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1 # Application of Game Theory to the interaction between
2 # plant viruses during mixed infections

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17 Running title: Games plant viruses play

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19 147 words in the abstract, 3073 words in the main text, 1 figure and 3 tables.

20 **ABSTRACT**

21

22 Natural mixed infections of plant viruses are frequent, often leading to
23 unpredictable variations in symptoms, infectivity, accumulation and/or vector
24 transmissibility. *Cauliflower mosaic caulimovirus* (CaMV) has been often found in
25 mixed infections with *Turnip mosaic potyvirus* (TuMV) in plants of the genus
26 *Brassica*. In this work we have addressed the effect of mixed infection in
27 infectivity, pathogenecity and accumulation of CaMV and TuMV in *Arabidopsis*
28 *thaliana* plants mechanically inoculated with cDNA infectious clones. In singly
29 infected plants TuMV accumulation was approximately 8-fold higher than that of
30 CaMV. In coinfected plants TuMV accumulated 77% more than in single
31 infections, while the accumulation of CaMV was 56% lower. This outcome
32 describes a biological game in which TuMV always plays the winner strategy,
33 leading to the competitive exclusion of CaMV. However, the infectivity for each
34 virus was not affected by the presence of the other and no symptom synergism
35 was observed.

36

36 **INTRODUCTION**

37

38 Infections of plants by two or more viruses are frequent in nature (Matthews,
39 1991) and have variable consequences, ranging from symptom amelioration to
40 synergistic exacerbation (Hammond *et al.*, 1999). Mixed infections can also modify
41 viral traits such as host range (Guerini & Murphy, 1999; Hacker & Fowler, 2000;
42 García-Cano *et al*., 2006), transmission rate (Rochow, 1970; Kuhn & Dawson, 1973;
43 Wintermantel *et al*., 2008), cellular tropism (Wege & Siegmund, 2007), or titer.
44 Most studies have focused on synergic diseases caused by two ssDNA virus or
45 ssRNA; particularly by a *Potyvirus* and other ssRNA virus. In most instances, the
46 titer of the non-potyvirus increases while that of the potyvirus is not altered
47 (Wang *et al*., 2002; Murphy & Bowen, 2006; Taiwo *et al*., 2007). This enhancement
48 has been explained by potyvirus HC-Pro-mediated RNA silencing suppression
49 (Pruss *et al*., 1997). Nevertheless these interactions not always produce synergic
50 diseases (Wang *et al*., 2004; Untiveros *et al*., 2007), and depending on the particular
51 combination of virus species, accumulation of the counterpart can also decrease
52 (Kokkinos & Clark, 2006).

53 Interaction between DNA and RNA viruses has received less attention, but it also
54 has unpredictable results depending on the species or strains involved (Hii *et al*.,
55 2002; Kokkinos, 2006; Pohl & Wege, 2007; Wege & Siegmund, 2007). *Cauliflower*
56 *mosaic caulimovirus* (CaMV) has a dsDNA genome, and is frequently found in
57 mixed infections with the ssRNA *Turnip mosaic potyvirus* (TuMV), particularly in
58 plants of the genus *Brassica* (Spak & Novikov, 1994; Raybould *et al*., 1999) leading
59 or not to symptom synergism (Hunter *et al*., 2002; Spence *et al*., 2007). Strikingly,

60 in *Brassica pervidis*, CaMV suppresses TuMV accumulation (Kamei *et al.*, 1969),
61 probably reflecting host and/or viral strain influence in the dynamic of the mixed
62 infections.

63 In mixed infections, each viral population changes the environment and becomes
64 part of the fitness landscape of the co-infecting population. For example in mixed
65 infections involving a potyvirus, HC-Pro-mediated silencing suppression subverts
66 host defenses facilitating infection by other viruses. Therefore, in mixed
67 infections, the fitness of each virus does not only depend on its adaptation to the
68 host, but also on the influence of its counterparts in a frequency-dependent
69 manner. These kinds of interdependent interactions can be seen as a sort of
70 biological games and, therefore, can be conveniently modeled and analyzed using
71 the mathematical framework provided by the Game Theory (Nowak & Sigmund,
72 2004). Game Theory means that the fitness of individuals in the population is not
73 constant but depends on the frequencies of different phenotypes (Nowak &
74 Sigmund 2004; Nowak, 2006). The theory considers a population of players
75 interacting according to the rules of a game. When involved in the game, each
76 player has a fixed strategy that practices when randomly interacting with other
77 players. Given that resources are limited, and therefore population growth is
78 density-dependent, the fitness (or payoff, in the jargon of the theory) of a given
79 individual depends on what strategy is playing against all other individuals in the
80 population. Table 1 shows the general expected payoff matrix for a two-player
81 two-strategy game. Briefly, in such games each player gets different fitness
82 depending on the frequency of the competing strategy in the population. The
83 entries of the matrix denote the fitness of the row player, i.e., player A has a fitness

84 *a* playing with another A but *b* when playing against a B player; player B has
85 fitness *c* when opposed to player A but *d* when facing another B individual. In a
86 well mixed population (i.e., no spatial structure exists in the system and thus all
87 encounters are equally likely to happen), the following four outcomes are possible:
88 *i*) If $a > c$ and $b > d$, then strategy A is the best to compete both against A and B
89 players, thus player A will dominate the population. *ii*) If $a < c$ and $b < d$, the
90 situation is reversed and B dominates. *iii*) If $a > c$ but $b < d$, then strategy A is
91 better when competing against player A but strategy B is better when playing with
92 B; a situation defining a coordinated game in which it is always better to mimic
93 the competitor's strategy and results in a monomorphic population (a situation
94 also known as strict Nash equilibrium). Finally, *iv*) if $a < c$ and $b > d$, both
95 strategies are the best competing to each other; defining a Hawk-Dove game that
96 leads to the co-existence of both players.
97 In this work we have addressed the effect of mixed infections in the accumulation,
98 infectivity and symptoms of CaMV and TuMV in *Arabidopsis thaliana* and applied
99 the basic formalism from Game Theory to make predictions about the long term
100 output of TuMV-CaMV interaction.
101
102 **METHODS**
103
104 **Infectious plasmids.** For infecting *A. thaliana* plants with TuMV, we used the
105 p35STunos infectious clone (Sánchez *et al.*, 1998). CaMV infections were started
106 with infectious clone pCaMVW260 (Schloelz & Shepherd, 1998). Both clones have
107 been described elsewhere. To prepare the standard for TuMV quantification (see

108 below) we used pT7Tu clone, a version of p35STunos carrying the T7 promoter
109 instead of the 35S promoter upstream TuMV genome (Sánchez *et al.*, 1998).
110 **Plants and inoculation procedures.** One month old seedlings of *A. thaliana* Col-0
111 were inoculated with TuMV p35STunos and/or CaMV pCaMVW260 infectious
112 cDNA clones prepared with PureYield™ Plasmid Maxiprep (Promega). Prior to
113 inoculation DNA concentration was adjusted to approximately 350 ng ml^{-1} with
114 water and mixed with carborumdun 10 mg ml^{-1}. Single infections were
115 established applying 1.36×10^{11} molecules of p35STunos or pCaMVW260 to each
116 of four leaves per plant. For mixed infections, leafs were inoculated with $1.36 \times$
117 10^{11} molecules of each infectious clone. A total of 17 plants were inoculated with
118 p35STunos, 20 with pCaMVW260 and 20 coinoculated with the mixture of both
119 clones, using a glass rod to spread the inoculum. Plants were maintained at 16 h
120 light, 24 ºC/20 ºC, day/night temperature until sample collection 14 dpi. After
121 collection, plants were weighted, inoculated leafs removed, and the rest grinded
122 into fine powder, split into aliquots and stored at –80 ºC.
123 **Nucleic acids extraction.** For RT-qPCR and qPCR assays, nucleic acids from up to
124 100 mg of tissue were purified using RNeasy® Plant Mini and/or the DNeasy®
125 Plant Mini Kits (Qiagen), respectively, following the manufacturer's instructions.
126 **RT-qPCR and qPCR assays.** To prepare the standard for TuMV quantification, a
127 full genome transcript of pT7Tu clone was synthesized using T7 RNA polymerase
128 (Roche). Template DNA was removed using TURBO DNA-free™ (Ambion).
129 Non-incorporated NTPs, products of template degradation and enzymes were
130 removed using RNeasy® Plant Mini Kit (Ambion). The transcription product was
131 visualized in agarose 1% gels and its concentration and purity were

132 spectrophotometrically determined. As standard for CaMV quantification, a
133 maxiprep of pCaMVW260 was used. To ensure reproducibility of the standard
134 curves, a single preparation of each standard was prepared.
135 To avoid introduction of experimental bias in single versus mixed infection
136 comparisons, each extraction round included samples of both types of inoculation.
137 After purification, nucleic acids were spectrophotometrically quantified by
138 triplicate. Typical yields of nucleic acids extractions from *A. thaliana* plants ranged
139 between 3.6 and 4.1 μg DNA per 100 mg of fresh tissue and 35 μg RNA per 100 mg
140 (DNeasy® and RNeasy® plant minikit handbooks, Qiagen). Therefore, to account
141 for this difference in yield and to express viral loads in comparable units, TuMV
142 accumulation was expressed as the number of viral RNA molecules in 100 ng of
143 total RNA whereas the accumulation of CaMV was expressed as the number of
144 DNA molecules in 10 ng of total DNA.
145 To titer TuMV, total plant RNA extracts were treated with DNase and their
146 concentration adjusted to 100 ng μl^{-1} with TURBO DNA-free™ (Ambion).
147 Aliquots of 1 μl of treated RNA were reverse-transcribed in three independent
148 reactions with TaqMan® (Applied Biosystems). To construct the standard curve,
149 equal volumes of six serial dilutions were also included in each plate
150 (concentrations: 5.72×10^5 - 1.79×10^9 molecules μl^{-1}). To ensure comparable
151 amplification dynamics of standards and samples, dilutions of the transcript were
152 performed in DNase-treated RNA (100 ng μl^{-1}) from healthy plants. Reaction
153 volumes were set up to 20 μl and an oligo $d(T)_{16}$ was used as primer to avoid the
154 reverse transcription of incomplete genomes. Each cDNA was amplified in a 20 μl
155 separate reaction containing: 1 μl cDNA reaction, 1× Power SYBR® PCR Master

156 Mix (Applied Biosystems) and 50 nM of the primers qTuMV-F 5'-
157 GGCACTCAAGAAAGGCAAGG-3′ and qTuMV-R 5'-
158 TTGTCGCGTTTTCCCTCTTC-3′. For CaMV quantification, sample DNA
159 concentrations were adjusted to 10 ng μl^{-1}. Each DNA was amplified in three
160 separate reactions using primers Ftaqcons-F 5'-
161 GATCCTCTGGAAACCCTAAAGCT-3′ and Ftaqcons-R 5'-
162 RGTYCKGTCTAAATTGATTC-3′. Standard DNA was prepared diluting
163 pCaMVW260 in DNA extracts from healthy plants at 10 ng μl^{-1} (pCaMVW260
164 concentrations: 1.20×10^5 - 3.74×10^8 molecules μl^{-1}). Amplification, data
165 acquisition and analysis were done using Applied Biosystems Prism™ 7000 or
166 7005 sequence detection systems.

167 For all runs, linear regression of the threshold cycle (C_t) with the log-transformed
168 number of molecules had $R^2 > 0.994$ and sample C_t values were within the
169 dynamic range of amplification. Efficiencies of RT-qPCR runs were of 73.3% and
170 74.8%, and of qPCR 77.8% and 77.3%. Minute differences among plates do not
171 impact quantification because sample C_t values are interpolated in standard
172 curves performed in the same plate. To evaluate the reproducibility of the assays,
173 the coefficient of variation between RT-qPCR or qPCR replicates of standard
174 dilutions were calculated, confirming that both protocols had world class
175 reproducibility (1.323% ± 0.307 and 1.606% ± 0.245, respectively).

176 **Statistical analyses.** All statistical analyses were performed with SPSS version
177 16.0.

178

179 **RESULTS AND DISCUSSION**

180

181 **Coinfection does not alter infectivity and symptoms of CaMV and TuMV**

182 First, we sought for any effect of coinfection on the efficiency of infecting *A.*
183 *thaliana* plants. Infectivity was measured as the ratio between the number of
184 infected plants determined by RT-qPCR and qPCR over the number of inoculated
185 plants. Table 2 shows the results of infectivity tests for both viruses in single
186 inoculation and in coinoculation experiments. The infectivity of TuMV, estimated
187 from single inoculation experiments was 0.895 ± 0.149, whereas the infectivity of
188 CaMV, was 0.864 ± 0.149 (in both cases the Laplace point estimator for small
189 samples has been used; ± 95% CI), thus in our inoculation conditions both clones
190 had the same ability to establish systemic infections. Using these two figures, it is
191 possible testing whether the observed distribution of cases (Table 2) significantly
192 departs from the null hypothesis of independent action. A goodness of fit test fails
193 to reject the null hypothesis (χ^2 = 2.188, 2 d.f., P = 0.335) and, therefore, we can
194 conclude that these two viruses do not interfere each other at the early stages of
195 the infection process.

196 Next we sought to analyze the effect of mixed infections in the symptoms. To do
197 so, the fresh weight of 10 mock-inoculated plants, singly infected and coinfected
198 plants was recorded 14 dpi, means and standard errors (SEM) were computed and
199 significance of differences assessed by a Tukey *post hoc* test. At the one side,
200 control and CaMV-inoculated plants have statistically homogeneous weights (P =
201 0.052), despite the fact that CaMV-infected plants were, on average 17.9% lighter
202 (2.558 ± 0.139 g) than healthy plants (3.115 ± 0.250 g). At the other side coinfected
203 and TuMV-inoculated plants were homogeneous (P = 0.983) and different from

204 the other group, with TuMV-infected plants being 43.2% lighter (1.768 ± 0.145 g)
205 than control plants and coinfected plants still being 4.4% smaller (1.690 ± 0.088 g)
206 than TuMV plants (although this difference was not significant). Therefore, the
207 strength of symptoms in coinfected plants was driven by TuMV and not
208 significantly influenced by the presence of CaMV.
209

210 **Coinfection exerts opposite effects on TuMV and CaMV accumulation**

211 Detection techniques used in most previous reports on mixed infections were
212 aimed to compare accumulation of each competing virus in singly versus
213 coinfected plants, but to our knowledge, relative accumulation among competing
214 viruses has been estimated only in a few instances (Scheets, 1998; Kokkinos &
215 Clarke 2006; Wintermantel *et al.*, 2008; Zeng *et al.*, 2007). To have an estimate of
216 the relative fitness of our competing viruses, TuMV was titered by reverse-
217 transcription followed by real-time PCR (RT-qPCR) and CaMV by qPCR using the
218 absolute quantification method. These techniques have been used to measure
219 viral load, and allow comparison of viral loads since it is expressed as number of
220 genomes relative to total nucleic acid (Bustin, 2000; Dhar *et al.*, 2008).
221 Figure 1 shows the mean virus load ± 1 SEM for each virus in single and mixed
222 infections. In single infections, TuMV accumulated $(1.865 \pm 0.897) \times 10^8$ molecules
223 whereas CaMV accumulation was 7.7-fold lower, $(2.411 \pm 0.167) \times 10^7$ molecules.
224 In mixed infections, TuMV load was $(3.307 \pm 0.125) \times 10^8$ molecules, a value that is
225 77.3% larger than the one obtained from TuMV-single infections (Mann-Whitney
226 test: $P < 0.001$). By contrast, the average load of CaMV in mixed infections was

227 (1.068 ± 0.092) × 10^7 molecules; i.e., 55.7% lower than the corresponding value
228 estimated from CaMV-single infections (Mann-Whitney test: $P < 0.001$).
229 Prior to performing the experiments here described, we expected an increase in
230 CaMV accumulation in doubly infected plants. The rational for this expectation
231 grounded in two premises: (*i*) the above mentioned beneficial effect exerted by
232 HC-Pro on the accumulation of coinfecting viruses and (*ii*) a previous report of
233 CaMV displacing TuMV in *B. pervidis* (Kamei *et al.*, 1969). This expectation proved
234 to be too naïve and here we provide evidences that in coinfected *A. thaliana* plants,
235 CaMV accumulated to a lesser level while TuMV accumulation was significantly
236 enhanced as a direct result of the interaction. The question that remains to be
237 answer is what the molecular determinants for this interaction are. CaMV encodes
238 for its own silencing suppressor, P6, which has a different mechanism of action
239 that HC-Pro. While HC-Pro binds siRNAs sequestering them from the RISC
240 (Lakatos *et al.*, 2006), P6 interacts with DRB4, a nuclear protein that facilitates
241 DCL4 antiviral activity (Haas *et al.*, 2008) and may not sequester siRNAs (Love *et*
242 *al.*, 2007). In addition, HC-Pro suppresses local silencing (Mallory *et al.,* 2001)
243 whereas P6 suppresses both local and systemic, and also may play other roles in
244 defense suppression, such as inhibition of ethylene signaling, sensitivity to auxin,
245 and gene expression in response to salicylic acid (Love *et al*., 2007). All these may
246 contribute to enhance TuMV local replication and colonization of distal parts of
247 the plant. Therefore, the observed reduced accumulation of CaMV in mixed
248 infections could be explained by two non-exclusive mechanisms, competitive
249 exclusion if TuMV uses shared resources more efficiently than CaMV, which is

250 supported by the higher accumulation of TuMV than CaMV in single infections,
251 and/or by TuMV triggering host responses affecting CaMV in a greater extent.
252 Our data showed that when coinoculated at equal concentrations, these two
253 unrelated viruses asymmetrically influence each other: whereas TuMV behaves as
254 a defector and benefits from the presence of CaMV, significantly increasing its
255 accumulation, CaMV behaves as a candid cooperator and pays a fitness penalty by
256 accumulating into significantly lower values. Nevertheless several prior studies
257 on animal viruses showed that initial conditions such as the relative frequency at
258 inoculation, the temporal order of inoculation (coinfection or superinfection) and
259 the spacing and order between superinfection events condition the outcome of
260 mixed infections (Alonso *et al.*, 1999; Miralles *et al.*, 2001; Carrillo *et al.*, 2007).
261 Hence, it would be of great interest to study the interaction of CaMV and TuMV
262 after different schemes of inoculation, especially superinfection which is the most
263 likely case in natural infections. These additional studies would also shed light on
264 the underlying mechanisms that determine the output of the interaction between
265 TuMV and CaMV in *A. thaliana*.
266

267 **Biological game between TuMV and CaMV predicts exclusion of CaMV**

268 To construct the pay-off matrix for TuMV and CaMV interaction, relative fitness
269 was computed as the ratio of the number of molecules accumulated in each case
270 and that obtained for CaMV in single infections (Table 3). Payoff values showed
271 that TuMV always shows higher fitness than CaMV, either competing with CaMV
272 ($c = 13.718 \pm 0.518 > a = 1.000 \pm 0.069$) or against itself ($d = 7.738 \pm 0.372 > b = 0.443$

273 ± 0.038), hence, CaMV strategy is unstable and will always be outcompeted,
274 rendering a strict Nash monomorphic equilibrium.

275 A different special case for the situation here observed is the well-known
276 prisoner's dilemma (Rapoport & Chammah 1965; Axelrod, 1984). This concept
277 was first introduced into virology by Turner & Chao (1999) to describe the
278 outcome of within-cell interactions between different genotypes of bacteriophage
279 ϕ6. In this game, the defector reaches its highest fitness by exploiting the
280 cooperator, while the latter pays the highest penalty when interacting with the
281 defector (i.e., $c > b$), as it is the case for TuMV and CaMV interaction. However,
282 the interaction between TuMV and CaMV departs from a prisoner's dilemma
283 because the fitness of TuMV in single infection is still higher than that of CaMV (d
284 $> a$), and a prisoner's dilemma requires a cost for mutual defection.

285 Although Game Theory has been applied to several biological problems, it has not
286 received too much attention from virologists. However, and just focusing in plant
287 viruses, the same interaction here described has been observed for several other
288 pairs of viruses (Hii *et al.*, 2002; Kokkinos & Clark 2006; Zeng *et al.*, 2007;
289 Wintermantel *et al.*, 2008). In other interactions, however, both viruses gain an
290 advantage (Scheets, 1998; Hii *et al.*, 2002), pay a cost (Wintermantel *et al.*, 2008),
291 one of them gains whereas the other is not apparently affected (Wang *et al.*, 2002,
292 2004; Wege & Siegmund, 2007), or one pays a cost while the other remains
293 unaffected (Pohl & Wege 2007). Since mixed viral infections are frequent in
294 nature, and the fitness of each virus depends not only on its own strategy but also
295 on that of its counterpart, Game Theory offers a valuable tool for studying such
296 interactions.

297

298 **ACKNOWLEDGEMENTS**

299

300 We thank R. Froissart (CNRS-INRA-CIRAD-ENSAM, Montpellier, France) for
301 comments, fruitful discussion, providing clone pCaMVW260, and the sequence of
302 primers Ftaqcons-F and Ftaqcons-R. F. Sánchez (INIA, Madrid, Spain) for
303 providing p35S-Tunos and pT7-Tu clones. A. Cuadrado and F. de la Iglesia for
304 excellent technical assistance. Helpful comments from our labmates and from
305 three anonymous reviewers are also welcomed. This work has been supported by
306 the Spanish MICINN grants BFU2006-14819-C02-01/BMC and HF2005-0284
307 (S.F.E.) and by a *Juan de la Cierva* contract (S.M.).

308

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436

Table 1: General payoff matrix for the interaction 437

between strategies A (focal) and B (opponent). 438

	Opponent	
Focal	**A**	**B**
A	*a*	*b*
B	*c*	*d*

438

Table 2: Infectivity of TuMV and CaMV in experiments of single and mixed infections. Values are reported as number of infected plants over the total number of plants inoculated. Infections were determined by RT-qPCR (TuMV) or qPCR (CaMV).

	TuMV	CaMV	TuMV and CaMV	None
Single infection	16/17	18/20		3/20
Mixed infection	1/20	0/20	19/20	0/20

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440

Table 3: Observed payoff matrix (relative accumulation ± 1 SEM) for the interaction between CaMV (focal) and TuMV (opponent) during mixed infections.

	Opponent	
Focal	**CaMV**	**TuMV**
CaMV	1.000 ± 0.069	0.443 ± 0.038
TuMV	13.718 ± 0.518	7.738 ± 0.372

441

441 **Figure 1:** Accumulation of CaMV and TuMV in singly and coinfected *A. thaliana*

442 plants determined by qPCR and RT-qPCR respectively.