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Interspecies crosstalk between *Magnaporthiopsis maydis* and *Fusarium verticillioides* in mutually infected maize plants

Galia Shofman[1,2] & Ofir Degani[1,2]✉

Maize late wilt disease (LWD), caused by the fungus *Magnaporthiopsis maydis*, affects crops in Israel, Egypt, Spain, Portugal, India, and other countries. This study examines the interaction between *M. maydis* and *Fusarium verticillioides*, another post-flowering stalk rot pathogen. A confrontation assay revealed antagonism between the species. *Fusarium verticillioides* extrolites significantly hindered *M. maydis* in cross-cultivation. In a 40-day growth room test, introducing *F. verticillioides* at sowing and *M. maydis* one week later reduced plant growth to 44% of the control. Vice versa or simultaneous inoculation also stunted growth significantly (55% and 67%, respectively). Real-time PCR confirmed these growth responses by tracking both pathogens' DNA in plant roots. Similar trends were observed in a semi-field, full-season potted trial. *Magnaporthiopsis maydis* showed higher aggressiveness with a 40% plant survival rate compared to the minor influence of *F. verticillioides*. At harvest, *M. maydis* DNA levels were up to 1000-fold higher than *F. verticillioides*. Co-inoculation during seeding improved healthy plant rates from 10% (*M. maydis* alone) to 30%. Pre-infection with *F. verticillioides* followed by *M. maydis* resulted in 80% asymptomatic plants at harvest and significantly decreased *M. maydis* infection, though plant growth indices (particularly the yield) remained low. This study highlights the interactions between these pathogens, potentially limiting LWD damage.

Keywords *Cephalosporium maydis*, Crop protection, Disease control, Fungus, *Harpophora maydis*, Late wilt, Microbiome, Pathogenicity, Real-time PCR

Maize (*Zea mays*), similar to other plants, faces many threats from various species of pathogens that exist within intricate communities[1] that also encompass an array of non-pathogenic microorganisms. A plant's pathobiome can be defined as a group of coexisting phytopathogens that mutually influence each other and the plant itself[2]. These assemblages consist of pathogens occupying the same ecological niche and having collaborative or competitive relationships as they vie for the plant's resources. This intricate phytopathogens' crosstalk can lead to disease outcomes that differ significantly from single infections[3].

Recent studies have demonstrated that various fungi and bacteria colonize maize grains[4]. Among these, certain species are recognized phytopathogens, such as *Alternaria alternata* and *Fusarium proliferatum*, which may act as opportunistic pathogens causing disease under favorable conditions. Interactions between these microorganisms occur in agricultural fields' soil before planting, which can profoundly impact disease severity[5]. An intriguing example comes from cotton (*Gossypium hirsutum*), where interactions between *Fusarium oxysporum*, a causative agent of wilt disease, and *Magnaporthiopsis maydis*, an opportunistic root-surface inhabitant, produced unexpected results[6]. These inter-species relationships led to a notable reduction in the severity of cotton wilt disease. The most significant reduction occurred when *M. maydis* was introduced to the soil before *F. oxysporum*. Co-inoculation of the plants with both fungi simultaneously provided some protection compared to *F. oxysporum* alone. However, introducing *M. maydis* to the ground after *F. oxysporum* had little to no protective effect[6]. Another study also documented similar antagonistic interactions between *M. maydis* and *Macrophomina phaseolina*, the causal agent of charcoal rot disease in cotton plants[3,7].

A synergistic inter-species influence that causes more crop damage is more frequently recognized[8]. In the Israeli agricultural context of the 1990s, the wilting phenomenon observed in commercial maize fields was initially attributed to the presence of *Fusarium verticillioides*, given its prevalence in the affected plant samples[9]. Subsequently, the application of Koch's postulates and molecular assays established that the principal etiological

[1]Plant Sciences Department, MIGAL – Galilee Research Institute, Tarshish 2, 1101600 Kiryat Shmona, Israel. [2]Faculty of Sciences, Tel-Hai College, Upper Galilee, 1220800 Tel-Hai, Israel. ✉email: d-ofir@migal.org.il; ofird@telhai.ac.il

agent responsible for the dehydration symptoms was *M. maydis*[10]. Conversely, *F. verticillioides* was identified as a secondary invader or opportunistic pathogen capable of developing within these compromised maize plants[11].

The *M. maydis* fungus (also referred to as *Cephalosporium maydis*[12] and *Harpophora maydis*[13]) is the causal agent of late wilt disease (LWD) in maize[14]. This disease has been reported so far in only 10 countries[7] and significantly impacts susceptible maize plants during maturity[9]. Recent findings have identified *M. maydis* in several secondary hosts, including cotton (Goliath or Pima cv.)[15]. The pathogen is associated with other plant pathogenic fungi such as *F. verticillioides*, the causal agent behind stalk rot, and *M. phaseolina*, which causes post-flowering stalk rot diseases in maize[16,17]. Indeed, dehydration, the main symptom of LWD, arises due to an inadequate water supply and may also be caused by those two secondary phytoparasites[16,18].

Fusarium verticillioides, a globally distributed fungal pathogen, can induce ear and kernel rot in maize crops, leading to substantial economic repercussions within the grain industry[19]. Under elevated temperature and humidity conditions, *F. verticillioides* exhibits rapid proliferation within maize. The fungus, like other members of the *Fusarium* genus, produces several mycotoxins, the most prominent of which are fumonisins, aflatoxins, ochratoxin A, zearalenone, DON, and T-2 and HT-2 toxins. These toxins cause significant contamination of food and feed, especially in cereals[20,21].

The dominance of secondary pathogens in the severity of LWD has been poorly investigated. Its importance for crop protection can be illustrated through a specific case study. In a field experiment conducted during the summer of 2018, it was observed that a field infested with *M. maydis* was simultaneously afflicted by another fungal disease caused by *F. verticillioides* and *F. oxysporum*[18]. This co-infection resulted in exacerbated dehydration and reduced crop yield. However, when plants remained unaffected by *Fusarium* spp., applying chemical treatments via drip irrigation substantially mitigated the LWD symptoms.

To corroborate the synergistic impact between *M. maydis* and *F. verticillioides*, research demonstrated that the interactions between these fungi could significantly influence maize plants by facilitating Fusarium ear rot disease infection[11]. The infection by *M. maydis* contributed to an escalated systemic infection by *F. verticillioides*, progressing from the plant stalks to the ears and kernels. This phenomenon was attributed to chemical and physiological changes, such as the production of sugars and gums within the xylem of maize stalks. Consequently, the involvement of additional pathogens may intensify LWD symptoms and supersede the protective effects of chemical treatments[11].

While accumulated scientific knowledge suggests that the outcome of *M. maydis* and *F. verticillioides* co-infection on LWD consequence is potentially disastrous, their intimate interactions in the plants are poorly understood. The current study addresses these aspects from in vitro confront tests and antifungal metabolites secretion inhibition assessment to *in planta* evaluation of the fungi infection order. The in vivo assays evaluated the impact of fungal crosstalk on maize plants during sprout development (up to 40 days at the beginning of the V4 fourth leaf stage in a growth room) and the plants' maturation phases (full-season open enclosure plants, up to 84 days, R3 milk stage). To this end, the sprouts were exposed to the fungal colonists, either concurrently or sequentially, before, during, and after sowing. The sprouts' survival rates, phenological progression, and indices related to the plants' health were assessed to appraise the consequences of these pathogenic interactions. Moreover, a molecular analysis using quantitative real-time PCR (qPCR) was employed to closely examine both fungi DNA within the host plant's roots or first internode, thereby allowing for a comprehensive assessment of their combined influence within the host environment.

Materials and methods

Origin and growth of *Magnaporthiopsis maydis*

Source of the fungus isolates

The *M. maydis* isolates Hm2 (CBS 133165, CBS-KNAW Fungal Biodiversity Center in Utrecht, The Netherlands) and Hm7[9,22] were obtained from diseased maize plants collected from commercial fields in northern Israel. These strains had previously undergone scrutiny for their pathogenicity, physiological behavior, colony morphology, and microscopic traits[10]. The pathogen's morphological and microscopic features closely resembled strains reported in Egypt and India[12,23]. DNA analysis based on PCR[10] and qPCR [22] verified the authenticity of these strains and virulence degree. Based on infection experiments conducted in growth room sprouts and net house full-season plants, Hm2 was ranked 6th and Hm7 12th (highest virulence degree) regarding aggressiveness among the 16 *M. maydis* isolates tested[22]. The strain of *F. verticillioides* (Saccardo Nirenberg) (ATCC 204499) in this work, designated Fv, was purchased from the American Type Culture Collection (ATCC).

Growth of the fungi

The fungal species were grown on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) at 28 ± 1 °C under dark conditions. Transferring the fungus to a new growth dish was done by extracting a 6-mm-diameter agar disk from the colony margins and planting it on a new plate that was kept afterward at the above conditions for 4–6 days. To set the growth rate of each fungus, the colonies' diameter was measured on two perpendicular planes and included five repetitions of each isolate. The examination was done twice to verify the results. For submerged cultures, 10 fungal disks were sown in an Erlenmeyer flask containing 150-ml potato dextrose broth (PDB; Difco Laboratories, Detroit, MI, USA). The flasks were plugged with a breathable cover and incubated for 14 days, shaken in the dark at 150 rpm at 28 ± 1 °C.

Plate confrontation assay

The assay analyzed the interaction of two fungal species on the same plate through a dual culture[4]. Six-mm disks from the margins of 5–7-day-old colonies were placed on opposite sides of a 90-mm diameter Petri dish, positioned at the halfway point between the center and the edge of the plate. The setup included three repetitions for each pair of isolates to ensure the consistency of the results. As a control group, disks from the same isolate

were placed similarly. The Petri dishes were then incubated at a steady temperature of 28 ± 1 °C in the dark for 4 to 5 days. Daily checks were conducted to monitor the growth progression of the colonies until they met. In this assay, a black line appearing where the colonies meet indicates antagonistic interactions between the species, resulting in growth restriction.

Secreted metabolite effect on colony growth

The fungal isolates were cultured in a liquid medium for two weeks, as noted above. The isolation of the secreted metabolites was previously described[22]. Initially, the growth liquid underwent filtration to separate the fungal solids utilizing a Büchner funnel equipped with two Whatman no. 2 papers connected to a vacuum pump through a Büchner flask. Subsequently, the pH of the filtrate was adjusted to align with the PDB medium's pH of 5.6 ± 0.2. A secondary filtration was conducted through a 0.22-micron vacuum filter (VWR International, Radnor, PA, USA) to sterilize the solution and eliminate any fungal and spore presence. To prepare the solid growth medium, 39 g of PDA powder was added to 1 L fungal extract, and the resultant medium was dispensed into 50 mm diameter Petri dishes. The medium was sterilized to eliminate any possibility of fungal particles (spores, sclerotia, or hypha segments). The subject fungi were cultivated in six repeats on media infused with isolate extracts. Control groups were grown on standard PDA media. The growth rate, indicative of growth inhibition, was monitored throughout four days.

Sprouts pathogenicity evaluation

Inoculation method

The inoculation protocol consists of pre-sowing soil inoculation with sterilized infected wheat grains and complementary infection by fungal colony agar disks. First, sterilized infected wheat grains were used for fungal spreading in the soil, guaranteeing a uniform inoculation load as possible, as previously described[24]. This is a prevalent technique for exerting maximum inoculation pressure under controlled conditions. This approach is useful when dealing with pathogens that exhibit limited saprophytic capabilities, such as *M. maydis*.

The wheat seeds were soaked in tap water overnight and then filtered and dried in a fume hood on paper towels for one hour. The seeds were sterilized in an autoclave for half an hour at 120°C. The sterilized wheat grains (150 g) were incubated with 10 *M. maydis* or *F. verticillioides* colony agar disks in disinfected plastic boxes (0.5 l volume). The colony agar disks (6-mm-diameter each) were prepared as described above. Each box was encased in aluminum foil to ensure dark conditions. The pathogen-inoculated wheat grains were incubated for three weeks in the dark at a temperature of 28 ± 1 °C. The seeds were mixed every few days by shaking to ensure uniform fungal colonization.

The growth room pot assay architecture (describing the different treatments) is elaborated in the Supplementary file, Table S1. Soil inoculation was performed in various combinations before, during, and after seeding. First, the soil was inoculated by adding and mixing 12 g of sterilized and infected wheat seeds to the top 5 cm of the ground in two-liter pots. The inoculated soil was kept moist for two weeks until the maize was sown. Second, on the sowing day, four *M. maydis* or *F. verticillioides* mycelium disks (6 mm) cut from the edge of a 5–7-day colony (two from each pathogen or four from one type) were added to each seed with the sowing[25]. Lastly, three disks were added to each seedling one-week post-sowing. Small plastic tubes were buried adjacent to each seed on the sowing day to ensure proximity to the sprouts during inoculation. One week later, the disks were placed in the holes left by the test tubes and covered with soil.

Growth room trial conditions

The experiment included *M. maydis* (strain Hm2) and *F. verticillioides* (ATCC 204499), applied individually and in dual fungi inoculation (simultaneously or in sequence). The treatments were compared to a non-infected (healthy) control. All experimental groups were conducted in nine biological repetitions (81 pots in total). Each pot was 2.5 L (upper diameter 16 cm, height 17 cm, lower diameter 12 cm). Two-liter pots were filled with commercial, non-sterilized garden soil (Saham, Givat Ada, Israel). The composition of the ground consisted of 65% coco coir, 20% peat, 10% tuff (volcanic stones varying from 4 to 10 mm in size), and 5% Multicote® (slow-release fertilizers manufactured by Pt. Multigreen Indonesia, Jakarta, Indonesia), all in a weight-to-weight ratio. The ground was mixed with 30% Perlite (no. 4) for aeration. In each pot, five corn seeds of the cv. Prelude (SRS Snowy River Seeds, Australia, sold by Green 2000 Ltd., Israel) were seeded to a depth of 4 cm. According to the seed company and our experience, this cultivar can reach maturity at 79 days and has cob dimensions of 18.9 (length) –5.3 (width) cm. The plants were developed in a growth room in an artificial light regime of 16 h and 8 h of night (using automatic lighting control), with 45–50% humidity at 28 ± 3 °C (regulated with an air conditioner). Each pot was watered routinely every two days with 100 ml of tap water[25]. The plants' phenological development stages were determined according to Abendroth et al.[26]. Thinning was conducted on day 21 post-sowing (V2 second leaf stage), leaving a single representative plant growing in each pot until the trial's conclusion. The experiment ended after 40 days (at the beginning of rapid vegetative growth phase V4, the fourth leaf stage).

The growth room trials' assessment of plant growth and health

The plants ' late wilt disease severity and growth parameters were measured during two stages of the plant's growing period: on the thinning day and at the experiment's end. These assessments included survival percentage, plant height, above-ground parts' fresh weight, and phenological stage (leaf count). Also, root tissue samples from all the experiments' plants were taken on both sampling dates and freeze-stored (at – 20 °C) for future DNA extraction.

Virulence in a semi-field, open enclosure, full-season pots trial

The full-season study (84 days, R3 milk stage) followed the above methodology with a minor modification: the pre-sowing inoculation (14 days before sowing) involved placing six disks in the designated seed planting area instead of sterilized infected wheat seeds' inoculation. The trial was conducted in 10-L pots (upper diameter 27.5 cm, height 25 cm, lower diameter 21 cm) placed in an open enclosure at the Avni Etan Experimental Farm located in the Golan Heights (north-eastern Israel, 32° 49′ 03.3″ N 35° 45′ 46.4″ E). The experiment's local peat soil was from a field with no known history of LWD infection. This infection is assumed to be minor and lacks significant influence if found to exist. It was mixed with 20% Perlite no. 4 for aerating the ground. Each pot was sown with five Prelude cv. seeds. The seeds were pre-treated with a mixture of thiram, captan, carboxin, and metalaxyl-M, a standard general pesticide treatment. Seed samples were tested before sowing to ensure high vitality. Computerized drip line irrigation was carried out using two liters per pot daily. We regularly (at least once a week) monitored the open enclosure pot experiment and closely tracked the plants' health to detect other diseases that could influence the experiment. Still, no pest control prevention treatments were needed or applied.

The experiment's detailed timetable is seeding – 03/08/2022, thinning to one plant/pot – 14/09/2022 (42 days after sowing, DAS), maize flowers fertilization – 28/09/2022 (56 DAS) and harvest – 26/10/2022 (84 DAS). During the maize cultivation season, the average temperature was 24.5°C, with a minimum of 12.6°C and a maximum of 40.8°C. The fertilization day of maize flowers was set based on the timing of pollination. This time point was determined by observing the emergence of silk (the female flower parts) from the ear of the maize plant. Meteorological data were collected by the Avni Etan Experimental Farm station. The average humidity was 66.1%, with a minimum of 18.0% and a maximum of 95.7%. No precipitations were recorded.

The semi-field trials' assessment of plant growth and health

The plants' phenological development stages were determined according to Ref.[26], and the number of surviving plants was determined on each sampling day. On day 42 post-sowing (V4 stage), thinning to one plant per pot was performed. At this halfway point, middle sampling records of various plant development parameters included height, above-ground parts' fresh weight, number of leaves, and flower count. The harvest occurred on day 84 (Stage R3), where height, weight, number of leaves, and ear weight were recorded.

Furthermore, signs of wilt disease were documented, focusing on the number of dry leaves and presenting symptoms in both lower stems (first above-ground internode) and ears. The evaluation of wilt within various plant components and the plant as a whole was categorized into four groups: dead, severe (showing clearly noticeable signs of dehydration), mild (displaying minor signs of dehydration), and healthy[27]. At mid-season and harvest sampling, all plants' roots or first above-ground internodes (respectively) were cut and kept frozen at – 20 °C until DNA extraction and purification.

Molecular analysis of *M. maydis*

qPCR detection was applied to track *M. maydis* and *F. verticillioides* DNA levels in the plant tissues (roots up to day 42, V4 stage, and first above-ground stem internode at day 84, R3 stage)[25]. DNA was extracted from the tissues of 8–10 plants per treatment. The plants' parts were washed thoroughly with running tap water and then incubated in 1% sodium hypochlorite (NaOCl) and sterile tap water for 10 min each. The plants' parts were cut into ca. 2 cm sections, and the total weight of each repetition was set to 0.7 g.

The fungal DNA was isolated and extracted according to a previously published protocol[28] with slight modifications[9]. Briefly, 0.7 g of maize plant tissue was ground with 4 ml of hexadecyltrimethylammonium bromide (CTAB). Afterward, 1.2 ml was transferred to the microfuge test tubes and heated at 65°C for 20 min. The tubes were centrifuged at 13,000 rpm at room temperature (24°C) for 5 min, and the supernatant (700 µl) was moved to new tubes with an equal volume of chloroform/isoamyl-alcohol (24:1). The last cleaning stage was repeated twice. The upper segment (300 µl) was transferred to a new microfuge test tube with 200 µl of previously cooled isopropanol, and the test tubes were maintained at 20°C for 20–60 min. The DNA was then concentrated at 12,000 rpm for 20 min at 4 °C, and the liquid was removed. The DNA precipitate was redissolved with 0.5 ml of ethanol. After an additional concentration and ethanol wash cycle, the DNA precipitate within the tubes was desiccated in a sterile environment overnight. Subsequently, the DNA was dissolved in 100 µl of ultra-pure water and maintained at – 20°C until utilized for qPCR.

The molecular detection method is based on a standard qPCR protocol[29] optimized to detect *M. maydis* DNA using species-specific primers[30]. The A200 primers are designed explicitly for *M. maydis*, amplifying a segment initially identified as specific through amplified fragment length polymorphism (AFLP)[30]. The primers used for *F. verticillioides* recognition amplify a section unique to this fungus species that encodes for the calmodulin protein[31,32] (Supplementary file, Table S2). The mitochondria's last enzyme in the cellular respiratory electron transport chain, the cytochrome C oxidase COX gene, served as a housekeeping reference to normalize the relative amount of DNA in the samples examined[33,34]. The COX gene was amplified using the COX F/R primer set (Table S2). The relative gene abundance was calculated according to the ΔCt model[29] and the same efficacy was assumed for all samples.

All qPCR reactions were performed in four technical repeats. Amplifications were made using the CFX 384 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and 384-well plates. The reaction volume was 5 µl/well. Each well contained 0.25 µl of the forward and backward primers (at 10 µM concentration), 2.5 µl of iTaq™ Universal SYBR Green Supermix solution (Bio-Rad Laboratories Ltd., Hercules, CA, USA), and 2 µl of diluted DNA sample. Reaction conditions were 95°C for 60 s (pre-cycle activation phase), followed by 40 cycles of 95°C denaturation phase for 15 s, 60°C for 30 s (annealing and extension), and finally, the formation of a melting curve.

Statistical analysis

The growth room and semi-field potted experiments were conducted in a fully randomized design. Analysis and statistical processing were performed using GraphPad Prism software, version 10 0.3 (275) 09/2023 (GraphPad Software Inc., San Diego, CA, USA). Some statistical processing was conducted using the R program, version 4.3.1. All data presented (in vitro and in vivo trials) were analyzed utilizing a Shapiro–Wilk normality test followed by a one-way analysis of variance (ANOVA) and then the Fisher's Least Significant Difference (LSD) test, which restricted comparing the experimental groups against a single control group at a significance level of $p < 0.05$. When normality distribution couldn't be identified ($p < 0.05$), the Kruskal–Wallis nonparametric and posterior Dunnett's tests were applied in several cases.

Due to the difficulty of uniformly inoculating the plants, high variability in the pot assay results (high standard error values) was expected. This made it challenging to obtain statistically significant differences. To maintain the power of the test and reduce the risk of type II errors, we opted not to adjust for multiple tests and used Fisher's Least Significant Difference (LSD) test.

Results

To gain a clearer understanding of the effects of co-infection by *M. maydis* and *F. verticillioides* on LWD outcomes, this study conducts in vitro confrontation tests and evaluates the inhibition of antifungal metabolite secretion. Additionally, it examines the sequence of fungal infections in plants before, during, and after seeding.

In vitro experiments

Growth rate and plate confrontation assay

Both fungi can develop easily on a rich medium (PDA) at a similar growth rate of ca. 12–12.8 mm/day (Fig. 1A). *Fusarium verticillioides* growth was significantly ($p < 0.05$) faster in the first three days. The plate confrontation assay evaluated the *F. verticillioides-M. maydis* co-relationships. When seeded together on the two poles of a growth plate, a dark line formed between the colonies' edges, implying antagonistic interactions (Fig. 1B). Some space in this dividing line between the pathogens is also apparent, suggesting that secreted antifungal metabolites from either competitor or both are involved in the mutual fungal growth repressions.

Secreted metabolite effect on colony growth

The influence of fungal extrolites on the growth of the other species colonies was assessed on day 4 of post-sowing. In this test, the growth medium embedded with the secreted fungal metabolites was autoclave-sterilized. Such a high temperature of 120°C destroys heat-sensitive metabolites' structure and function. Thus, the secreted metabolite assay performed here can reveal only a partial picture of the non-direct *M. maydis-F. verticillioides* crosstalk. For comparison, all isolates were cultivated on a regular PDA medium. Figure 2 depicts the average radius of the colonies and their photos. It can be observed that *M. maydis* isolate Hm2 does not exert a significant inhibitory impact on itself nor the *F. verticillioides* compared to the control group. Conversely, a significant ($p < 0.05$) growth delay of approximately 17% is observed in isolate Hm7 (grown on Hm2-secreted products). This isolate hindered its own growth by roughly 11% but didn't alter the development of the other isolates tested. *Fusarium verticillioides* exudates significantly influenced the *M. maydis* isolates (2 and 7) and to a lesser degree itself.

In vivo experiments

To uncover the impact of *F. verticillioides-M. maydis* interactions, we conducted two separate pots experiments: a half-season study in a growth chamber (40 days, Stage V4) and a full-season study (84 days, Stage R3) conducted in field conditions at the Avni Etan Experimental Farm (Supplementary file, Fig. S1). These experiments explored various inoculation methods: singular pathogen inoculations, combined inoculations involving both pathogens; and sequential infection, where one pathogen was introduced either 14 days before sowing or on the sowing day, followed by the introduction of the other pathogen on the seeding day or one week thereafter (respectively). Growth and health evaluations and qPCR molecular tracking of the pathogens inside the host tissues followed these protocols. The data obtained were compared to healthy control plants in uninfected soil. No random effect with statistical significance was found in the pots' setting.

Sprouts pathogenicity evaluation

The growth chamber treatments slightly impacted the sprout above-soil surface emergence (Supplementary file, Table S3). Even the double inoculation (with both pathogens) with the seeding was harmless in this measure. Still, the sequence inoculation started from the sowing day marked reduced emergence (statistically significant at growth day 4 in the Mm 0 + Fv + 7 treatment). At the mid-sampling (day 21**,** Stage V2), the least pronounced effect was observed when the inoculation occurred two weeks before sowing (Fig. 3A–D). In contrast, introducing the pathogens with the seeding (mutually or in sequence) resulted in the most significant ($p < 0.05$) development delay and the highest mortality rate. In addition, survival was impaired (31-34% mortality without statistical significant) by the following treatments: *F. verticillioides* 14 days before seeding and ordered infection with the two pathogens, which started on that day. The most severe disease symptoms were recorded in the seeding inoculation treatment when *F. verticillioides* preceded *M. maydis*.

At the end of the growth room trial (day 40**,** Stage V4), adding the pathogens one after the other was more damaging than simultaneous infection (Fig. 3E–H). Still, those treatments only reached statistically significant differences from the control in the survival measure. Here, too (as in the mid-sampling), *F. verticillioides* inoculation followed by *M. maydis* was harmful, as indicated by the significantly low survival rate caused when this sequence was applied 14 days before sowing. Furthermore, ordered infection on the sowing day, regardless of the pathogen implementation sequence, led to significant sprout death (75%). Although these treatments were

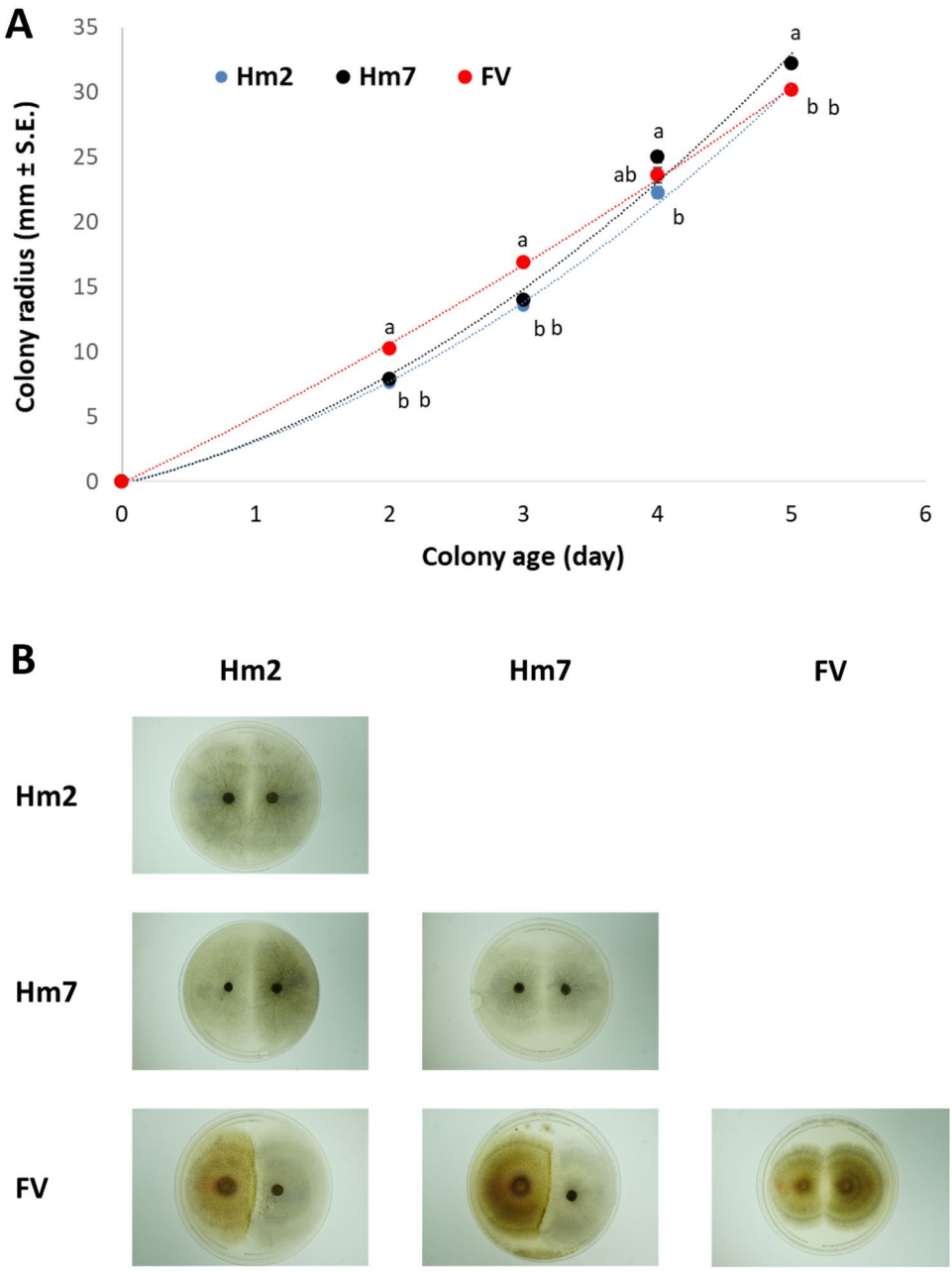


Fig. 1. Colony growth rate (**A**) and plate confrontation assay (**B**). The fungal colonies were incubated at 28 ± 1°C in the dark on potato dextrose agar (PDA) medium. The growth assay (**A**) included six biological repeats. Error bars indicate standard error. Different letters (a-b) above the chart's bars represent an ANOVA test significant difference ($p < 0.05$) in each growth day. The plate myco-antagonism test (**B**) aimed at assessing the interaction between *Magnaporthiopsis maydis* isolates (Hm2 and Hm7) and *Fusarium verticillioides* (ATCC 204499, FV), which were cultured separately and together on media plates for five days until colony confluence was achieved. In this assay, the isolates' names on the upper X-axis represent the right isolate on the plate, while those on the Y-axis represent the left isolate. The presence of a black band in the meeting area between the colonies indicates species antagonistic interactions (growth restriction).

not statistically significant regarding growth measures, they caused a 46–50% decrease in the surviving plants' fresh weight.

Comparative analysis summarizing the total impact of all measures collected in both sampling days of the growth room trial (Supplementary file, Table S4) highlights that the development process was most substantially disrupted when sprouts were first inoculated with one pathogen, followed by the other (*F. verticillioides* first was more influencing). The mutual infection was less impactable. These events were much more severe when applied from sowing onwards than two weeks earlier. An additional conclusion is that *F. verticillioides* was more aggressive at this growth phase than *M. maydis* when applied alone 14 days before planting.

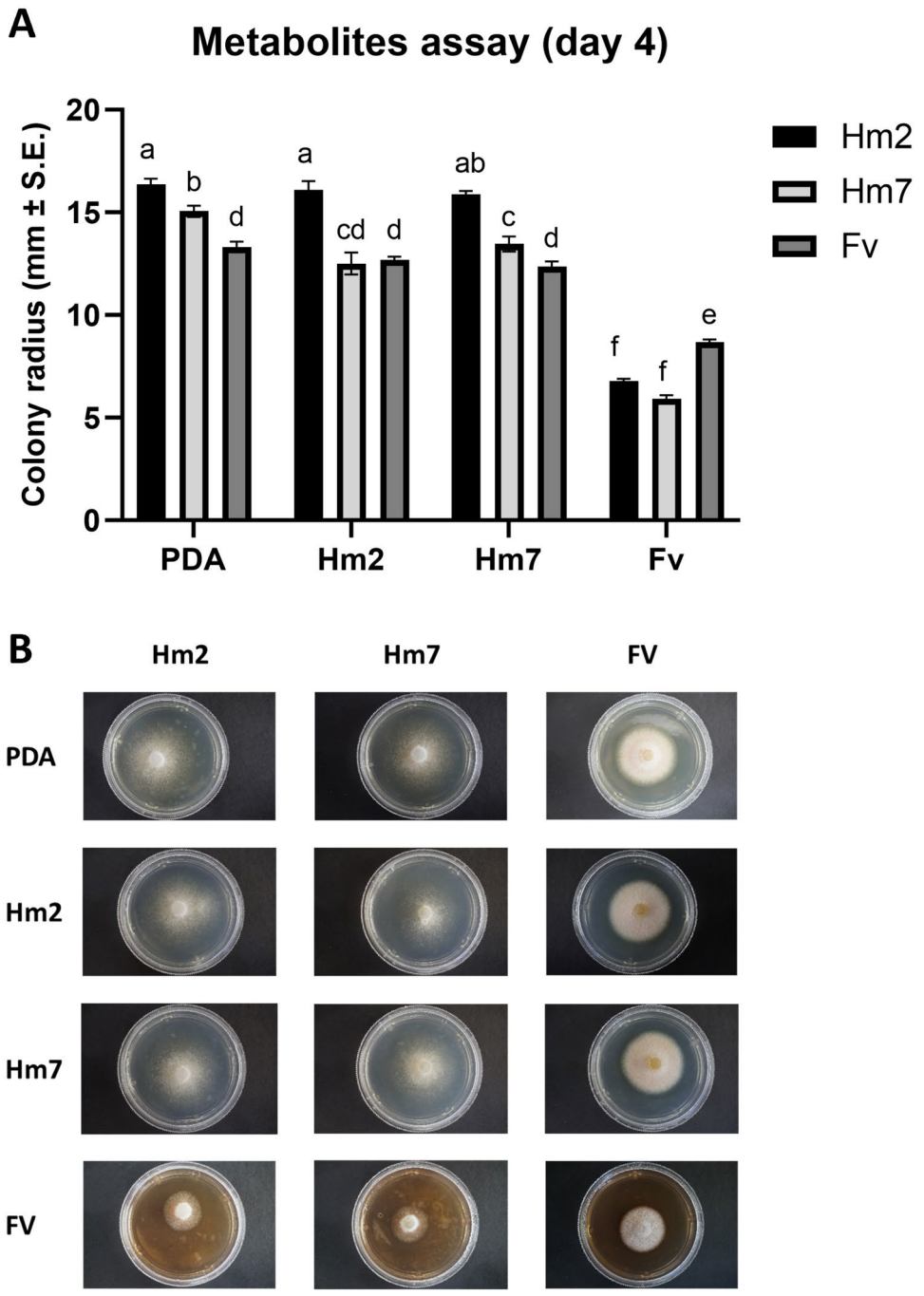
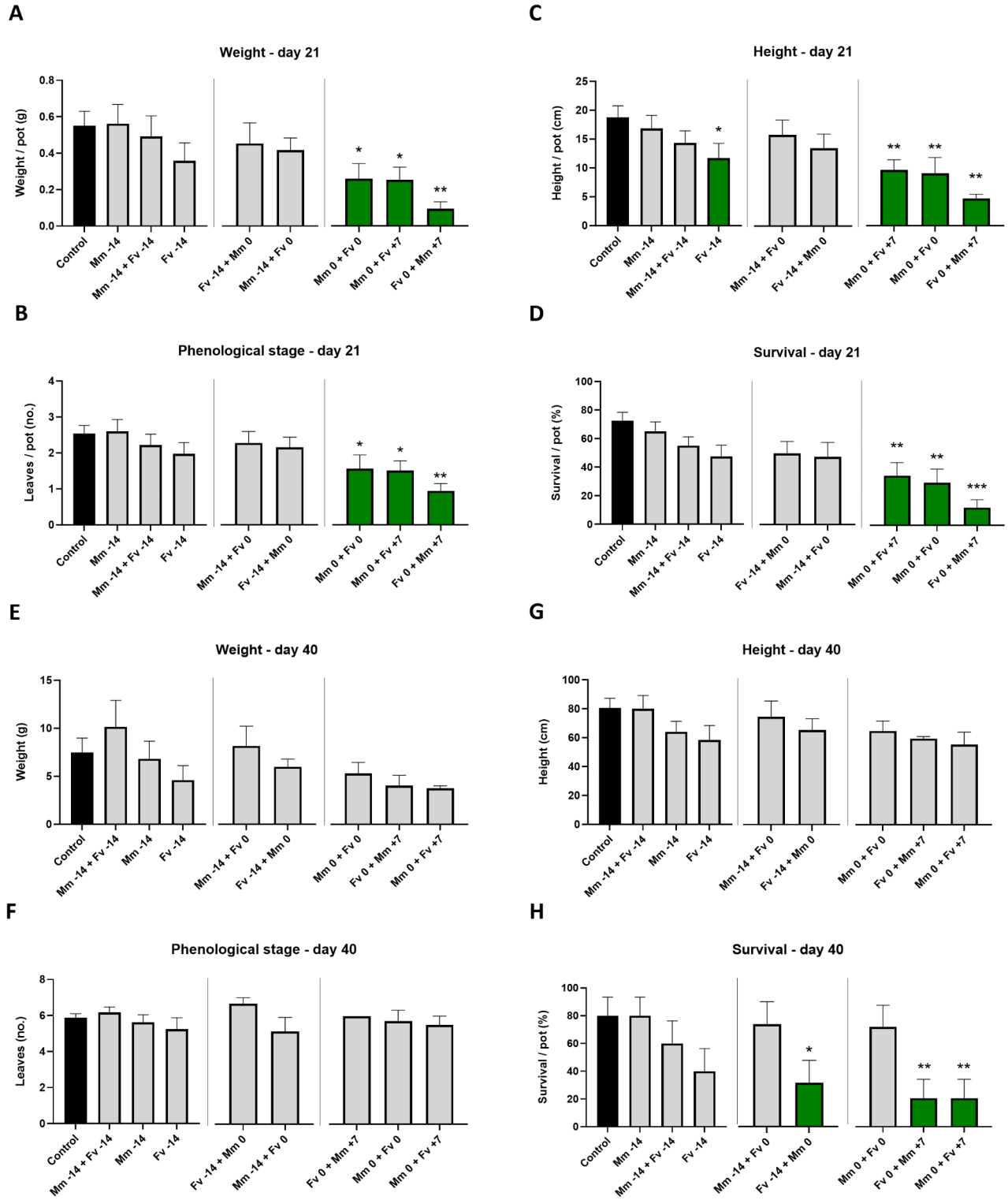


Fig. 2. Secreted metabolites plate inhibition assay's colony radius (**A**) and photos (**B**). Colony growth rate of *M. maydis* isolates Hm2 and Hm7 and *F. verticillioides* (FV) was observed on a rich PDA medium supplemented with fungal metabolites derived from the three isolates. The X-axis in (**A**) and Y-axis in (**B**) represent the fungal growth products-based substrate. The other axis displays the various isolates cultivated on that growth medium. As a control, all isolates were grown on a regular PDA medium. Incubation took place in a dark environment at 28 ± 1°C for four days. Each isolate was subjected to four replicates, and the average colony radius was measured at the end of the cultivation period. Error lines represent a standard error. Different letters (a-f) above the chart's bars represent an ANOVA test significant difference ($p < 0.05$).

The qPCR assessment of the pathogens' DNA inside the plants' roots provides another insight into the infection order effect (Fig. 4). Comparing the *M. maydis* (Mm) DNA fluctuations to those of *F. verticillioides* (Fv) revealed almost identical patterns with statistical significance in some treatments. At day 21 (Stage V2), those were peaked in the Fv -14 + Mm 0 (*M. maydis* DNA) and Fv 0 + Mm + 7 (*F. verticillioides* DNA) treatments. At the end of the growth room experiment, the simultaneous dual infection 14 days pre-sown or at seeding evoked



an intense *M. maydis* infection level. This was the exact picture in the *F. verticillioides* results, with one exception. This species' DNA levels were also highest in the Mm -14 + Fv 0 treatment.

Virulence in a semi-field, open enclosure, full-season pots trial

The advantages of an open enclosure, full-season pots trial over a commercial field trial allow us complete control over soil composition, amount of water, fertilizer, and, more importantly, infection order and load. The semi-field study also permits adding a control group of healthy plants and a random design to distribute the treatments while preventing unwanted mutual influence between them. In fields, even those known to harbor the pathogen, there is an inherent variability in pathogen distribution and resulting disease manifestation[7,9]. Consequently, pot cultivation aids in obtaining a robust and homogenous infection to the greatest extent feasible.

◂**Fig. 3**. The growth room experiment development and survival indices on days 21 (**A**-**D**) and 40 (**E**–**H**) after sowing (Stages V2 and V4). Prelude cv. maize sprouts were grown under controlled conditions and the flowing growth values were estimated: (**A**,**E**) wet weight, (**B**,**F**) phenological stage (number of leaves), (**C**,**G**) plant height, and (**D, H**) the serving plants' percentage. Control—uninfected healthy plants. The plants were inoculated separately, in combination, or sequentially by *M. maydis* (Mm) and *F. verticillioides* (Fv). These protocols were applied 14 days before sowing (-14) and at seeding (0). In the sequential infection, which started from sowing, the second inoculation occurred a week later (+7). Each value is a mean of 4–10 repetitions (plants per treatment). Error lines represent a standard error. The statistical significance of variance between each group and the control (highlighted in black) was tested using the one-way analysis of variance (ANOVA) assessment and the Fisher's Least Significant Difference (LSD) posthoc test. When normality distribution couldn't be identified (**D**,**H**), the Kruskal–Wallis nonparametric and posterior Dunnett's tests were applied. Significance differences are represented by green bars and asterisks above the chart bars (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$).

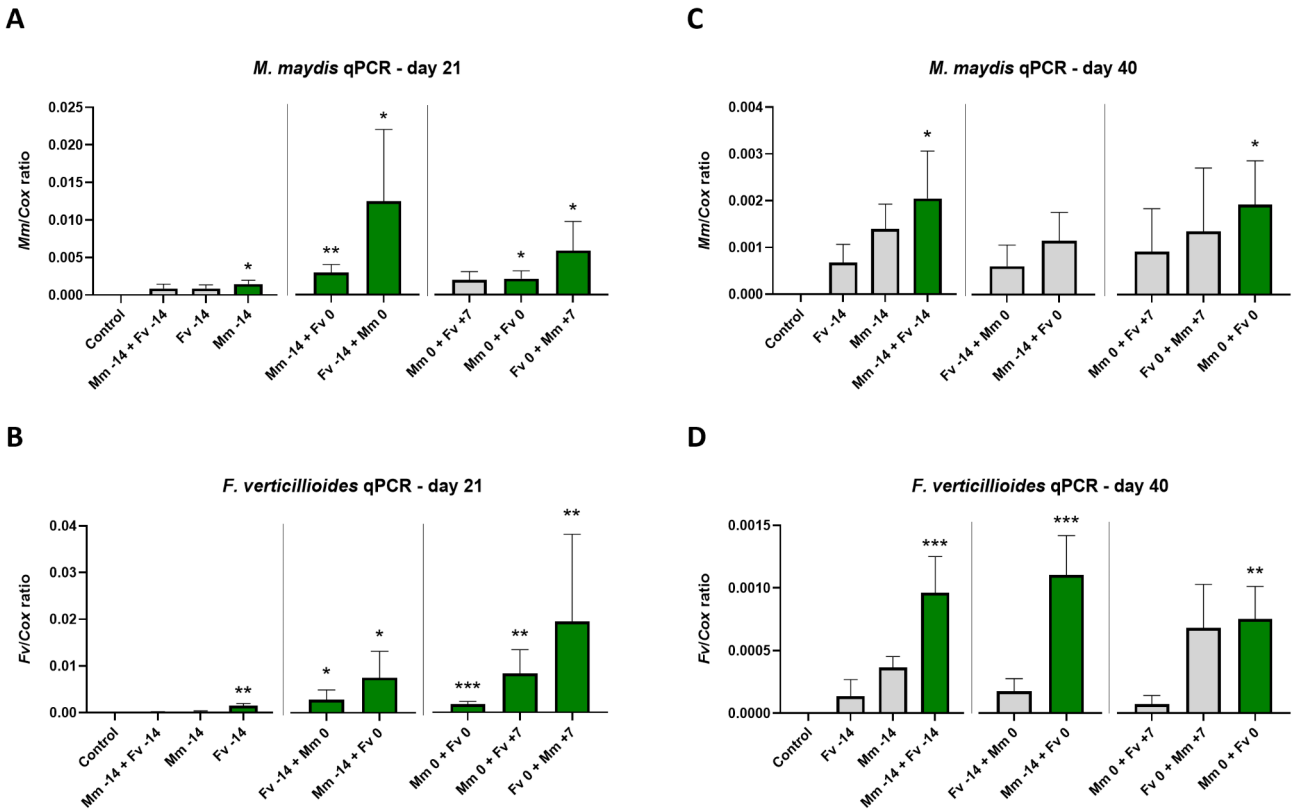


Fig. 4. Growth room experiment quantitative real-time PCR (qPCR) analysis days 21 and 40 after sowing (Stages V2 and V4). The plants were inoculated separately, in combination, or sequentially by *M. maydis* (Mm) and *F. verticillioides* (Fv). These protocols were applied 14 days before sowing (-14) and at seeding (0). In the sequential infection, which started from sowing, the second inoculation occurred a week later (+7). The Y-axis shows the ratio of the specific *M. maydis* or *F. verticillioides* DNA (**A**,**B**, day 21, Stage V2, and **C**,**D**, day 40, Stage V4) to the housekeeping gene-encoding cytochrome C oxidase (COX). Values represent an average of 8–10 repetitions (plants per treatment). Error lines represent a standard error. The statistical significance of variance between each group and the control (highlighted in black) was tested using the one-way ANOVA (or Kruskal–Wallis assay, **A**,**B**) and is represented by green bars and different asterisks above the chart bars (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$).

This study aimed at understanding the impact of a combined infection compared to *M. maydis* or *F. verticillioides* alone, or in order, on both 14 days before sowing (day -14) and with the sowing (day 0). Unexpected for the mid-season sampling, day 42 (Stage V4), the overall plants' growth in most infection treatments was better than the non-infected control group (Fig. 5). On that day, the average flower/pot was 1.35, indicating stress-induced flowering response. Still, one treatment group did not record such a response. In all growth and survival parameters measured, the plants that were inoculated on the sowing day with each pathogen alone or with both simultaneously had reduced growth significantly ($p < 0.05$) compared to the plants that were inoculated 14 days pre-sowing or the sequence infection that started from day 0 onwards. In some measurements, such as weight, the enhanced growth-related stress response was less evident.

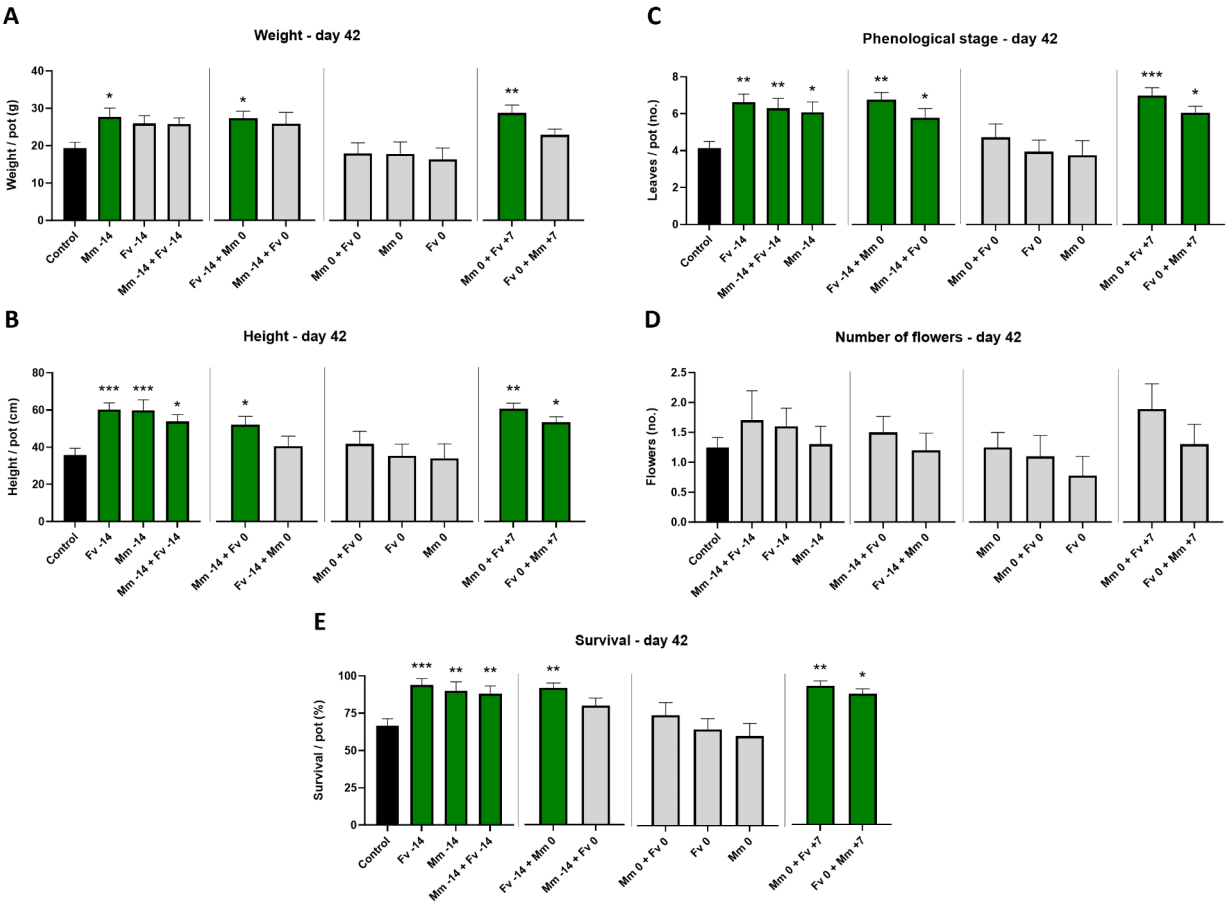


Fig. 5. Semi-field experiment growth indices on day 42 after sowing (Stage V4). The plants were inoculated separately, in combination, or sequentially by *M. maydis* (Mm) and *F. verticillioides* (Fv). These protocols were applied 14 days before sowing (-14) and at seeding (0). In the sequential infection, which started from sowing, the second inoculation occurred a week later (+7). (**A**) wet biomass, (**B**) plant height, (**C**) phenological stage (leaves count), (**D**) number of flowers, and (**E**) the surviving plants' percentage. Control—uninfected healthy plants. Each value is a mean of 8–10 repetitions (average/pot per treatment). Error bars signify the standard error. The statistical significance of variance between each group and the control (highlighted in black) was tested using the one-way ANOVA (or Kruskal–Wallis assay, **E**) and is represented by green bars and different asterisks above the chart bars (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$).

The mid-season stress-related growth enhancement was not observed at harvest (day 84, Stage R3, Fig. 6). In these mature plants, the disease was notable – up to 90% symptomatic plants and 30% growth reduction (as will be elaborated below). In most measures, the control of healthy plants achieved the highest health score. Regarding the growth indexes, the infection protocols applied from the sowing (sole inoculation, mutual, and ordered) marked the most severe decrease. Of these (day 0), the most significant was inoculation with *M. maydis* at sowing and subsequently with *F. verticillioides* a week later (Mm 0 + Fv + 7). A meaningful growth compromise is also recorded when the latter fungus precedes the former in the soil (Fv 0 + Mm + 7). Additionally, on one occasion (height valuation), the 14 days of pre-sowing *M. maydis* and *F. verticillioides* add-on led to significant growth suppression.

Notably, applying *F. verticillioides* in conjunction with *M. maydis* on the day of sowing not only reduced plant dehydration and mortality but also mitigated the severity of damage to the ears. In contrast, when both pathogens were present together 14 days before sowing, the growth alone was predominantly compromised. Likewise, such a conclusion can be drawn from summarizing all measures collected in both sampling days of the semi-field trial (Supplementary file, Table S5). A comparative evaluation of the growth and survival indices in the semi-field practice was made to summarize the effects of the two fungi singly or in combination. On day 84 (Stage R3), the total summarization of the indices ranked Mm 0 as the most influential (30% growth reduction), followed by Mm 0 + Fv + 7 (21%), Fv 0 + Mm + 7 (21%), and the double infection with the seeding (20%). The inoculation protocols with Fv 0 and the Mm -14 + Fv 0 were ranked fourth and fifth (12%).

The least influential treatments were *M. maydis* or *F. verticillioides* alone two weeks prior to sowing and the dual infection on that day (0, 4, and 7% growth suppression, respectively). Thus, the soil pre-infection was far less harmful than applying the pathogens during seeding. The two mid-influencing sequenced treatments

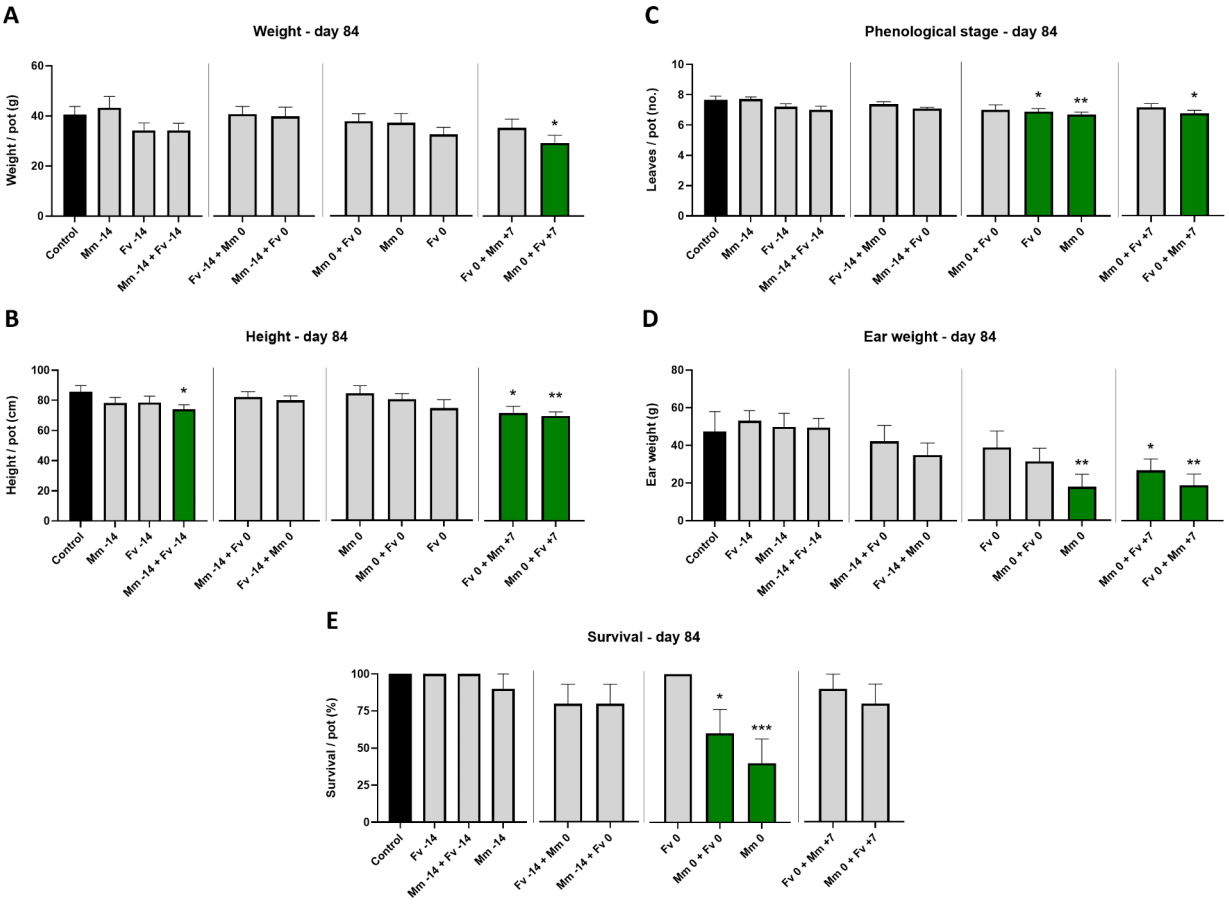


Fig. 6. Semi-field experiment growth indices on day 84 after sowing (Stage R3). The plants were inoculated separately, in combination, or sequentially by *M. maydis* (Mm) and *F. verticillioides* (Fv). These protocols were applied 14 days before sowing (-14) and at seeding (0). In the sequential infection, which started from sowing, the second inoculation occurred a week later (+7). (**A**) fresh weight, (**B**) plant height, (**C**) phenological stage (number of leaves), (**D**) ear weight, and (**E**) the surviving plants' percentage. Control—uninfected healthy plants. Each value is a mean of 9–10 repetitions (plant per treatment). Error bars signify the standard error. The statistical significance of variance between each group and the control (highlighted in black) was tested using the one-way ANOVA (or Kruskal–Wallis assay, **C**,**E**) and is represented by green bars and different asterisks above the chart bars (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$).

started with one pathogen applied 14 days before sowing, and the second inoculation event was performed on the seeding day.

The ear, stem, and leaf dehydration symptoms on day 84 after sowing (Stage R3) generally agreed with the growth and survival data. Those evaluations in total (Supplementary file, Figs. S2, S3) or separately (Fig. 7) pointed out that introducing *M. maydis* at sowing alone (65% severely diseased plants), together (40%), or the first in a sequence with *F. verticillioides* (20%) led to the harshest disease (Supplementary file, Fig. S3). Even two weeks before sowing, if *M. maydis* was applied first or second (at sowing), the harshly diseased plant percentages were high (20%), although dead plants (10%) were only identified in the Fv -14 + Mm 0 treatment (Supplementary file, Fig. S3).

Comparing the plants' different organs' wilt symptoms by their deviations from the control group showed that dry leaves were the most significant. In contrast, ear symptoms were relatively minor (Fig. 7). In all organs tested, adding *M. maydis* to the seeds on the sowing day was the worst for plant health. In contrast, *F. verticillioides* alone had a neglectable health risk in this experiment. Additionally, the two weeks' pre-sowing soil inoculation was also less influential.

Magnaporthiopsis maydis qPCR tracking on day 84 (R3 stage) closely aligns with the data of growth, survival, and symptoms (Fig. 8). In particular concerning disease symptoms (ear, stem, and leaf dehydration), Mm 0 was observed to be the most aggressive, followed by Mm 0 + Fv + 7 (*M. maydis* first at sowing and *F. verticillioides* second a week later). Reduction in infection was pronounced when the latter fungus precedes the former in the soil. Still, infection levels remained high when they were inoculated simultaneously. This suggests that introducing *F. verticillioides* undermines the virulence of *M. maydis* compared to infection with only *M. maydis*. To support

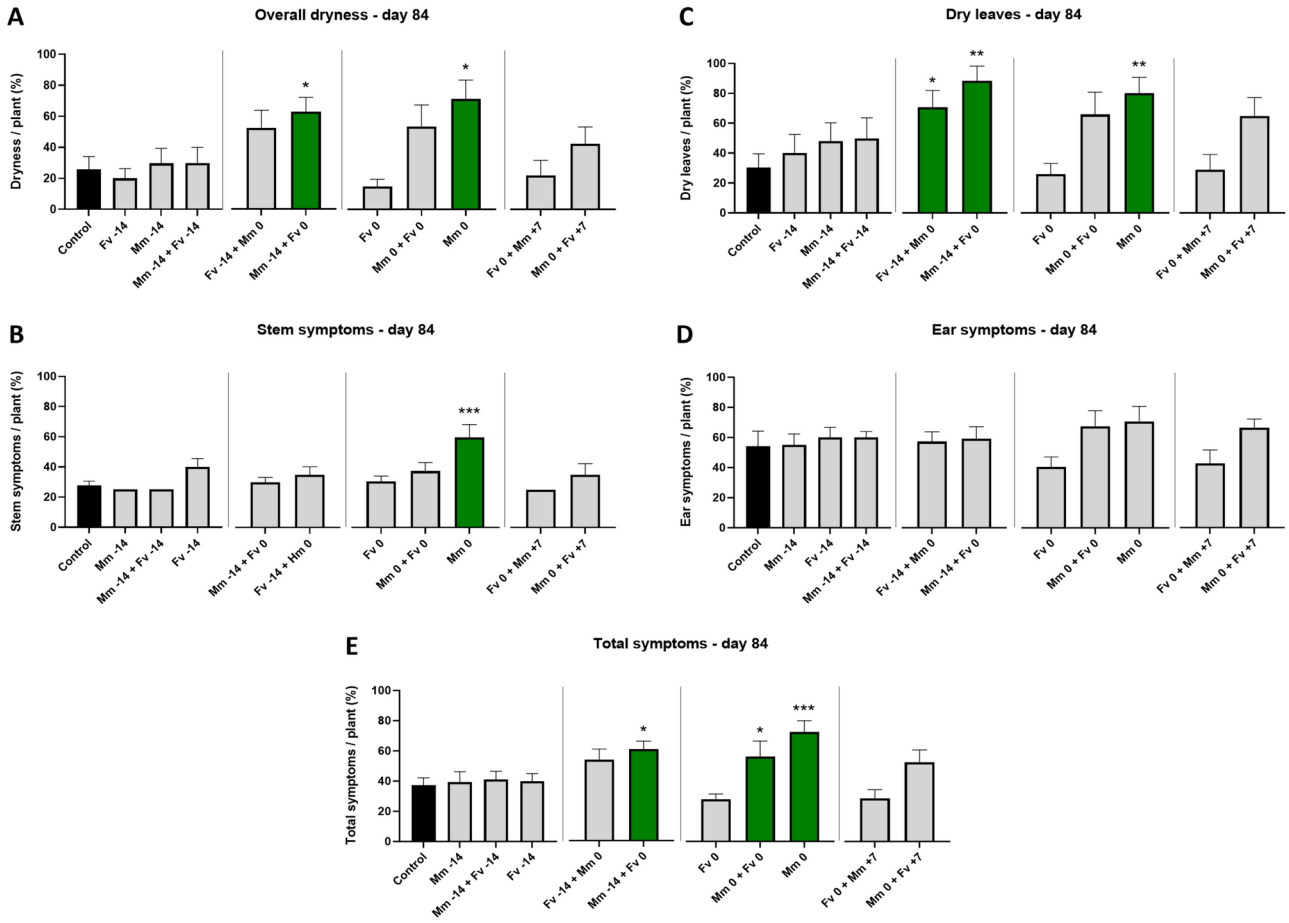


Fig. 7. Semi-field experiment day 84 after sowing (Stage R3) – comparison of the plants' different organs wilt symptoms. The plants were inoculated separately, in combination, or sequentially by *M. maydis* (Mm) and *F. verticillioides* (Fv). These protocols were applied 14 days before sowing (-14) and at seeding (0). In the sequential infection, which started from sowing, the second inoculation occurred a week later (+7). The overall plant health and its various body parts symptoms were estimated according to the four categories: 1—healthy, 2—mild, 3—severe, and 4—dead[34]. (**A**) general disease symptoms, (**B**) stem symptoms, (**C**) dry leaves, and (**D**) ear symptoms. The classification of the lower stem (first above-ground internode) and the cobs' spathes disease symptoms are described in Ref.[27]. The average score of all these (total symptoms) is presented in (**E**). Each value is a mean of 9–10 repetitions (plant per treatment). Error bars signify the standard error. The statistical significance of variance between each group and the control (highlighted in black) was tested using the one-way ANOVA (or Kruskal–Wallis assay, **A**-**C**) and is represented by green bars and different asterisks above the chart bars (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$).

this, the up to 1,000-fold higher *M. maydis* DNA levels in the plants' tissues compared *to F. verticillioides* DNA at the season-ending (day 84, Stage R3) imply that the late wilt agent was the major influencer.

Comparing the DNA variations of the two sampling days reveals an interesting picture. At mid-season sampling (day 42**,** Stage V4), the two pathogens' tracking had similar patterns with significant DNA levels in the sequence inoculation that started 14 days before sowing. Intriguingly, *M. maydis* DNA peaked when *F. verticillioides* was the first in this order, while the latter peaked when *M. maydis* was ahead. Yet, the two pathogens had a varied DNA pattern within the plant host tissues at harvest. At that stage, *M. maydis* infection levels were highest when the pathogen was added with sowing. In contrast, those of *F. verticillioides* rose at the 14-day pre-sowing treatments, particularly in the mutual infection with *M. maydis*.

Discussion

This study aimed to determine the role of *M. maydis* and *F. verticillioides* co-inoculation events on the severity of maize late wilt disease (LWD). The research was inspired by prior studies, which examined similar scenarios between *M. maydis* and *F. oxysporum*[6] and *M. phaseolina*[35]. Late wilt disease in maize represents a significant phytopathological challenge threatening agricultural yield in affected areas[36]. The *M. maydis* pathogen may lead to potential yield loss as high as 100% in food and fodder crops in highly infected areas planted with susceptible maize hybrids. The fungus is part of a group of soil-borne pathogens causing diseases collectively known as the post-flowering stalk rot complex[17], which reduces the yield and quality of crops. *Fusarium verticillioides*, the

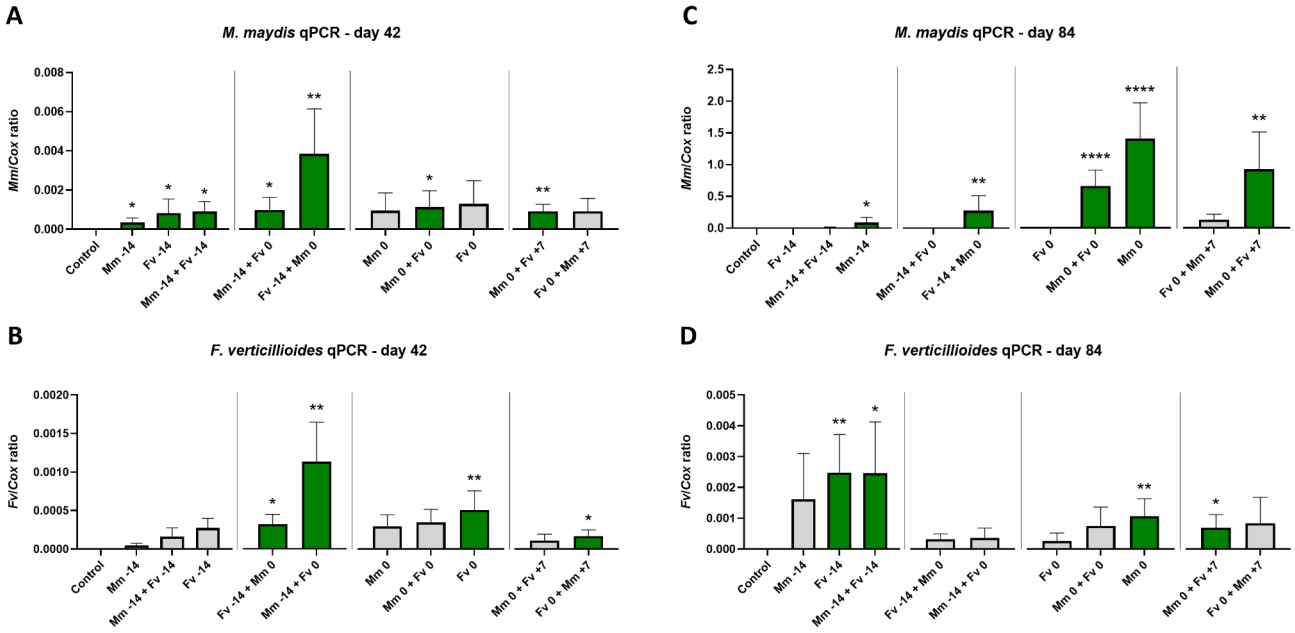


Fig. 8. Semi-field experiment qPCR days 42 and 84 after sowing (growth Stages V4 and R3, respectively). The plants were inoculated separately, in combination, or sequentially by *M. maydis* (Mm) and *F. verticillioides* (Fv). These protocols were applied 14 days before sowing (-14) and at seeding (0). In the sequential infection, which started from sowing, the second inoculation occurred a week later (+7). The Y-axis shows the ratio of the specific *M. maydis* or *F. verticillioides* DNA ((**A**, **B**), day 42, and (**C**, **D**), day 84) to the housekeeping gene-encoding cytochrome C oxidase (COX). Values represent an average of 9–10 repetitions (plants per treatment). Error lines represent a standard error. The statistical significance of variance between each group and the control (highlighted in black) was tested using the Kruskal–Wallis test and is represented by green bars and different asterisks above the chart bars (*$p < 0.05$, **$p < 0.005$, ****$p < 0.00005$).

Fusarium stalk and ear rot causal agent, another member of this complex, induces several deleterious diseases in maize, including seedling blight, stalk, ear, and seed rot[37]. Both fungi are soil- and seed-borne and can be found together in soil or plant residue.

The current study focuses on revealing the role of each of these pathogens in co-infection scenarios. Co-colonization by fungi is likely the rule rather than the exception[38]. So far, it has been generally assumed, according to several studies[10,11], that these pathogens act synergistically to enhance crop damage. Yet, the degree of dominance of each of the two pathogens in the co-infection event and the impact of the inoculation sequence on host colonization during growth has remained obscure. Thus, it would be valuable to investigate further the underlying mechanisms and potential interactions between these pathogens to understand their collective impact on plant health and development, as was done here.

As Saleh et al.[39] noted, *M. maydis* is slow-growing compared to other, more fast-growing fungi, specifically *Fusarium* spp. Indeed, *F. verticillioides* grows significantly faster than *M. maydis*. The first indication of *F. verticillioides-M. maydis* mutual inhibition or competition can be drawn from the plate's confrontation and secreted metabolites assays. A typical response between two species that inhibit each other's growth on an artificially rich medium is the formation of a dark borderline between them[40]. The appearance of hyphal granules, dark gel-like structures and vacuoles observed in fungal interactions may indicate cell death due to mycoparasitism or nutrient deprivation due to intense competitive interactions. A brown-black line demarking the combating fungal strains could be linked with melanin, 1,8-dihydroxynaphthalene (DHN), a defense against environmental stresses[41]. It was reported that in *Armillaria mellea*, melanized hyphal cells derived from different fungal species of the complex constituted the black line[40]. This demarcation area was flanked on each side by the vesicular cells forming the pseudosclerotial plates characteristic of each respective species.

The formation of a dark borderline between *M. maydis* and *F. verticillioides* implies antagonism between the two fungi. In line with this, the secreted metabolites assay results suggested that *F. verticillioides* extrolites inhibit *M. maydis* growth. Indeed, *Fusarium* spp. produces toxins[20] that are important in pathogen-pathogen interactions. Previous work focusing on maize kernels and in vitro evaluations[42] have demonstrated that interactions between *F. verticillioides* and *Aspergillus flavus* reduce fumonisins and aflatoxins biosynthetic gene expression profiles. Such an interaction could exist here and should be explored in future studies.

The full-season study was conducted here in its current form due to practical limitations. Still, it would be beneficial to conduct such studies in two Agricola cycles that would provide a better understanding of the two pathogens under different environmental conditions. Indeed, environmental factors are the third important aspect (together with host plant susceptibility and pathogens' composition and aggressiveness) that should be studied to comprehensively understand disease impact and risks. The current study focuses on the biotic

environmental factors determining LWD severity and outcome. More studies are needed to unfold the role of pathobiome members in the host and the environment affecting LWD, like the works that explored *M. maydis* -*M. phaseolina* interactions[3,35].

An additional essential angle is deepening our understanding of the pathogen populations' intraspecies interactions. Significant variations in growth patterns and aggressiveness among *M. maydis* isolates exist[22,43,44]. Moreover, virulent strains of *M. maydis* are dispersed throughout Israel, affecting plant growth and disease symptoms differently. With this scientific understanding progress, significant knowledge gaps remain regarding the roles and impacts of other environmental (biological and physical) factors on the disease triangle. Such gaps in understanding are inspiring targeted studies.

The sequence and timing of pathogen introduction proved to play a pivotal role in influencing growth rates and plant health. Differences between the growth room and the semi-field full-season trial are expected. Such differentiations arise from the two experiments' varied growth conditions, including pot size, light intensity and composition, soil type, humidity, temperature, etc. Therefore, comparing the experiments according to sheer numeric values is less critical than contrasting the influence of all treatments on the maize plants.

The two experiments had quite different outcomes on days 40–42 post-seeding (Stage V4). The plants developed more slowly in the optimal controlled atmosphere in the growth room and reacted to the pathogens' infiltration primarily by growth repression. In contrast, in the open enclosure trial on the same growth day, the overall plants' growth in most infection treatments was better than in the non-infected control group. This response is likely related to the plant's promotion of persistence and propagation under the pathogen stress related to insufficient water supply[45]. Indeed, plants can adjust their developmental progress in reaction to various stressors. Within the context of pathogen-induced stress, plants may instigate the onset of flowering as an expedient reproductive strategy to secure the production of offspring[46]. Notably, on day 42 of growth, the mean number of flowers per pot was 1.35, signifying an opportune moment for activating this response. To the best of our knowledge, this is the first report on enhanced development response to *M. maydis*- and *F. verticillioides*-induced stress.

Exploring this behavior further and revealing its triggers and mechanisms would be very interesting, especially since the response was temporary and didn't affect the season-ending disease outburst. Other plant host-fungal pathogen studies (for example, *F. oxysporum* accelerated flowering response in Arabidopsis[47,48]) could provide essential clues. Pathogen infection generally increases plant hormone levels (salicylic acid, jasmonic acid, and ethylene), accelerating flowering[47]. The weaker inoculation impact of the pre-sowing treatment could have been overcome by the plants that accelerated their growth. In contrast, the stronger infection outcome at sowing may mask this effect, as happened here.

Therefore, comparing the concluding sampling days of both trials is more scientifically appropriate. In particular, this entails the comparative analysis of the results obtained on growth room day 42 (Stage V4) in contrast to those from semi-field day 84 (Stage R3). Indeed, both experiments supported each other and led to similar conclusions. The soil inoculation at sowing in general, and with *M. maydis* alone in particular, led to the most severe wilt disease. Moreover, this pathogen is probably more aggressive than *F. verticillioides*, but the latter has a better saprophytic ability, enabling it to survive better in bare soil, as proposed earlier[49]. Another important conclusion is that antagonistic interactions between the two pathogens do exist and could result in decreased disease outcomes, mainly if the two pathogens are introduced to the host simultaneously. Still, should *M. maydis* successfully infiltrate a plant and instigate a disease, it is conceivable that subsequent *F. verticillioides* establishment may exacerbate the damage, as evidenced by existing reports[10,11]. Also, we must consider that, like *M. maydis*[22], *F. verticillioides* can undergo pathogenic variations[50] and virulent strains of the fungus may significantly alter the results.

Egyptian researchers[11] compared the impact of inoculating maize with *F. verticillioides* alone and with *M. maydis* on maize ears' quantity, weight, and rot. They found that disease severity was considerably higher in the combined inoculation. Another observation was that when maize was inoculated with *M. maydis* and started exhibiting disease symptoms, subsequent infection with *F. verticillioides* resulted in a less severe disease than when using *F. verticillioides* alone. Our observations align with these findings.

A central inquiry emerges regarding why *M. maydis* inoculated 14 days before sowing (Mm -14) is less virulent than when it is inoculated on the day of sowing (Mm 0). One might anticipate that pre-soil-establishment of *M. maydis* at -14 days will lead to a more intense sprouts' infection. Such a pattern appears evident with *F. verticillioides*. It's plausible that soil conditions weren't conducive for *M. maydis* proliferation, as anticipated earlier[49]. Consequently, its potency may have been compromised when it endured a two-week waiting period before the plant colonized. When introduced concurrently with the seeds, *M. maydis* would bypass dependence on soil conditions and directly infect the maize. Combining inoculations of *M. maydis* 14 days before sowing and *F. verticillioides* on the day of sowing (Mm -14 + Fv 0) could provide an opportunity for *F. verticillioides* to flourish unimpeded by *M. maydis*, making the disease primarily attributable to *F. verticillioides*. This hypothesis is supported by the DNA analysis of this treatment at the mid-season sampling. Still, it warrants further validation.

Second to the *M. maydis* sole infection with seeding, the most pronounced impediment to plant development across all parameters was observed in two treatments where plants were exposed to one fungal species on the day of sowing and a week later to the second species. Despite a similar 80–90% survival rate in these treatments, the plants displayed minor disease symptoms when *F. verticillioides* was the first in the soil. So, upon *F. verticillioides* colonization of the host plant, it most likely inhibits the colonization of *M. maydis*, thereby attenuating the progression of the disease. Still, this sequence of events hindered the host plant growth. These new data raise questions regarding the two pathogens' intimate interactions within the host, requiring further investigation.

A former study on cotton found that *M. maydis* could successfully infect the plants when it precedes *M. phaseolina* in the soil[35]. This scenario, similar to the relationships of the two pathogens studied here, implies a repeat pattern. It would be very interesting to investigate the interactions of *M. maydis* with other post-flowering

stalk rot disease complex members and expand the research to include more than two pathogens. In situ studies could illuminate the pathogens' competition for space or nutrition resources. The qPCR used here could provide a sensitive method to track multiple pathogens inside plant tissues during pathogenesis using species-specific primers[51,52].

The lifestyle of each pathogen should be considered since *M. maydis* is hemibiotrophic in nature[7]. At the same time, *F. verticillioides* produces toxins that kill the host, tending towards necrotrophic behavior[53]. It should be emphasized that while the latter was studied extensively due to its wide distribution and hosts[54], *M. maydis* is far less familiar to the phytopathologists' community. Consequently, this late wilt pathogen's secreted metabolites[55], including potential toxins, are yet to be discovered. One such metabolite (6-Pentyl-α-Pyrone) was recently reported[25], revealing the importance of this research direction.

Following this, the pathogens could sometimes be dormant, i.e., opportunists. So, their interactions and the stresses they cause to the host plant can be much more complex since their surrounding (host and environment) dependent activity could be crucial[35]. In addition, the soil microbiome and conditions that impact the ability of each pathogen to survive on the bare ground, especially for long periods, have a major influence on disease outcome, as demonstrated here. The microbiome community can be manipulated by the enrichment of plant-friendly members or organic and other compounds[56,57]. This will eventually provide the crops with better immunity to diseases, as discussed in many studies (see, for example Ref.[58]). While the soil microbiome is one line of defense, the plants' protective endophytes are another barrier[4,59]; both are essential for future scientific studies.

Conclusions

Maize late wilt disease (LWD), caused by the phytopathogenic fungus *M. maydis*, is endemic to Israel, Egypt, Spain, Portugal, India, and a few other countries, remaining unfamiliar to the rest of the world. The consequences of LWD can be severe, and the development of effective measures is an ongoing scientific mission. The traditional research paradigm, which focused on a single leading causal agent, has now been replaced by a more holistic approach that considers pathogenesis within the context of the phytobiome concept. This work investigated the co-interactions between *M. maydis* and another post-flowering stalk rot species complex member, *F. verticillioides*. The study is the first to report antagonism that can occur under specific conditions between the two fungal species. It also reveals the significant impact of inoculation sequence, both before and during growth, on disease outcomes. The results lay the groundwork for further exploration of these interactions and the underlying mechanisms that drive them. They also raise critical questions, such as: Do these interactions push *M. maydis* toward a more biotrophic lifestyle (a phenomenon recently reported in the literature)? Does competition for resources, such as nutrients and space, lead the two pathogens to occupy distinct niches within the host? How might this interplay between pathogens and other organisms influence pest management strategies to enhance crop safety and yield? Advanced research techniques, such as green or red fluorescent protein tagging, Laser Ablation Electrospray Ionization (LAESI), and metagenomics, could provide valuable tools to address these questions.

Data availability

All data generated or analyzed during this study are included in this published article.

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Author contributions
Conceptualization, G.S. and O.D.; data curation, G.S. and O.D.; formal analysis, G.S. and O.D.; funding acquisition, O.D.; investigation, G.S. and O.D.; methodology, G.S. and O.D.; project administration, O.D.; resources, O.D.; supervision, O.D.; validation, G.S. and O.D.; visualization, G.S. and O.D.; writing (original draft), O.D.; writing (review and editing), G.S. and O.D. All authors have read and agreed to the published version of the manuscript.

Declarations

Competing interests
The authors declare no competing interests.

Ethics approval and consent to participate
All methods were performed according to the Ministry of Agriculture and Rural Development, Consultation Service (Shaham, Beit-Dagan, Israel), and the Israel Northern Research and Development (Northern R&D, Kiryat Shmona, Israel) guidelines and regulations. These authorities authorized and supervised the experimental research and semi-field study on plants.

Additional information
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Correspondence and requests for materials should be addressed to O.D.

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