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# Reduction of leaf area and symptom severity as proxies of disease-induced plant mortality: The example of the *Cauliflower mosaic virus* infecting two *Brassicaceae* hosts

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ABSTRACT

Disease induced effects on host survival are important to understand the evolution of parasitic virulence and host resistance/tolerance. Unfortunately, experiments evaluating such effects are in most cases logistically demanding justifying the measurement of survival proxies. For plant hosts commonly used proxies are leaf area and the nature and severity of visual qualitative disease symptoms. In this study we tested whether these traits are indeed correlated to the host mortality rate induced by viral infection. We infected *Brassica rapa* and *Arabidopsis thaliana* plants with different natural isolates of *Cauliflower mosaic virus* (CaMV) and estimated over time the development of symptoms and the relative reduction of leaf area compared to healthy plants and followed plant mortality. We observed that the mortality of infected plants was correlated with the relative reduction of leaf area of both *B. rapa* and *A. thaliana*. Measures of mortality were also correlated with the severity of visual qualitative symptoms but the magnitude of the correlations and the time frame at which they were significant depended on the host plant: stronger and earlier correlations were observed on *A. thaliana*.



## 1. Introduction

Diseases affect host fitness through modifications of host life history traits. For horizontally transmitted parasites a host life history trait commonly measured is the reduction of host survival due to parasite infection (e.g. de Roode et al., 2008; Ferguson and Read, 2002; Jensen et al., 2006). Evaluation of host survival is indeed relevant to our understanding of the ecology and evolution of host–parasite interactions since host survival directly impacts the time during which the parasites may be transmitted, and hence parasite fitness (e.g. Anderson and May, 1982; Frank, 1996; Levin, 1996).

Because measures of host survival are often logistically complicated, it has become common use to measure various potential proxies such as reduction of size (Jensen et al., 2006), biomass (de Roode et al., 2008), wing asymmetry (Agnew and Koella, 1997). When the hosts are plants such proxies may be modifications of plant height, leaf biomass or area, or the nature and severity of visual qualitative disease symptoms such as chlorosis and/or mosaic, necrosis, host stunting or other leaf disorders. Measures of leaf area reduction and visual qualitative symptom severity are non-destructive and can be taken repeatedly during the course of infection.

Surprisingly, at least for viral plant diseases very few experiments evaluated in the same biological system the reductions of host survival, leaf area, and/or severity of visual qualitative symptoms (Table 1). More importantly, no study tested statistically the relationship between these potential proxies with plant survival. Moreover, despite the broad host range of most plant viruses (Hull, 2001), we found only one study reporting the effect of infection of a luteovirus on both survival and leaf area of different host species (Malmstrom et al., 2005). However, even this study did not report on the correlation between the two traits.

In the present manuscript, we aimed at testing whether the relative reduction of leaf area and the severity of visual qualitative symptoms are indeed correlated with disease induced host mortality when two natural host plants (*Brassica rapa* and *Arabidopsis thaliana*) were singly infected by several viral natural isolates of the *Cauliflower mosaic virus* (CaMV).

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**Table 1**
Studies reporting concomitant measures of reductions of leaf area, severity of symptoms, and/or host survival induced by plant viruses on their host.

| Virus | Number of virus genotype tested | Host plant species | Survival relative to healthy plants | Leaf area relative to healthy plants | Evaluation of symptoms | References |
|---|---|---|---|---|---|---|
| *Banana bunchy top virus* | 1 | *Musa acuminata* | • | ↓ | + | Hooks et al. (2008) |
| *Barley yellow dwarf virus* [-PAV] | 1 | *Wild grass species* | $\leq_{pe}$ | $\leq_{py}$ | • | Malmstrom et al. (2005) |
| *Cucumber mosaic virus* | 10 | *Cucumis melo* | ↓ | • | + | Betancourt et al. (2011) |
| *Lettuce mosaic virus* | 9 | *Lactuca sativa* | • | ↓ | + | Bos et al. (1994) |
| *Indian peanut clump virus* | pop° | *Triticum aestivum* | ↓ | • | + | Delfosse et al. (1999) |
| *Rice yellow mottle virus* | 2 | *Oryza sativa spp.* | + | $\downarrow_{vp}$ | + | Fargette et al. (2002) |
| *Tobacco leaf curl virus* | pop | *Eupatorium chinense* | ↓ | ↓ | • | Yahara and Oyama (1993) |
| *Tobacco leaf curl virus* | pop | *Eupatorium makioni* | $\leq_{e}$ | ≥ | + | Funayama et al. (1997) |
| *Cauliflower mosaic virus & Turnip rosette virus* | pop | *Brassica nigra* | • | $\downarrow_{ve}$ | + | Thurston et al. (2001) |
| *Turnip mosaic virus & Turnip yellow mosaic virus* | pop | *Brassica oleracea* | ↓ | = | + | Maskell et al. (1999) |
| *Turnip mosaic virus* | 1 | *Brassica juncea* | • | ↓ | + | Guo et al. (2005) |

↓: Decrease of the considered trait compared to healthy plants.
≤: Decrease or no change of the considered trait compared to healthy plants.
≥: Increase or no change of the considered trait compared to healthy plants.
=: No change of the considered trait compared to healthy plants.
+: Authors report variation on the trait.
$^{p}$: Variation according to the plant genotype.
$^{y}$: Variation according to the year of experiment.
$^{e}$: Variation according to the environment.
$^{v}$: Variation according to the viral genotype.
°: Pop corresponds to a viral population isolated from the field without any further characterization.
•: Data not available.

## 2. Materials and methods

### 2.1. Plant growth conditions

Seeds of *Brassica rapa-rapifera* (L.) *cv*. 'Just Right' and *A. thaliana* ecotype Col-0 were sown in batches in a glasshouse (24 ± 2 °C, 13 h light). Seven days after seeding, seedlings were transplanted into individual 9 cm × 9 cm pots for *B. rapa* or 5.5 cm × 5.5 cm for *A. thaliana* (24 pots per batch) with Huminsubstrat Neuhaus N2, pH 5.5–6.5 (Klasman Deilmann GmBH, Geeste, Germany). Plants were then immediately transferred in a phytotron with 9 h light (3600 lux) at 22 ± 1 °C, 15 h night at 20 ± 1 °C, and 80 ± 5% relative humidity. They were also irrigated with 15:10:30 NPK + oligonutrients. Plants continued to grow as rosettes after 6 weeks; none of the infected plants initiated flowering while less than one percent of healthy plants did under these growth conditions.

### 2.2. Viral isolates and inoculation

CaMV is a reverse-transcribing pararetrovirus with double stranded DNA (dsDNA) virus transmitted exclusively horizontally by aphids (Mink, 1993; Tomlinson and Walker, 1973). We evaluated proxies of survival and decrease in host survival induced by twenty viral isolates of CaMV provided by Dr. Nadia S. Al-Kaff (John Innes Institute, UK; Al-Kaff and Covey, 1994, 1995; Cecchini et al., 1998). All these isolates were collected worldwide in the field either on *Brassica* spp. or on unspecified hosts (Table S1; Hull, 1980). After its sampling in nature, each viral population was passaged twice on *B. rapa-rapifera* (L.) *cv*. 'Just Right' and stored as dehydrated material in the John Innes Institute. We used this dehydrated material to initiate the viral populations of each isolate. Samples of each dehydrated plant infected by a single viral isolate were first suspended into inoculation buffer (Tris 10 mM; $MgCl_2$ 2.5 mM; pH 7.5). We then inoculated 50 μl of each solution on the fourth or fifth leaf of four weeks old turnips (*B. rapa-rapifera cv*. 'Just Right') by using an abrasive powder (Carborundum, Sigma). These singly-inoculated plants were grown in a glasshouse. Thirty days post-inoculation (dpi) all systemically infected leaves were harvested and stored at −80 °C. This material was then used as source of inoculum for the experiments. We thus first suspended samples of leaves of infected plants into inoculation buffer and quantified (qPCR, SYBR-green® technology, MxPro3005, Stratagene) the viral DNA with one set of primers to target the virus (F – 1487 5′-AACAACCTCATTGAGATTGTAGGA-3′ and R – 1553 5′-TCCGAAGGGTCTTTGCTTAG-3′). For each isolate, a dilution factor was calculated as the ratio of the quantification of the isolate relative to the least concentrated isolate. Each isolate was diluted with inoculation buffer according to the dilution factor except for the least concentrated isolate. Inoculation of each of the twenty CaMV isolates was performed as described above on *B. rapa* on 6 plants. For logistic reasons, only a subset of nine CaMV isolates were inoculated on all leaves (approximately eight developed leaves) of four weeks old *A. thaliana* (15 per isolate). All inoculated plants for the experiments were grown in the phytotron and their positions were randomized. Control plants corresponded to mock-inoculated plants, i.e. inoculated with samples of healthy *B. rapa* ground into inoculation buffer.

### 2.3. Proxies and host mortality

We followed the survival of individual plants and measured host mortality directly as hazard ratios of infected relative to healthy plants. We also measured host mortality of each isolate as the percentage of plants infected by this isolate which had died at the date where between isolate variance of mortality was maximal. That date was determined a posteriori by following mortality over time.

We measured throughout the infection two proxies of survival, namely: (*i*) the reduction of leaf area due to viral infection and (*ii*) the severity of qualitative symptoms by visually rating symptoms.

#### 2.3.1. Mortality

The mortality of plants (infected and control) was followed every one to five days after 33 dpi and 35 dpi (no plant had died before that date) until 228 dpi and 98 dpi (the death of all infected plants) for *B. rapa* and *A. thaliana*, respectively. Plants were considered as dead when the apex was yellow, old leaves were dried and no new leaves were growing anymore. For *B. rapa*, the mortality rate per isolate and controls was estimated on 6 plants while for *A. thaliana*, the mortality rate per isolate was estimated on 6–15 infected plants (i.e. depending on the infectivity of the different isolates) and on 51 plants for uninfected controls. For each group of plants Kaplan–Meier survival parameters were estimated.