Insight Into Late Wilting Disease of Cucumber Demonstrates the Complexity of the Phenomenon in Fluctuating Environments

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Abstract

Some diseases are caused by coinfection of several pathogens in the same plant. However, studies on the complexity of these coinfection events under different environmental conditions are scarce. Our ongoing research involves late wilting disease of cucumber caused by coinfection of *Cucumber green mottle mosaic virus* (CGMMV) and *Pythium* spp. We specifically investigated the role of various temperatures (18, 25, 32°C) on the coinfection by CGMMV and two predominant *Pythium* species occurring in cucumber greenhouses under Middle Eastern climatic conditions. During the summer months, *Pythium aphanidermatum* was most common, whereas *P. spinosum* predominated during the winter–spring period. *P. aphanidermatum* preferred higher temperatures while *P. spinosum* preferred low temperatures and caused very low levels of disease at 32°C when the 6-day-old seedlings were infected with *P. spinosum* alone. Nevertheless, after applying a later coinfection with CGMMV

on the 14-day-old plants, a synergistic effect was detected for both *Pythium* species at optimal and suboptimal temperatures, with *P. spinosum* causing high mortality incidence even at 32°C. The symptoms caused by CGMMV infection appeared earlier as the temperature increased. However, within each temperature, no significant influence of the combined infection was detected. Our results demonstrate the complexity of coinfection in changing environmental conditions and indicate its involvement in disease development and severity as compared with infection by each of the pathogens alone.

Keywords: damping-off, greenhouse, minor pathogen, multiple infection, soilborne pathogen, synergistic effect, Tobamovirus, oomycetes, vegetables, epidemiology, disease development and spread, disease management, viruses and viroids

Studies on plant diseases commonly refer to a single pathogen interacting with a single host plant. However, the important role of pathogens coinfecting the same host plant has been recognized in intra-kingdom as well as cross-kingdom interactions (Lamichhane and Venturi 2015; Tollenaere et al. 2017). These complex multispecies interactions, existing in the wild and in agroecosystems, may result in diverse epidemiological outcomes, including antagonistic and synergistic effects (Abdullah et al. 2017; Ataga et al. 1999; Tollenaere et al. 2017). Although an increasing number of studies recognizes the importance of these complexes, the interaction of such pathosystems with environmental conditions is still poorly understood (Marçais et al. 2017).

One such system involves the combined infection of *Cucumber green mottle mosaic virus* (CGMMV) and *Pythium* spp. that causes extensive collapses in mature greenhouse cucumbers (Philosoph et al. 2018). CGMMV was first described in 1935 (Ainsworth 1935) and belongs to the genus *Tobamovirus*, family *Virgaviridae* (Adams et al. 2009). It has been reported in Japan, Israel, Greece, and China (Antignus et al. 1990; Inoue 1967; Varveri et al. 2002;

Zhang et al. 2009) and more recently in Canada (Ling et al. 2014), California, U.S.A. (Tian et al. 2014), and Northern Territory, Australia (Tesoriero et al. 2015). CGMMV is considered a major pathogen of cucurbits worldwide (Dombrovsky et al. 2017). CGMMV's natural hosts include melon (*Cucumis melo*), watermelon (*Citrullus lanatus*), and cucumber (*Cucumis sativus*) (Lovisolo 1980). The highly stable viral particles can persist in CGMMV-contaminated seeds (Liu et al. 2014; Reingold et al. 2015), contaminated soil from a previous growing cycle (Reingold et al. 2016), and wild vegetation (Antignus et al. 1990). The virus spreads by direct mechanical contact with a range of contaminated sources, including seeds, propagation stock materials, and soil (Choi 2001; Wu et al. 2011). Typical symptoms of CGMMV in cucurbits include mottle and mosaic patterning of infected leaves and fruit distortions (Choi 2001; Lee 1996), often resulting in reduced yield and lower market value (Dombrovsky et al. 2017; Nontajak et al. 2014; Park et al. 2005).

In the last decade, the phenomenon of late wilting has increased in trellised cucumber greenhouses during CGMMV epidemics, resulting in extensive collapse and growth inhibition of plants more than 3 weeks after transplanting (Antignus et al. 2001; Philosoph et al. 2018). Similar phenomena have been reported in different countries including Nigeria (Ayo-John et al. 2014) and Norway (Sundheim et al. 2008). In Israel, the emerging wilting phenomenon that leads to the collapse of plants appears mostly in defined patches in greenhouses, often accompanied by visible damage to the lower stem tissue, and were demonstrated to be tightly linked with coinfection by *Pythium* spp. (Philosoph et al. 2018). Moreover, in the aforementioned study, there was almost no collapse of plants that were infected with CGMMV alone. These results led to the understanding that the collapse is caused by the interaction between these two pathogens.

The genus *Pythium* includes major pathogens of cucumber (Ben-Yephet and Nelson 1999; Zitter et al. 1996), and can cause up to 75% mortality during the first 2 weeks after transplanting (Al-Sadi et al. 2007, 2008). Plant-pathogenic *Pythium* species may cause diverse disease symptoms (Coffua et al. 2016; Hendrix and Campbell

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1973; Schroeder et al. 2013), ranging from symptomless to severe damping-off and root rot, depending on the host, environmental conditions, and plant age (Martin and Loper 2010). These pathogens may also colonize mature hosts, including cucumber plants, where symptoms include minor root rot and growth retardation. However, some studies suggest that with additional stress, some *Pythium* spp. may result in extensive collapse in infected plots (Al-Sadi et al. 2010) including viral infection stress (Nitzany 1966; Philosoph et al. 2018).

In recent years, the question of how coinfection may affect disease dynamics has gained increased recognition (Marçais et al. 2017). One of the fields that needs further studies is the role of environmental effects on the combined infection process. Climatic conditions and especially temperature may have a strong impact on the outcome of infection and pathogen populations, but very little is known about their relative importance during the coinfection process (Marçais et al. 2017; Vaumourin and Laine 2018).

Our previous study showed that two major species of *Pythium* are commonly isolated from collapsed mature plants coinfecting with CGMMV (Philosoph et al. 2018). The first, *Pythium spinosum* was used to demonstrate the late wilting phenomena under controlled environment at room temperature (24°C) and is known from other areas for causing damage at mild temperatures (Al-Sadi et al. 2007; Gherbawy and Abdelzahr 2002). Additionally, *P. aphanidermatum* was also detected in collapsed coinfecting plants and is most common worldwide under much warmer conditions (temperatures as high as 30°C [Al-Sadi et al. 2007; Watanabe et al. 2008]). This provides a case study in which two different species of *Pythium* alternate among the different growth seasons with potentially different ecological requirements during coinfection with CGMMV. The aim of the current study was to investigate the parameters involved in disease dynamics under coinfection with each of the two *Pythium* species and CGMMV, and to elucidate the influence of temperature (mild winter versus summer) on each of the two *Pythium* species with and without viral infection.

Materials and Methods

Data collection and pathogen isolates. *Pythium* isolates were collected between January 2014 and December 2015 from collapsed cucumber plants (Supplementary Table S1). These plants were also confirmed to be infected by CGMMV through enzyme-linked immunosorbent assay (ELISA) (Clark and Adams 1977) analysis for the presence of CGMMV coat protein as described by Shargil et al. (2016). Substrate hydrolysis time was 30 min. Optical density (OD) of samples was recorded at 405 nm by an ELISA reader (Anthos, Salzburg, Austria). In order to ensure that each collected plant was not infected by a mixture of two or more *Pythium* species, each isolate included combined hyphae of three different segments from root and crown root of each individual plant. DNA from 39 of these *Pythium* isolates was extracted with the commercial kit MaserPure yeast DNA (Epicenter, Madison, WI) as further described by Philosoph et al. (2018). The DNA was amplified by using primers ITS1 and ITS4 (White et al. 1990). Sequences were compared with the NCBI database, and representative isolates were deposited in GenBank under accession numbers MF116303 and MF118618. The 39 isolates were assigned into one of the four main growing seasons that are commonly practiced in the central Hefer Valley (Ahituv, geographical coordinates: 32.389988, 34.990965).

Temperature responses of *P. spinosum* and *P. aphanidermatum* isolates. In vitro tests were performed to evaluate temperature responses of *P. spinosum* and *P. aphanidermatum* isolates. Among the collection of 39 sequenced isolates, six isolates of *P. spinosum* and six isolates of *P. aphanidermatum* were randomly chosen for the mycelial growth experiments. Agar disks (8 mm diameter) from the margins of 48-h-old colonies were placed in the center of 90-mm agar plates containing potato dextrose agar (PDA) (Difco Laboratories). The plates were maintained at temperatures of 10, 15, 20, 25, 30, or 35 ± 1°C in incubators (Sanyo, MIR-154) under a 12-h light/dark regime. After 24 h, colony size was measured in two perpendicular directions and colony area was calculated as an ellipse

[$\Pi \times (R1 \times R2)$]. There were three replicates (petri dishes) per treatment (*Pythium* isolate × temperature) and the experiment was repeated once. Gaussian 3-parameter regressions [$f = a \times \exp(-0.5\{(x-x_0)/b\}^2)$] were computed for each treatment (Bunce 2008; Luo et al. 2001). Optimal temperatures were estimated after calculating the first derivatives of the regression equations using SigmaPlot 12 (Systat Software, San Jose, CA). The differences between the optimal temperatures of *P. spinosum* and *P. aphanidermatum* were compared with a *t* test ($\alpha = 0.05$).

Pathogen growth and inoculation. *P. spinosum* isolate PS-04-OB and *P. aphanidermatum* isolate PA-03-MA6 were cultured independently on potato dextrose agar (PDA; Difco) and incubated at 25°C for 2 days. From each isolate, 10 populated agar disks (10 mm) were excised from the 48-h-old colony edge and placed in a 500-ml Erlenmeyer flask containing 80 g of autoclaved pearl millet. The culture was incubated for 5 days at 25°C. *Pythium*-colonized millet seeds were homogenized in a Waring blender (Moulinex, France) for 30 s and adjusted to 0.25% (wt/wt) in potting mixture (70:30 peat:tuff ratio, Even-Ary, Israel). The control treatment consisted of a mix of 0.25% noncolonized sterilized millet seeds. CGMMV inoculation was conducted as previously described (Reingold et al. 2015). Briefly, each cucumber plant was mechanically inoculated by gently rubbing the cotyledons with extract from CGMMV-inoculated cucumber plants (Ah isolate, GenBank accession number KF155232). The extract was homogenized in phosphate buffer (0.01 M, pH 7) containing Carborundum dust (silicon carbide). The infection buffer was washed off from the cotyledons 5 min postinoculation. Plants with *Pythium* alone and the noninoculated control were similarly treated with virus-free buffer and Carborundum.

Plant growth conditions. Commercially disinfected cucumber seeds (cv. Kefir Zeraim Gadera, Israel) were sown in a nursery tray containing potting mixture in a pest- and disease-free greenhouse at 23 ± 1°C. The germinated seedlings were kept under a 12-h photoperiod, and were fertilized and irrigated with drippers twice a day (~0.1 liter per irrigation) with 5:3:8 NPK fertilizer (irrigation water planned to contain total N, P, and K concentrations of 120, 30, and 150 mg/liter, respectively; EC 2.2 dS/m), allowing for 25 to 50% drainage.

Aggressiveness of *P. spinosum* and *P. aphanidermatum* isolates at different temperatures in controlled-environment experiments. Plant seedlings were grown before inoculation as described above until 6 days after sowing. Four cucumber seedlings of uniform growth were transplanted into each pot (3 liter, 16-cm-diameter) containing potting mixture (peat:tuff 7:3 vol/vol, Shaham, Givat Ada, Israel). Treatments (at least 16 pots per treatment) were conducted in three different controlled-environment growth chambers (at least 192 plants in each chamber) at 18, 25, and 32°C. A Hobo Data logger U12-012 (Onset, Bourne, MA) was used to document the soil and air temperatures in each chamber. The soil temperatures at 5 cm depth were 18.3 ± 0.10, 24.4 ± 0.07, and 32.1 ± 0.07°C, respectively. In each chamber, three treatments were performed (for a minimum of 64 plants per treatment): (i) pathogen-free, (ii) inoculation with *P. spinosum*, (iii) inoculation with *P. aphanidermatum*. Damping-off incidence in the plants during the first 8 days, until mortality ceased, was used to draw disease-progress curves and calculate the area under the mortality progress curve (AUMPC, in % × days), representing the intensity of the entire epidemic in each treatment. AUMPC was determined as previously described (Jaiswal et al. 2014). The experiments were repeated twice.

Combined infection of CGMMV and *Pythium* spp. at different temperatures under controlled conditions experiments. These experiments were initiated with the surviving 14-day-old plants from the previous stage (i.e., the damping-off experiment) to mimic the scenario in commercial cucumber greenhouses in which *Pythium*-infested plants survive the first wave of seedling damping-off but still host *Pythium* in their tissues. Therefore, after the initial 8 days post-inoculation in the previous set of experiments, 96 surviving plants were selected from each of the growth chambers set the three temperatures (18, 25, and 32°C), which included 32 plants from each of the

following treatments: (i) pathogen-free, (ii) inoculation with *P. spinosum*, (iii) inoculation with *P. aphanidermatum*. For each of the three temperatures, the plants were used to create four new blocks, each containing 16 plants from each of the following treatments: (i) pathogen-free, (ii) inoculation with *P. spinosum*, (iii) inoculation with *P. aphanidermatum*, (iv) inoculation with CGMMV, (v) inoculation with both CGMMV and *P. spinosum*, and (vi) inoculation with both CGMMV and *P. aphanidermatum* (in total 96 plants in each chamber). During the experiment, the mortality rate was documented along with CGMMV symptom development. The experiment was repeated twice. As lengths of the two repeated experiments differed (23 and 40 days, respectively), we normalized the epidemic parameters using the Relative AUMPC (Jaiswal et al. 2014) for mortality and the relative area under the disease progress curve (RAUDPC) for viral symptoms. The documented mortality rate was also used to calculate the synergistic effect of CGMMV and *Pythium* spp. using the Abbott formula (Kosman and Cohen 1996; Levy et al. 1986) as described by Philosoph et al. (2018). At the end of the experiments, leaves from each treatment group were collected for CGMMV detection (~200 mg). The leaves were ground in a general extraction buffer (Bioreba, Switzerland) and the presence of CGMMV was detected by ELISA (Clark and Adams 1977) using commercial CGMMV antibodies (Agdia, U.S.A.) and CGMMV-positive and negative references. Substrate hydrolysis time was 20–40 min. Plates were recorded at 405 nm using an ELISA reader (Multiskan Fc, Thermo Scientific). Positive ELISA results were calculated based on a minimum ratio of three times of the Negative Reference (3xNR) as described by Shargil et al. (2016). In addition, the roots from all plants were examined for the presence of *Pythium* spp. using selective corn meal agar as described by Philosoph et al. (2018).

Statistical analysis. The controlled-environment chamber experiments were conducted twice. In each experimental set, the results of the experimental repeats were firstly pooled and analyzed jointly with ‘experiment’ as an additional block factor. As no significant differences were observed between the two experimental repeats, and there were furthermore no significant effects of the experiment factor and the other main interactions ($P > 0.391$) (Supplementary Tables S2 and S3), the data of repeated experiments were combined into one dataset for further analysis.

Data were analyzed by two-way analysis of variance (ANOVA) for *Pythium* aggressiveness, with *Pythium* species and temperature as main factors and their interactions. For the effect of combined infection at different temperatures, a three-way ANOVA model was applied with *Pythium* species, temperature, and CGMMV infection as main factors and their interactions using JMP 14 software (SAS Institute, Cary, NC). To enable ANOVA, percentage values were normalized by arcsine square-root transformation. Multiple comparisons of the means were conducted by using Tukey-Kramer honestly significant difference (HSD) test ($\alpha = 0.05$). To calculate the impact of the combined infection at different temperatures based on the percentage of viral symptomatic plants in each block (CGMMV, CGMMV + *P. spinosum*, CGMMV + *P. aphanidermatum*), within each temperature, a χ^2 test ($\alpha = 0.05$) was used to compare the proportion of CGMMV, CGMMV + *P. spinosum*, and CGMMV + *P. aphanidermatum* infected plants.

Results

Data collection and pathogen isolates. Two *Pythium* species, *P. spinosum* and *P. aphanidermatum* (>99.2% identity to NCBI accessions KU208387 and AY598622, respectively), were detected among the 39 sequenced isolates. All of the plants were infected by a single species. *P. spinosum* was detected between November and May—Israeli winter until late spring (Fig. 1A). *P. aphanidermatum* was detected between April and October (Fig. 1A)—late spring, summer, and autumn. The corresponding average temperatures are presented in Figure 1B.

Growth responses of *P. spinosum* and *P. aphanidermatum* under different temperatures. Differences between the in vitro growth responses of the *P. spinosum* and *P. aphanidermatum* isolates at different temperature were highly significant ($P < 0.001$). For the six

tested *P. spinosum* isolates, the optimal temperature range was $25.8 \pm 0.35^\circ\text{C}$ (Fig. 2A) and for the six *P. aphanidermatum* isolates, $32.8 \pm 0.37^\circ\text{C}$ (Fig. 2B).

Aggressiveness of *P. spinosum* and *P. aphanidermatum* isolates at different temperatures under controlled environment. The two main factors ‘temperature’ and ‘*Pythium* spp.’ and their interaction were significant ($P < 0.045$). For *P. spinosum*, the final mortality incidence (FMI) at 18°C was significantly higher than at 25°C and 32°C (Fig. 3); similarly the AUMPC values related to *P. spinosum* were significantly higher at 18°C compared with 25 and 32°C (Supplementary Fig. S1). For *P. aphanidermatum*, the FMI at 18°C was significantly lower than those at 25°C and 32°C (Fig. 3), with a similar pattern for the AUMPC values. While comparing the aggressiveness measures between the two *Pythium* spp. within each temperature, *P. spinosum* was more aggressive than *P. aphanidermatum* at 18°C while *P. aphanidermatum* was more aggressive than *P. spinosum* at 25°C and 32°C (Fig. 3).

The effect of combined infection of CGMMV and *Pythium* spp. on disease severity at different temperatures under controlled conditions. In these experiments, which were conducted on the 14-day-old surviving seedlings from the previous experiment, the three-way interaction of ‘*Pythium* spp.’ $\times$ ‘temperature’ $\times$ ‘CGMMV’ was significant ($P < 0.022$). For the plants infected with *P. spinosum* without CGMMV infection, mortality of seedlings was very low (FMI = 3.12%) and was only detected at 18°C and 32°C (Fig. 4A). However, during the combined infection with CGMMV, significantly higher mortality incidences were detected at each

Fig. 1. (A) Distribution of 19 *Pythium aphanidermatum* (PA) and 20 *P. spinosum* (PS) isolates during the four main growing seasons of cucumber in central Israel. (B) Average minimum and maximum temperatures of “Maor meteorological station” (Ahituv area) during the 2014–2015 growing seasons measured 0.5 m above the ground (<http://www.meteo.co.il>).

temperature ($P < 0.001$, Fig. 4A), resulting in a high synergistic effect (Table 1). The FMI of the combined infection were 56.25% at 18°C, 25% at 25°C, and an unexpected mortality of 43.75% at 32°C and with no significant differences between the temperatures (Fig. 4A). Similar pattern was detected for the RAUMPC values of the three temperatures (19.6 ± 6.28 , 8.1 ± 1.80 , and 19.4 ± 7.42 for 18, 25, and 32°C, respectively Supplementary Fig. 2A). The mortality of seedlings infected with *P. aphanidermatum* alone only occurred at 32°C, with a relatively low mortality rate of 12.5% (Fig. 4B). The FMI of the combined infection was temperature-dependent and was significantly lower at 18°C (6.25%). Final mortality incidence values were also significantly different between 25°C (31.2%) and 32°C (62.5%) (Fig. 4B). These results of the combined infection were also reflected with strong synergistic effect at 25°C and 32°C (Table 1). Similar pattern was detected for the RAUMPC values of the three temperatures (1.1 ± 0.92 , 13.3 ± 2.77 , and 26.4 ± 5.68 for 18, 25, and 32°C, respectively), but with no significant difference between 25°C and 32°C. No mortality was observed for the noninoculated control or for CGMMV infection alone.

Effect of temperature and combined infection with CGMMV and *Pythium* spp. on CGMMV symptom appearance. Temperatures affected the development of CGMMV symptoms (Table 2 and Supplementary Fig. S3). At 32 and 25°C, first symptoms appeared 6 days and 9 days post-CGMMV inoculation, respectively. A longer delay was observed at 18°C, where early symptoms were visualized on day 18 post-CGMMV inoculation. The influence of temperature (based on the RAUDPC values) was significantly higher as the temperature increased ($P \leq 0.005$). However, within each temperature, no significant influence of the combined infection was

detected for the RAUDPC of the viral symptoms with either of the *Pythium* spp. (Table 2). The ELISA OD reads for all the treatments with CGMMV ranged between 0.10 and 0.18 (compared with 0.011–0.013 for the NR and 0.016–0.024 for samples infected with *Pythium* spp. alone or noninoculated controls) demonstrating no differences regardless of temperature and the combined infection (Table 2). Values for all the treatments without the CGMMV infection (i.e., *Pythium* alone and noninoculated control) were below the value of 0.05 as expected from noninfected treatments).

Discussion

In the last 50 years, the importance of coinfection and pathogen complexes have been demonstrated in several pathosystems (Lamichhane and Venturi 2015). Most descriptions have focused on complexes of pathogens from the same kingdom [ascochyta blight in pea (Le May et al. 2009); soybean wilt (Barros et al. 2014); mosaic disease in cucurbits (Salvaudon et al. 2013)], while several studies have indicated that viral-fungal/oomycete interactions also play an important role in plant diseases (Ito et al. 2017; Koike and Yang 1971; Zitter 1995). Nonetheless, there are very few in-depth studies on viral-fungi or viral-oomycetes, with most of these focusing on a description of the phenomenon per se, such as *Pythium ultimum* and cucumber mosaic virus (CMV) in cucumber (Nitzany 1966), *Septoria apiicola* and celery mosaic virus (CeMV) in celery (Ataga et al. 1999), and *Peronosclerospora sorghi* and maize streak virus (MSV) in maize (Damsteegt et al. 1993). Important questions regarding the influence of environmental conditions on these complexed pathosystems have been studied to a lesser extent.

Here, we report on a broader aspect of the pathogen coinfection. Combined infection may result in not only a higher, synergistic effect at pathogens' optimal temperatures, but also unexpected new and severe symptoms occurring under suboptimal conditions for the pathogens that exhibit very minor damage when present as single infections.

Monoculture practice of cucumber cultivation in Israel enabled detection of the temporal dynamics of two different *Pythium* spp. *P. aphanidermatum* was dominant in the warmer summer months, whereas *P. spinosum* dominated during the comparably colder winter

Fig. 2. Mycelial growth area of (A) six *Pythium spinosum* isolates (PS) and (B) six *P. aphanidermatum* isolates (PA) grown in PDA plates and maintained at 10, 15, 20, 25, 30, or 35 ± 1°C. Colony area was calculated after 24 h. Gaussian 3-parameter regressions [$f = a \times \exp(-0.5((x-x_0)/b)^2)$] were calculated for each treatment. Optimal temperatures were estimated after calculating the first derivatives of the regression. There were three replicates (petri dishes) per treatment (*Pythium* isolate × temperature) and the experiment was repeated once. Values are the average of the experiments.

Fig. 3. Final mortality incidence (FMI) of cucumber plants infected with *Pythium* alone in three controlled-environment chambers set at 18, 25, and 32°C, upon inoculation with *Pythium spinosum* (PS) or *P. aphanidermatum* (PA). Six-day-old seedlings were inoculated with one of the *Pythium* spp. Final mortality incidence was calculated until day 8. No mortality was observed for the noninoculated control. Columns labeled by a common capital letter and small letter are not significantly different at $\alpha \leq 0.05$ according to Tukey-Kramer HSD test within PS and PA treatments, respectively. Asterisk denotes a significant difference at $\alpha \leq 0.05$ between the two *Pythium* spp. (PS versus PA) at the same temperature. Bars = standard error. The values represent the average of two experiments; each experiment contains at least 64 plants per treatment.

months. In addition, during the spring and autumn seasons, a mixed population of the two species was present. These results concur with previous reports on these pathogens from around the world (Al-Sadi et al. 2007; Gherbawy and Abdelzahr 2002; Stirling et al. 2004) and are in line with the *in vivo* and *in vitro* experiments conducted in our study. CGMMV epidemics persist during all seasons at the background of commercial cucumber greenhouses and under diverse environmental conditions (Dombrovsky et al. 2017), potentially providing an additional and essential pathogen for coinfection with either of the *Pythium* spp.

Each of the two tested *Pythium* spp. is known mostly as a causal agent of early damping-off exhibiting only minor symptoms in mature cucumber plants (>14 days after planting), while CGMMV infection causes mottle and mosaic symptoms. The fact that their combination resulted in the sudden occurrence of late wilting demonstrates that the late wilting disease of cucumber is caused by the coinfection of an oomycete and the virus. Late wilting disease on plants older than 14 days, involving CGMMV and *P. aphanidermatum*, demonstrated a similar pattern to the damping-off disease caused by *P. aphanidermatum* alone on 6-day-old seedlings with regards to the optimal temperature required for causing both diseases. One of the unexpected outcomes of this research was the fact that

P. spinosum, which caused almost no damage at high temperatures during the early damping-off epidemics on 6-day-old seedlings, became highly aggressive under the suboptimal condition of 32°C when CGMMV was introduced 14 days postplanting.

Wilting phenomena resulting from viral diseases associated with root necrosis have been reported in several cases (Duggal and Lakhanpal 1981; García-Jiménez et al. 2000; Pearson et al. 1993; Soler-Aleixandre et al. 2005), but their potential connection with minor soilborne pathogens, and their interaction with environmental conditions, have been underestimated. Gergerich and Dolja (2006) pointed out that under different environmental conditions, a plant's response to viral infection may range from symptomless to severe disease and plant death. It would be interesting to challenge known and future extensive collapse events related to viruses with the broader aspect of virus-minor pathogen systems while further considering environmental aspects in such pathosystems.

Fluctuating weather conditions, including sudden heat waves, are common in many parts of the world and their frequency rises (Cramer et al. 2018; Rosenzweig et al. 2001). These conditions are also quite common in the Middle East and the Mediterranean region, which are characterized by hot summers, cool winters, and relatively mild weather conditions during the spring and autumn that are commonly

Fig. 4. Final mortality incidence (FMI) of cucumber plants in the combined infection experiment with cucumber green mottle mosaic virus (CGMMV) and each of the *Pythium* sp. Experiments were set in three controlled-environment chambers at 18, 25, and 32°C, upon inoculation with (A) *Pythium spinosum* and CGMMV (PS+CG) or *P. spinosum* alone (PS), and cucumber plants upon inoculation with (B) *P. aphanidermatum* and CGMMV (PA+CG) or *P. aphanidermatum* alone (PA). Seedlings were inoculated with CGMMV 8 days after inoculation with one of the *Pythium* sp. Final mortality incidence was calculated from 10 days after *Pythium* spp. inoculation (i.e., 2 days after CGMMV inoculation) until the end of the experiment. No mortality was observed for the noninoculated control or for seedlings inoculated with CGMMV alone. Columns labeled by a common capital letter are not significantly different at $\alpha \leq 0.05$, according to Tukey-Kramer HSD test, between the different temperatures in (A) PS+CG or (B) PA+CG treatments. Within each temperature, asterisk denotes a significant difference at $\alpha \leq 0.05$ at (A) PS+CG versus PS or (B) PA+CG versus PA. Bars = standard error. The values represent the average of two experiments; each experiment contains 16 plants per treatment.

Table 1. Analysis of synergy factor (SF) based on relative area under the mortality progress curve (RAUMPC) values for combined infection with cucumber green mottle mosaic virus (CG) and *P. spinosum* (PS) or *P. aphanidermatum* (PA)

RAUMPC (% × days)						
<i>Pythium</i> spp.	Temperature (Celsius)	CG + <i>Pythium</i>	<i>Pythium</i>	Control	CG	SF
PS	18	19.59 ± 6.28	2.73 ± 0.02	0	0	7.17 ± 2.29
PS	25	8.12 ± 1.80	0	0	0	NCSF ^a
PS	32	19.41 ± 7.42	1.63 ± 0.01	0	0	11.9 ± 4.55
PA	18	1.07 ± 0.92	0	0	0	NS ^b
PA	25	13.32 ± 2.77	0	0	0	NCSF
PA	32	26.38 ± 5.68	4.79 ± 0.03	0	0	5.50 ± 1.18

^a NCSF, noncalculated synergistic factor, representing high synergistic values that cannot be calculated with the Abbott formula since *Pythium* RAUMPC values were 0.

^b NS = 'CG + *Pythium*' treatment was not significantly different from 0, therefore the SF value is also not significant.

interrupted by high-temperature events (above 30°C) (Fig. 1B) (Brunetti et al. 2004). These events might explain the occurrence, in specific greenhouses during the spring and autumn, of extended plant collapse, where *P. spinosum* and CGMMV are detected as causal agents, but not *P. aphanidermatum*.

Our findings also raise new questions about the mechanisms that underlie this synergistic damage and new symptoms. One of the main questions is the role of the virus itself in this complex pathosystem. Based on a previous report (Philosoph et al. 2018) and our current study, we concluded that late plant collapse of cucumber is a complex disease that is not caused by CGMMV alone. In addition, typical CGMMV symptoms (leaf mosaic and mottling) develop earlier at higher temperatures and vice versa for the colder temperatures. Interestingly, the fact that coinfection of CGMMV with *P. spinosum* leads to extended cucumber plant collapse under the latter's suboptimal condition of 32°C, whereas coinfection of CGMMV and *P. aphanidermatum* barely causes any plant collapse under its suboptimal condition at 18°C, suggests that CGMMV infection contributes an indirect weakening effect on the plant's defense system. Increased expression of viral particles that partially clog the plant xylem and phloem vessels (French and Elder 1999; Moreno et al. 2004; Reingold et al. 2016) may also contribute to the plants' susceptibility to collapse. Indeed, no statistical differences were observed in the appearance of the virus symptoms with or without the *Pythium* spp. and no significant differences were detected in the leaf tissues by ELISA. Nevertheless, there is a possibility that the viral and oomycetes interaction under the mixed infection (and the different temperatures) promotes the accumulation of viral particles or oomycetes mycelium in other plant tissues such as the roots and the lower parts of the transport system. This aspect needs to be further tested by a substantial quantitative and univocal methods such as Quantitative RT-PCR for both *Pythium* and CGMMV.

It is possible that the complex interaction with abiotic stress (e.g., heat) is an additional factor in the plant's response to viral infection, resulting in increased or decreased tolerance to pathogens (Patel and Franklin 2009). It has been found that infection with several independent viruses at high temperatures results in a reduction in plant hormone levels, such as salicylic acid (Malamy 1992), weakening the plant's defense mechanism and inhibiting its ability to cope with

the viral pathogen (Malamy 1992; Yalpani et al. 1991). In addition, the different hormonal pathways that are influenced by these stresses might negatively interact with the expression of other important defense systems and weaken the plant's reaction during the combined infection (Thaler et al. 2012). These hypotheses need to be further investigated using a high-throughput transcriptomic sequencing platform to reveal the complexity of the plant's defense mechanisms against plant pathogens during single infections and coinfections, e.g., with oomycetes or fungi, under fluctuating environmental conditions.

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Table 2. Calculated impact of relative area under the disease progress curve (RAUDPC) based on the percentage of plants with foliar symptoms of cucumber green mottle mosaic virus (CGMMV). Treatments included infection with CGMMV alone (CG); or with combined infection of CGMMV with *P. spinosum* (CG+PS) or *P. aphanidermatum* (CG+PA) at different temperatures.

Treatments	RAUDPC average ^a	ELISA OD ^b 405 nm average and range ^c
CG-18°C	19.78	0.09 (0.088-0.102)
CG+PS-18°C	23.03 ns ^d	0.14 (0.125-0.155)
CG+PA-18°C	19.78 ns	0.11 (0.089-0.146)
Average	20.9 ± 1.08	0.12 ± 0.013
CG-25°C	48.96	0.12 (0.115-0.124)
CG+PS-25°C	49.58 ns	0.14 (0.147-0.147)
CG+PA-25°C	47.33 ns	0.16 (0.136-0.180)
Average	48.6 ± 0.67	0.14 ± 0.011
CG-32°C	63.29	0.10 (0.099-0.106)
CG+PS-32°C	62.93 ns	0.13 (0.114-0.147)
CG+PA-32°C	56.06 ns	0.13 (0.107-0.177)
Average	60.8 ± 2.35	0.12 ± 0.009

^a The data include the average of two experiments. In each experiment, each treatment included 16 plants.

^b OD = Optical density.

^c The ELISA results were considered positive for a minimum ratio of three times that of the Negative Reference (3xNR); the NR ranged in our experiments between 0.011 and 0.013. The ELISA values for our noninoculated control plants or those with *Pythium* spp. alone ranged between 0.016 and 0.024.

^d ns = nonsignificant difference; Within each temperature, χ^2 test ($\alpha = 0.05$) was used to compare the proportions of CG, CG+PS, and CG+PA, infected plants. ($\alpha = 0.05$).

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