Application of game theory to the interaction between plant viruses during mixed infections

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Natural mixed infections of plant viruses are frequent, often leading to unpredictable variations in symptoms, infectivity, accumulation and/or vector transmissibility. Cauliflower mosaic caulimovirus (CaMV) has often been found in mixed infections with turnip mosaic potyvirus (TuMV) in plants of the genus Brassica. This study addressed the effect of mixed infection on infectivity, pathogenicity and accumulation of CaMV and TuMV in Arabidopsis thaliana plants inoculated mechanically with cDNA infectious clones. In singly infected plants, TuMV accumulation was approximately 8-fold higher than that of CaMV. In co-infected plants, there was 77 % more TuMV accumulation compared with single infections, whilst the accumulation of CaMV was 56 % lower. This outcome describes a biological game in which TuMV always plays the winner strategy, leading to the competitive exclusion of CaMV. However, the infectivity of each virus was not affected by the presence of the other, and no symptom synergism was observed.

INTRODUCTION

Infection of plants by two or more viruses is frequent in nature (Matthews, 1991) and has variable consequences, ranging from symptom amelioration to synergistic exacerbation (Hammond et al., 1999). Mixed infections can also modify viral traits such as host range (Guerini & Murphy, 1999; Hacker & Fowler, 2000; García-Cano et al., 2006), transmission rate (Rochow, 1970; Kuhn & Dawson, 1973; Winternmantel et al., 2008), cellular tropism (Wege & Siegmund, 2007) and titre. Most studies have focused on synergistic diseases caused by two single-stranded (ss) DNA or ssRNA viruses, particularly by a member of the genus Potyvirus and another ssRNA virus. In most instances, the titre of the non-potyvirus increases, whilst that of the potyvirus is not altered (Wang et al., 2002; Murphy & Bowen, 2006; Taiwo et al., 2007). This enhancement has been explained by potyvirus HC-Pro-mediated RNA-silencing suppression (Pruss et al., 1997). Nevertheless, these interactions do not always produce synergistic diseases (Wang et al., 2004; Untiveros et al., 2007) and, depending on the particular combination of virus species, accumulation of the counterpart virus may also decrease (Kokkinos & Clark, 2006).

Interactions between DNA and RNA viruses have received less attention, but they also have unpredictable results depending on the species or strains involved (Hii et al., 2002; Kokkinos, 2006; Pohl & Wege, 2007; Wege & Siegmund, 2007). Cauliflower mosaic caulimovirus (CaMV) has a double-stranded DNA genome and is frequently found in mixed infections with the ssRNA turnip mosaic potyvirus (TuMV), particularly in plants of the genus Brassica (Spak & Novikov, 1994; Raybould et al., 1999), which may or may not lead to symptom synergism (Hunter et al., 2002; Spence et al., 2007). Strikingly, in Brassica perviridis, CaMV suppresses TuMV accumulation (Kamei et al., 1969), probably reflecting host and/or virus strain influence in the dynamic of the mixed infection.

In mixed infections, each viral population changes the environment and becomes part of the fitness landscape of the co-infecting population. For example, in mixed infections involving a potyvirus, HC-Pro-mediated silencing suppression subverts host defences, facilitating infection by other viruses. Therefore, in mixed infections, the fitness of each virus depends not only on its adaptation to the host, but also on the influence of its counterparts in a frequency-dependent manner. These kinds of interdependent interaction can be seen as a sort of biological game and therefore can be conveniently modelled and analysed by using the mathematical framework provided by game theory (Nowak & Sigmund, 2004). Game theory means that the fitness of individuals in the population is not constant, but depends on the frequencies of different phenotypes (Nowak & Sigmund, 2004; Nowak, 2006). The theory considers a population of players interacting according to the rules of a game. When involved in the game, each player has a fixed strategy that they practice when interacting randomly with other players. Given that resources are limited, and therefore population growth is density-dependent, the fitness (or pay-off, in the jargon of the theory) of a given individual depends on what strategy they are using against all other individuals in the population. Table 1 shows the general expected pay-off matrix for a two-player, two-strategy game. Briefly, in such a frequency-dependent interaction can be seen as a sort of biological game and therefore can be conveniently modelled and analysed by using the mathematical framework provided by game theory (Nowak & Sigmund, 2004). Game theory means that the fitness of individuals in the population is not constant, but depends on the frequencies of different phenotypes (Nowak & Sigmund, 2004; Nowak, 2006). The theory considers a population of players interacting according to the rules of a game. When involved in the game, each player has a fixed strategy that they practice when interacting randomly with other players. Given that resources are limited, and therefore population growth is density-dependent, the fitness (or pay-off, in the jargon of the theory) of a given individual depends on what strategy they are using against all other individuals in the population. Table 1 shows the general expected pay-off matrix for a two-player, two-strategy game. Briefly, in such
games, each player has a different fitness depending on the frequency of the competing strategy in the population. The entries in the matrix denote the fitness of the player in each row, i.e. player A has fitness $a$ playing with another A, but fitness $b$ when playing against a B player; player B has fitness $c$ when facing player A, but fitness $d$ when facing another B individual. In a well-mixed population (i.e. no spatial structure exists in the system and thus all encounters are equally likely to happen), the following four outcomes are possible. (i) If $a>c$ and $b>d$, then strategy A is the best to compete against both A and B players; thus, player A will dominate the population. (ii) If $a<c$ and $b<d$, the situation is reversed and B dominates. (iii) If $a>c$ but $b<d$, then strategy A is better when competing against player A, but strategy B is better when playing with B—a situation defining a coordinated game in which it is always better to mimic the competitor’s strategy and resulting in a monomorphic population (a situation also known as strict Nash equilibrium). Finally, (iv) if $a<c$ and $b>d$, both strategies are equally competitive, defining a hawk–dove game that leads to the co-existence of both players.

In this work, we addressed the effect of mixed infections on the accumulation, infectivity and symptoms of CaMV and TuMV infection in Arabidopsis thaliana and applied the basic rules of game theory to make predictions about the long-term results of TuMV–CaMV interactions.

### METHODS

#### Infectious plasmids.

For infection of A. thaliana plants with TuMV, we used the p35STunos infectious clone (Sánchez et al., 1998). CaMV infections were carried out with infectious clone pCaMVW260 (Schoelz & Shepherd, 1998). Both clones have been described previously. To prepare a standard for TuMV quantification (see below), we used the pT7Tu clone, a version of p35STunos carrying the T7 promoter instead of the 35S promoter upstream of the TuMV genome (Sánchez et al., 1998).

#### Plants and inoculation procedures.

One-month-old seedlings of A. thaliana strain Col-0 were inoculated with TuMV p35STunos and/or CaMV pCaMVW260 infectious cDNA clones prepared with a PureYield Plasmid Maxiprep kit (Promega). Prior to inoculation, the DNA concentration was adjusted to approximately 350 ng ml$^{-1}$ with water and mixed with carborundum (10 mg ml$^{-1}$). Single infections were established by applying 1.36 x 10$^{11}$ molecules of each infectious clone. A total of 17 plants was inoculated with p35STunos, 20 with pCaMVW260 and 20 co-inoculated with the mixture of both clones, using a glass rod to spread the inoculum. Plants were maintained at 16:8 h light:dark and 24:20 °C day/night temperature until sample collection at 14 days post-infection (p.i.). After collection, plants were weighed, inoculated leaves were removed and the rest were ground into fine powder, split into aliquots and stored at $-80\,^\circ$C.

#### Nucleic acid extraction.

For quantitative PCR (qPCR) and RT-qPCR assays, nucleic acids from up to 100 mg tissue were purified by using DNeasy Plant Mini and/or RNeasy Plant Mini kits (Qiagen), respectively, following the manufacturer’s instructions.

#### RT-qPCR and qPCR assays.

To prepare the standard for TuMV quantification, a full-genome transcript of clone pT7Tu was synthesized using T7 RNA polymerase (Roche). Template DNA was removed using a TURBO DNA-free kit (Ambion). Unincorporated NTPs, products of template degradation and enzymes were removed using an RNeasy Plant Mini kit (Qiagen). The transcription product was visualized on a 1% agarose gel and its concentration and purity were determined spectrophotometrically. A maxiprep of pCaMVW260 was used as a standard for CaMV quantification. To ensure reproducibility of the standard curves, a single preparation of each standard was prepared.

To avoid the introduction of experimental bias in single versus mixed infections and to ensure each extraction round included samples of both types of inoculation. After purification, nucleic acids were quantified spectrophotometrically in triplicate. Typical yields of nucleic acid extractions from A. thaliana plants were 3.6-4.1 μg DNA per 100 mg fresh tissue and 35 μg RNA per 100 mg fresh tissue (DNeasy and RNase Plant minikits; Qiagen). Therefore, to account for this difference in yield and to express viral loads in comparable units, TuMV accumulation was expressed as the number of viral RNA molecules in 100 ng total RNA, whereas the accumulation of CaMV was expressed as the number of DNA molecules in 10 ng total DNA.

To titrate TuMV, total plant RNA extracts were treated with DNase and their concentration was adjusted to 100 ng μl$^{-1}$ with a TURBO DNA-free kit (Ambion). Aliquots of 1 μl treated RNA were reverse-transcribed in three independent reactions with TaqMan Reverse Transcription Reagents (Applied Biosystems). To construct a standard curve, equal volumes of six serial dilutions were also included in each plate (concentrations of 5.72 x 10$^2$–1.79 x 10$^9$ molecules μl$^{-1}$). To ensure comparable amplification dynamics of standards and samples, dilutions of the transcript were carried out using DNase-treated RNA (100 ng μl$^{-1}$) from healthy plants. Reaction volumes were set up in a volume of 20 μl and an oligo(dT)$_{16}$ primer was used to avoid the reverse transcription of incomplete genomes. Each cDNA was amplified in a separate reaction containing: 1 μl cDNA reaction, 1 x Power SYBR PCR Master Mix (Applied Biosystems) and 50 nM of the primers qTuMV-F (5’-GGGACTCAAGAAGGCAAGG-3’) and qTuMV-R (5’-TTGTCGCCGTTTTCCCTCTTC-3’). For CaMV quantification, sample DNA concentrations were adjusted to 10 ng μl$^{-1}$. Each DNA was amplified in three separate reactions using primers Ftaqcons-F (5’-GATCTCTTGAAAACCCTAAAGCT-3’) and Ftaqcons-R (5’-RTGYCKGCTCT-AAATTTGATCC-3’). Standard DNA was prepared by diluting pCaMVW260 in DNA extracts from healthy plants at 10 ng μl$^{-1}$ (pCaMVW260 concentrations of 1.20 x 10$^2$–3.74 x 10$^8$ molecules μl$^{-1}$). Amplification, data acquisition and analysis were carried out using an Applied Biosystems Prism 7000 or 7005 sequence detection system.

For all runs, linear regression of the threshold cycle ($C_t$) with the log-transformed number of molecules had $R^2$>0.994, and sample $C_t$ values were within the dynamic range of amplification. The efficiency of RT-qPCR reactions was 73.3–74.8 % for different runs, whereas for qPCR, efficiencies were 77.3–77.8 %. Minute differences among plates did not affect quantification because sample $C_t$ values were

### Table 1. General pay-off matrix for the interaction between strategies A (focal) and B (opponent)

<table>
<thead>
<tr>
<th>Focal</th>
<th>Opponent</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td>$a$</td>
<td>$b$</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>$c$</td>
<td>$d$</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

Co-infection does not alter infectivity of CaMV and TuMV or symptoms of infection

First, we looked for any effect of co-infection on the efficiency of infecting Arabidopsis thaliana plants. Infection was measured as the ratio between the number of infected plants determined by RT-qPCR and qPCR compared with the number of inoculated plants. Table 2 shows the results of infectivity tests for both viruses in single-inoculation and in co-inoculation experiments. The infectivity of TuMV as estimated from single-inoculation experiments was 0.895 ± 0.149, whereas that of CaMV was 0.864 ± 0.149 (in both cases, the Laplace point estimator for small samples has been used, ±95% confidence interval). Thus, under our inoculation conditions, both clones had the same ability to establish systemic infection. Using these two figures, it was possible to test whether the observed distribution of cases (Table 2) departed significantly from the null hypothesis of independent action. A goodness-of-fit test failed to reject the null hypothesis ($\chi^2 = 2.188$, 2 degrees of freedom, $P = 0.335$) and therefore we concluded that these two viruses did not interfere with each other during the early stages of the infection process.

Next, we sought to analyse the effect of mixed infections on the symptoms. To do this, the fresh weight of ten mock-inoculated plants, both singly infected and co-infected, was recorded at 14 days post-inoculation (dpi). Means ± SEM were computed and the significance of differences was assessed by a Tukey post-hoc test. Control and CaMV-inoculated plants were found to have statistically homogeneous weights ($P = 0.052$), despite the fact that CaMV-infected plants were, on average, 17.9% lighter (2.558 ± 0.139 g) than healthy plants (3.115 ± 0.250 g). In contrast, co-infected and TuMV-inoculated plants were homogeneous ($P = 0.983$) and different from the other group, with TuMV-infected plants being 43.2% lighter (1.768 ± 0.145 g) than control plants and co-infected plants being 4.4% lighter (1.690 ± 0.088 g) than TuMV plants (although this difference was not significant). Therefore, the strength of symptoms in co-infected plants was driven by TuMV and was not influenced significantly by the presence of CaMV.

Table 2. Infectivity of TuMV and CaMV in experiments of single and mixed infections

<table>
<thead>
<tr>
<th>Infection</th>
<th>TuMV</th>
<th>CaMV</th>
<th>TuMV and CaMV</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single</td>
<td>16/17</td>
<td>18/20</td>
<td>3/37</td>
<td></td>
</tr>
<tr>
<td>Mixed</td>
<td>1/20</td>
<td>0/20</td>
<td>19/20</td>
<td>0/20</td>
</tr>
</tbody>
</table>

Co-infection exerts opposite effects on TuMV and CaMV accumulation

Detection techniques used in most previous reports on mixed infections have been aimed at comparing the accumulation of each competing virus in singly versus co-infected plants, but to our knowledge, relative accumulation among competing viruses has been estimated only in a few instances (Scheets, 1998; Kokkinos & Clark, 2006; Zeng et al., 2007; Wintemantel et al., 2008). To determine an estimate of the relative fitness of our competing viruses, TuMV was titrated by RT-qPCR and CaMV by qPCR using an absolute quantification method. These techniques have been used to measure and to allow a comparison of viral load, as it is expressed as the number of genomes relative to total nucleic acid (Bustin, 2000; Dhar et al., 2008).

Fig. 1 shows the mean ± SEM virus load for each virus in single and mixed infections. In single infections, TuMV accumulated $1.865 \times 10^6 \pm 0.897 \times 10^6$ molecules, whereas CaMV accumulation was 7.7-fold lower ($2.411 \times 10^5 \pm 0.167 \times 10^5$ molecules). In mixed infections, TuMV load was $3.307 \times 10^6 \pm 0.125 \times 10^6$ molecules, a value that was 77.3% higher than that obtained from TuMV single infections (Mann–Whitney test: $P < 0.001$). By contrast, the mean load of CaMV in mixed infections was $1.068 \times 10^6 \pm 0.092 \times 10^6$ molecules; i.e. 55.7% lower than the corresponding value estimated from CaMV single infections (Mann–Whitney test: $P < 0.001$).

Prior to performing the experiments described here, we expected an increase in CaMV accumulation in doubly infected plants. The rationale for this expectation was

![Viral load (molecules)](http://vir.sgmjournals.org)
grounded in two premises: (i) the above-mentioned beneficial effect exerted by HC-Pro on the accumulation of co-infecting viruses, and (ii) a previous report of CaMV displacing TuMV in B. perviridis (Kamei et al., 1969). This expectation proved to be too naïve and here we provide evidence that, in co-infected A. thaliana plants, CaMV accumulated to a lower level, whilst TuMV accumulation was enhanced significantly as a direct result of the interaction. The question that remains is: what are the molecular determinants for this interaction? CaMV encodes its own silencing suppressor, P6, which has a different mechanism of action from HC-Pro. Whilst HC-Pro binds small interfering RNAs (siRNAs), sequestering them from the RNA-induced silencing complex (Lakatos et al., 2006), P6 interacts with DRB4, a nuclear protein that facilitates DCL4 antiviral activity (Haas et al., 2008), and may not sequester siRNAs (Love et al., 2007). In addition, HC-Pro suppresses local silencing (Mallory et al., 2001), whereas P6 suppresses both local and systemic silencing and may also play other roles in defence suppression, such as inhibition of ethylene signalling, sensitivity to auxin and gene expression in response to salicylic acid (Love et al., 2007). All of these may contribute to enhance TuMV local replication and colonization of distal parts of the plant. Therefore, the observed reduced accumulation of CaMV in mixed infections could be explained by two non-exclusive mechanisms: (i) competitive exclusion if TuMV uses shared resources more efficiently than CaMV, which is supported by the higher accumulation of TuMV compared with CaMV in single infections, and/or (ii) by TuMV triggering host responses affecting CaMV to a greater extent.

Our data showed that, when co-inoculated at equal concentrations, these two unrelated viruses influenced each other asymmetrically: whilst TuMV behaved as a defector and benefited from the presence of CaMV, increasing its accumulation significantly, CaMV behaved as a candid cooperator and paid a fitness penalty by accumulating at significantly lower levels. Several prior studies on animal viruses have showed that initial conditions, such as the relative frequency of the two viruses at inoculation, the temporal order of inoculation (co-infection or superinfection) and the spacing and order between superinfection events, affect the outcome of mixed infections (Alonso et al., 1999; Miralles et al., 2001; Carrillo et al., 2007). Hence, it would be of great interest to study the interaction of CaMV and TuMV after different schemes of inoculation, especially superinfection, which is the most likely case in natural infections. These additional studies would also shed light on the underlying mechanisms that determine the result of the interaction between TuMV and CaMV in A. thaliana.

### Biological game between TuMV and CaMV predicts exclusion of CaMV

To construct the pay-off matrix for TuMV and CaMV interaction, relative fitness was computed as the ratio of the number of molecules accumulated in each case compared with the number obtained for CaMV in single infections (Table 3). Pay-off values showed that TuMV always showed higher fitness than CaMV, either competing with CaMV ($c=13.718 \pm 0.518 > a=1.000 \pm 0.069$) or against itself ($d=7.738 \pm 0.372 > b=0.443 \pm 0.038$); hence, the CaMV strategy is unstable and will always be outcompeted, rendering a strict Nash monomorphic equilibrium.

A different special case for the situation observed here is the well-known prisoner’s dilemma (Rapoport & Chammah, 1965; Axelrod, 1984). This concept was first introduced into virology by Turner & Chao (1999) to describe the outcome of within-cell interactions between different genotypes of bacteriophage $\phi 6$. In this game, the defector reaches its highest fitness by exploiting the cooperator, whilst the latter pays the highest penalty when interacting with the defector (i.e. $c>b$), as is the case for TuMV and CaMV interaction. However, the interaction between TuMV and CaMV departed from a prisoner’s dilemma because the fitness of TuMV in a single infection was still higher than that of CaMV ($d>a$), and a prisoner’s dilemma requires a cost for mutual defection.

Although game theory has been applied to several biological problems, it has not received much attention from virologists. However, just focusing on plant viruses, the same interaction described here has been observed for several other pairs of viruses (Hii et al., 2002; Kokkinos & Clark, 2006; Zeng et al., 2007; Wintermantel et al., 2008). In other interactions, however, both viruses gain an advantage (Scheets, 1998; Hii et al., 2002), both viruses pay a cost (Wintermantel et al., 2008), one of them gains whereas the other is not apparently affected (Wang et al., 2002, 2004; Wege & Siegmund, 2007) or one pays a cost whilst the other remains unaffected (Pohl & Wege, 2007).

As mixed viral infections are frequent in nature and the fitness of each virus depends not only on its own strategy, but also on that of its counterpart, game theory offers a valuable tool for studying such interactions.

### Table 3. Observed pay-off matrix (relative accumulation $\pm 1\text{SEM}$) for the interaction between CaMV (focal) and TuMV (opponent) during mixed infections

<table>
<thead>
<tr>
<th>Focal</th>
<th>Opponent</th>
<th>TuMV</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaMV</td>
<td>1.000 ± 0.069</td>
<td>0.443 ± 0.038</td>
</tr>
<tr>
<td>TuMV</td>
<td>13.718 ± 0.518</td>
<td>7.738 ± 0.372</td>
</tr>
</tbody>
</table>

### ACKNOWLEDGEMENTS

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