mld is the ratio between the total number of plants infected by each strain (alone or in mixed infection). Before the gap, only symptomatic plants were sampled; on the last two seasons, blind sampling was conducted. Inset: the slope of the linear regression of ln(*RR*) versus time is a measure of the fitness difference between the two strains (last two points excluded because of the different sampling procedure).
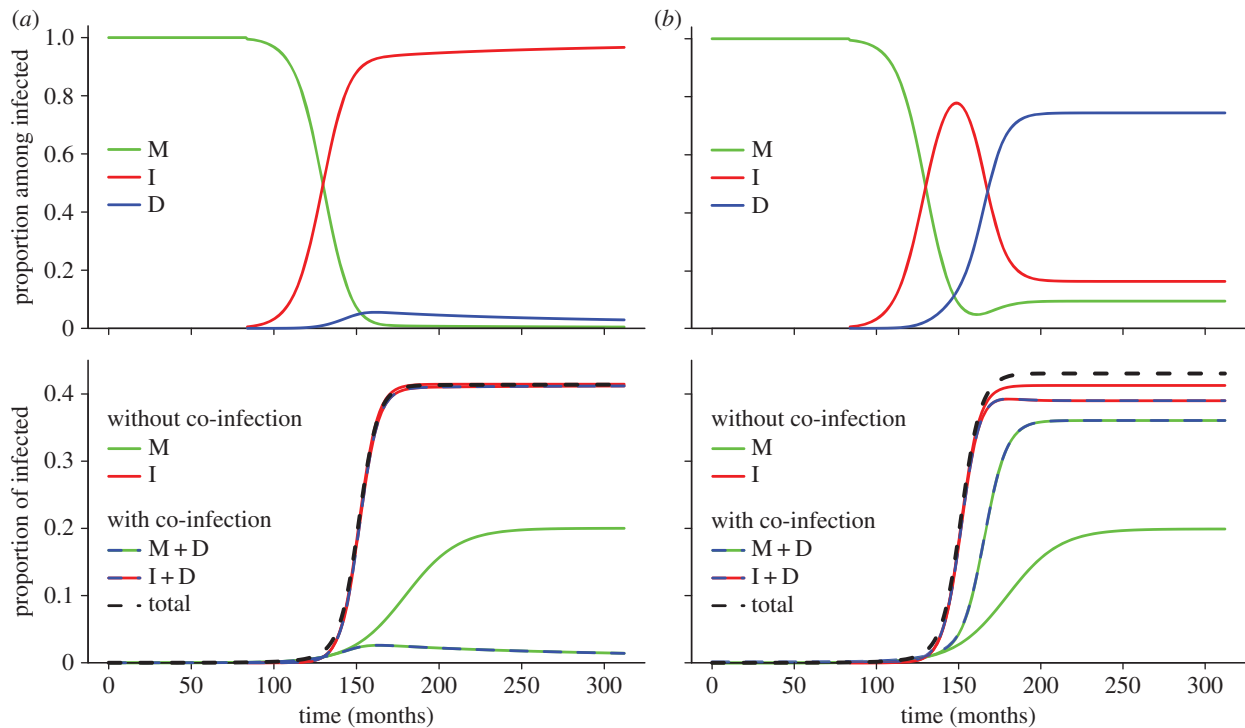


**Figure 5.** Modelled temporal dynamics of the two *Tomato yellow leaf curl virus* strains using the set of parameters described in the electronic supplementary material. (*a*) Dynamics assuming a total transmission rate from co-infected plants equal to that of the most transmissible strain in single infection. (*b*) Dynamics using the transmission rates measured in our experiments. M, TYLCV-Mld; I, TYLCV-IL; D, double (mixed) infections. The top panels represent the proportion of infected plants in each category (the three curves sum to 1). In the bottom panels, bicolour curves represent the proportion of plants infected by each strain (single or mixed infection) and the dashed curve shows the total prevalence of TYLCV; plain curves are provided as a benchmark and represent prevalence in single-strain epidemics.

TYLCV-IL is only slightly reduced; consequently, the net amount of disease at the epidemiological equilibrium should be higher than if the Mld strain had been fully displaced by the IL strain (figure 5*b*, bottom panel).

## 4. Discussion

Our field survey revealed that interstrain competition led to the rapid—but incomplete—displacement of the resident TYLCV-Mld by the newcomer TYLCV-IL. After checking that the competing strains were the non-recombinant offspring of the introduced TYLCV-Mld and TYLCV-IL, we discovered that TYLCV-Mld benefited from mixed infections for two traits: accumulation within tomato plants, and transmission by *B. tabaci*. Finally, with quantitative models, we were able to estimate key parameters of the complex interaction between the two strains and to link our experimental results with TYLCV epidemiological dynamics.

## (a) Relationship between within-plant viral load and transmission rate

A link between within-plant viral load and transmission rate is generally accepted even if the studies on circulative viruses are rare [33]. Here, we demonstrated on TYLCV, a circulative non-propagative virus, that in single infection the IL strain accumulates better (faster and in greater amounts) within the plant than the Mld strain and is also better transmitted. We also demonstrated that in the mixed infections, the two strains had similar accumulation dynamics within the plant and similar transmission rates. In that sense, within-plant viral load could appear, *a posteriori*, as a good proxy for the transmission rate of this circulative virus. The simplest explanation would be a mass-action mechanism (more virus genomes in the plant implying more virions in the insect implying a higher transmission rate). However, we ruled out this straightforward mass-action putative mechanism, because a sixfold difference in within-plant viral load between the two strains did not result in a significant difference in their accumulation in *B. tabaci*. The observed link between within-plant virus load and transmission is thus less direct, or maybe fortuitous. In fact, the key factor explaining the displacement of TYLCV-Mld by TYLCV-IL is that, although the two strains reach the same virus load in the vector, the transmission rate (i.e. the ability to be inoculated and to initiate an infection) is 37% higher for TYLCV-IL than for TYLCV-Mld. Epidemiological factors other than transmission rate, such as extended host range [7,34], might be involved in the shift of viral populations. However, as previously described in Florida [35], in Réunion Island TYLCV does not require alternative hosts during intercropping or overwintering periods, because tomato plants are cultivated all year long. Thus, although the 37% difference in transmission rates between the two strains might not be the only factor involved in the observed displacement of the Mld strain, it is sufficient to explain it, as shown by the model.

## (b) Benefits of mixed infections through unilateral facilitation

Another striking result of the field survey is the persistence of TYLCV-Mld predominantly in mixed infections promoting virus–virus interactions (figure 1 and table 1). Mixed infections of plant viruses are common in nature, and a number of important virus diseases of plants are the outcome of interactions between causative agents [34,36,37]. Mixed infections can lead to a variety of within-host virus–virus interactions such as synergism or interference [36,38] that may induce changes in the genetic structure of viral populations. Here, we demonstrated that the presence of another strain alters the within-plant accumulation kinetics of both strains. TYLCV-Mld benefits from mixed infections and accumulates much better (i.e. faster and in greater amounts) than alone, at the expense of TYLCV-IL, which has a lower viral load compared with the single infection. TYLCV-Mld appeared to be better transmitted from co-infected plants than from single-infected plants, but the presence of TYLCV-Mld did not significantly hinder the transmission of TYLCV-IL. Taken together, these observations highlight the benefits of mixed infections for the natural spread of TYLCV-Mld. Furthermore, contrary to the widespread assumption that different virus strains share the same resources that determine the

host or vector carrying capacity, our transmission experiments and the resulting predicted dynamics point to an increase in the total amount of TYLCV. Therefore, the total amount of disease at epidemiological equilibrium would be higher than if the severe strain had wiped out the mild strain. The molecular basis of the assistance conferred by TYLCV-IL to TYLCV-Mld remains unknown. Heteroencapsidation would be the prime suspect if the two strains differed genetically in the capsid genes (V1 ORF), but they differ mainly in the C4 ORF [21]. Interestingly, the C4 ORF of TYLCV-IL is better than the C4 ORF of TYLCV-Mld at suppressing gene silencing (a key antiviral defence of the host plant) [39]. Thus, in co-infection, the C4 ORF of the IL strain might transcomplement the Mld genome, which might explain why the Mld strain accumulates more in the presence of the IL strain. Directly testing this hypothesis is an interesting research perspective.

## (c) Transmission bottleneck for a circulative virus

In addition to their major role in the dissemination of viruses, vectors also impact the evolutionary dynamics of viral populations, through selection and genetic drift. However, to the best of our knowledge, there are only two formal estimates of the bottleneck size during vector transmission, both for non-circulative plant viruses [40,41]. Here, we provide the first estimate of the number of effectively transmitted viral genomes for a circulative non-propagative virus, and we show that this number is twice as high for the IL strain as for the Mld strain. But the most striking feature of the transmission process is the huge bottleneck between the seven million viral genomes quantified in the vector and the one and two effectively transmitted viral genomes for the two strains. These estimates are surprisingly low, and similar to those calculated for viruses transmitted in a non-circulative manner [40,41]. Indeed, applying our estimation approach to the data published by Moury *et al*. [40] and Betancourt *et al*. [41] provided estimates of 1.03 for potato virus Y and 2.78 for cucumber mosaic virus (not shown), which are close to the estimates provided by the authors. This third estimate of the effective number of viral particles transmitted by an insect vector confirms that plant viruses are submitted to narrow population bottlenecks during the horizontal transmission process, even in the circulative transmission pathway.

## (d) Modelling the long-term coexistence of the Mld and IL strains

In our models, all estimates of the relative success of the two strains ($R_0$ ratio, relative prevalence, selection coefficient) indicate that the IL strain is much fitter than the Mld strain when co-infections are rare. Given that the two strains share the same host, applying the ecological principle of competitive exclusion [6] in a naive way would predict the complete displacement of TYLCV-Mld by TYLCV-IL. Using a frequency-independent pay-off matrix in the context of evolutionary game theory [11,42,43] would also predict that the Mld strain should have gone the way of the dodo, because the IL strain was shown to be fitter than the Mld strain both in an IL population and in a Mld population. However, density and frequency dependence, as well as complex population dynamics, preclude using simple fitness estimates [32]. Our experimental results imply that the mean fitness of each genotype depends on the

proportion of mixed infections (i.e. on the frequency of the two genotypes in the host population), which varies over time. The relative fitness of the two strains thus also varies over time. Indeed, although $s$ remained relatively constant throughout the first years of the epidemics in the field ($R^2 = 0.94$ in figure 4 inset), the observed concavity of $\ln(RR)$ versus time points to a decreasing selective advantage of the IL strain over time. In the presence of frequency-dependent selection, making even qualitative predictions concerning the long-term dynamics of two strains is no trivial task. The epidemiological model indicates that the two strains should, in fact, coexist in the long run, which is at odds with what one would intuitively conclude from their estimated relative fitness in the single infections. This rare documented case of frequency-dependent selection highlights the interest of epidemiological models parametrized with the experimental transmission probabilities of the co-occurring genotypes measured in single and mixed infections. Note that, as in many population genetics models, changes in allele frequencies are assumed to occur much faster than the production of new genotypes. In the long term, such new (mutant or recombinant) genotypes might alter the current dynamics and might even evolve new types of interactions such as antagonism or synergy.

In conclusion, while most virology research has traditionally focused on the properties of individual virus species, our study highlights the complex interplay between two strains of a pathogen, where unilateral facilitation is responsible for the epidemiological assistance and maintenance of the less fit strain.

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