



Article

New Antifungal Compound, 6-Pentyl- α -Pyrone, against the Maize Late Wilt Pathogen, *Magnaporthiopsis maydis*

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Abstract: 6-pentyl- α -pyrone (6-PP) is a powerful *Magnaporthiopsis maydis* antifungal compound, recently discovered when the potent growth medium of *Trichoderma asperellum* was analyzed. Despite its high potential in plate assay, it was not inspected for plant treatment prevention. Late wilt disease, caused by the fungus *M. maydis*, threatens commercial maize production in high-risk areas. Thus, the search for control options against the pathogen is one of the top priorities in Israel, Egypt, and other countries. Disease-resistant maize genotypes can reduce the damages. Yet, aggressive variants of the fungus can overcome host resistance. The current study aimed at inspecting *T. asperellum* and its secreted metabolite, pure 6-PP, against the pathogen in plants over a full growth period. First, adding *T. asperellum* directly to seeds with sowing provides significant protection to sprouts (up to 42 days) in a growth room, with more than two-fold growth promotion and reduced pathogen root infection (detected by real-time PCR). The same procedure applied in a commercial field was less beneficial in rescuing the plants' growth and yield. Still, it reduced the cobs' symptoms by 11% and resulted in nine-fold lower levels of the pathogen's DNA in the stem tissue. Second, the *T. asperellum* purified 6-PP compound (30 μ g/seed) was used in seed coating and tested against the *T. asperellum* secretory metabolites' crude (diluted to 50%). At the season's end, these treatments improved plant biomass by 90–120% and cob weight by 60%. Moreover, the treatments significantly ($p < 0.05$) reduced the symptoms (up to 20%) and pathogen infection (94–98%). The current study's results reveal the potential of 6-PP as a new fungicide against *M. maydis*. Such a treatment may protect maize plants from other soil diseases.

Keywords: biological control; *Cephalosporium maydis*; crop protection; fungus; *Harpophora maydis*; late wilt; maize; pathogenicity; real-time PCR; *Trichoderma asperellum*



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1. Introduction

The world's corn (*Zea mays* L., maize) harvested area is the third largest after wheat and rice (FAOSTAT, 2020 Food and Agriculture Commodity Production data), reflecting its importance globally. Maize late-wilt disease (LWD) can cause acute damage to susceptible hybrids in infected areas under favorable conditions. Such a scenario prevails in Egypt [1,2], Israel [3], Portugal [4], Spain [5], India [6], and other less impacted regions [7,8]. LWD is typified by rapid dehydration of fodder and sweet maize, typically from the tasseling stage until just before maturity [9,10]. The disease's causal agent is the fungus *Magnaporthiopsis maydis*, which is recognized by two additional synonyms, *Cephalosporium maydis* and *Harpophora maydis* [11–13]. The fungus is a hemibiotroph, soil-borne [14], and seed-borne [15], and can be spread as sclerotia, conidia, or hyphae on plant remains. The pathogen can survive for prolonged periods in the soil or by establishing inside alternative host plants, such as *Lupinus termis* L. (lupine) [16], *Setaria viridis* (green foxtail), *Citrullus lanatus* (watermelon), and *Gossypium hirsutum* L. (cotton) [17,18]. It may act as an endophyte in those secondary hosts [19] but also as an opportunist, causing severe disease in some environments and under host-associated conditions [20,21].

The *M. maydis* pathogenesis in LWD-sensitive corn hybrids is well-described in the academic literature [22]. Sabet et al. [23] reported that the roots' penetration and inoculation occur throughout the first 21 days post-seeding. At the end of this period, the pathogen appears in the vascular system (the xylem) and spreads up in the shoot. At tasseling (ca. day 50–60, near the male flowering), the pathogen could be detected in the stem, its DNA levels peaked [24], and the first shoot symptoms were revealed shortly later [10]. On ca. day 70, the last week or two before harvest, the fungus hyphae and secreted substances impeded the water supply and, in severe cases, led to the host's acute dehydration and death [25]. The symptoms were increased under drought stress [5,26,27]. Economic losses due to LWD were up to 40% in Egypt [28], 100% in Israel [24], and 51% in India [29]. A similar but delayed disease mode appears in resistant genotypes, and *M. maydis* can inoculate and be established in the seeds of these apparently healthy plants and increase its dispersal [3].

Nowadays, there is a necessity to improve our toolkit to investigate the pathogen, restrict its spread, and limit its impact [24]. Past efforts to control LWD had relied on using agricultural (flood following and balanced soil fertility) [30–32], biological [1,33–35], physical (solar heating) [36], allelochemical [37,38] and chemical solutions [39,40], tillage, cover crop or crop rotation [4,41,42], as well as other options [43–45], with various levels of success. Despite these efforts, the most efficient, environmentally friendly, and cost-effective way today to contain the disease is relying on resistant maize hybrids [6,46–49]. Yet, the presence of highly violent *M. maydis* isolates [50,51] that may overcome host resistance pushes scientists to seek other approaches.

In Israel, maize is an important crop, with a harvested area of 3,820 ha and volume production of 73,419 tons (FAOSTAT, 2020 Food and Agriculture Commodity Production data). The average production quantity of maize crops in metric ton yield per metric hectare exhibited an increasing trend, from 5–7 in 1980–1984 to 10–15 in 1990–2000 and 20–25 in 2010–2020 (Figure 1). Effective LWD risk management may be a factor in this success, mainly by avoiding sensitive maize cultivars' growth [52].

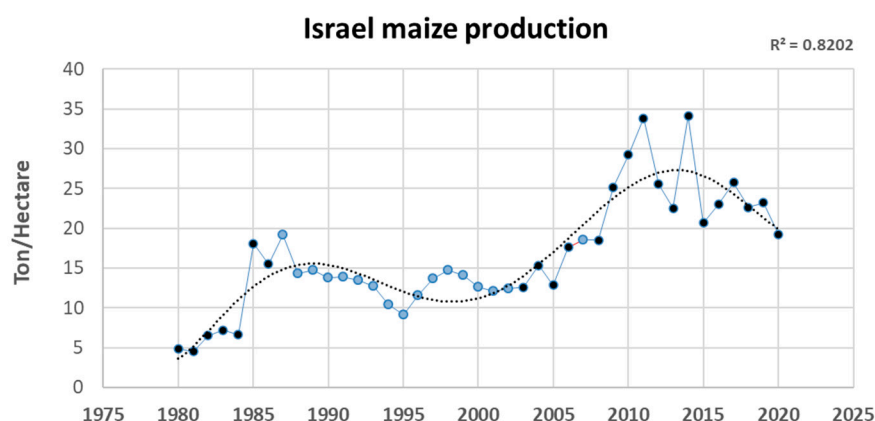


Figure 1. Israel maize production per metric hectare between the years 1980 and 2020. Data from FAOSTAT, 2020 Food and Agriculture Commodity Production. Values in dark blue are official data. Values in light blue are unofficial data or estimations. The trend line is a sixth-order polynomial.

It was demonstrated that *M. maydis* is present in the host tissues of successfully chemically treated plants [52]. This may result in the development of the fungus's fungicide immunity. The most effective fungicide against the late-wilt agent is Azoxystrobin [52,53]. Still, the rapid expansion of this fungicide tolerance has become increasingly problematic. Furthermore, the antifungals' widespread use creates a major concern—their remaining toxicity and influence are endangering human health and the environment [54].

Several studies have already demonstrated microorganism-based LWD bio-control (most recently [1,34,55–58]). A particularly studied method is based on plant growth-promoting rhizobacteria, which could also enhance plant health (summarized by [59]). An

alternative approach is using *Trichoderma* species against the LWD pathogen. Members in this genus can develop endophytic mutualistic interactions with various plant species [60], while other species have been used as a bio-protective barrier against phytoparasitic fungi [61]. Still, many knowledge gaps exist regarding the effectiveness of *Trichoderma* spp. against *M. maydis*. Therefore, their full potential as a biopesticide is yet to be explored. To demonstrate this potential, it was shown [33] that adding *Chlorella vulgaris* (microalgae) extracts to each of the *Trichoderma* species *T. koningii* and *T. virens* was an efficient treatment against LWD, resulting in a 72% disease frequency reduction in the greenhouse and 2.5-fold higher grain yield in the field.

Over the past few years, we tested nine *Trichoderma* isolates versus *M. maydis*. Three of these isolates revealed control ability against the maize pathogen: *Trichoderma asperelloides* (T.203) and *Trichoderma longibrachiatum* (T.7407 from a marine sponge); and *Trichoderma asperellum* (strain P1), an endophytic symbiont isolated from the seeds of an LWD-sensitive maize cultivar [57,58]. Growth-room seedlings pathogenicity trials show that all three *Trichoderma* isolates promoted the inspected plants' growth indices, reduced LWD dehydration signs, and caused a significant reduction in *M. maydis* DNA inside the host tissues. Similar results were achieved in a full-season field trial (82 days) [55].

In follow-up work, we isolated a potent inhibitor chloroform extract (approx. 400 mg/L) from *T. asperellum* growth medium, capable of entirely preventing *M. maydis* growing [56]. Liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) purification steps identified the secreted active ingredient as 6-pentyl- α -pyrone (also called pyrone 6-pentyl-2H-pyran-2-one or 6-PP). The pure compound exhibited high antifungal efficiency versus the LWD pathogen. Based on reports in the literature, this active ingredient is identified as a key bioactive compound of various *Trichoderma* species [62].

This metabolite plays a pivotal role in the biocontrol capability of the producing species. It displays multiple actions against phytoparasitic fungi, such as restricting their spore germination, mycelium pigmentation, and hyphal growth [63]. 6-PP also reduces fusaric acid production by *Fusarium moniliform* and mycotoxin deoxynivalenol by *Fusarium graminearum* [64]. For commercial production, a large amount of 6-PP can be generated by *T. asperellum* by utilizing forced aeration on a solid-state fermentation system [62]. Because 6-PP is a food-grade volatile compound, it may also be applied post-harvest to shield crops in storage.

The current study aimed at developing a biological environmentally friendly control strategy against *M. maydis* LWD based directly on *T. asperellum*, its secreted metabolites' crude, or pure 6-PP. To this end, we conducted growth room trials in seedlings (up to 42 days) and full-season experiments in a net house or the field. Tracking *M. maydis*'s DNA within the host tissues using quantitative real-time PCR (q-PCR) enables direct evaluation of the treatments' potential.

2. Materials and Methods

2.1. Rationale and Research Design

This three-year study examined the biocontrol potential of *Trichoderma asperellum* (P1 strain) and its secreted metabolites, specifically the 6-pentyl- α -pyrone (6-PP, Sigma-Aldrich, CAS number 27593-23-3, Buchs, Switzerland) purified active ingredient, against the maize late wilt pathogen, *Magnaportheopsis maydis*. A test series was conducted to evaluate the potential of this new green biopesticide. These trials started by directly applying *T. asperellum* with the seeding in sprouts' pathogenicity evaluation (growth room conditions up to 42 days) and under field conditions for the entire growing season. The following step examined the *T. asperellum* secreted metabolites (growth medium crude) against the purified active ingredient, 6-PP, in a semi-field environment (net house) full-season experiment. While the growing room provides a controlled environment, the net house offers a better field-like environment while maintaining more uniform environmental conditions and better regulation of soil composition, inoculation load, and water management. Both trials

enable adding non-infected plants as a negative control. Therefore, it is an important step designed to achieve a complete image. Field experiments, while providing a more actual commercial growth environment, are unstable regarding physical and climatic conditions, pathogen existence, and other factors (e.g., soil composition and microbiome), as well as the cross-influence between treatments.

The preliminary steps—inspecting *T. asperellum* biocontrol potential using the sprout pathogenicity assay (in a growing room up to 42 days from sowing, DAS) and the field trial—were conducted using the late-wilt-susceptible maize genotype Prelude (a sweet maize cultivar produced by SRS Snowy River Seeds, Australia, marketed by Green 2000 Ltd., Bitan Aharon, Israel). The *T. asperellum* secreted metabolites’ examination in the net house was made using the sweet maize genotype Megaton cv. (from Limagrain, Saint-Beauzire, Puy-de-Dôme, France, supplied by Hazera Seeds Ltd.). These two cultivars were tested previously for their susceptibility to the late wilt pathogen [41,52] and identified as LWD-highly sensitive hybrids. A special effort was devoted to maintaining similar conditions in the growth room and mesh house experiments. Both trials were conducted based on the same soil conditions, inoculation method, and controls.

2.2. Fungal Species and Growth Conditions

All *M. maydis* isolates (Table 1) chosen for the current work were examined in our lab for their aggressiveness towards LWD-susceptible maize hybrids. They were found to have moderate pathogenicity levels (Ofir Degani, personal communication). The *T. asperellum* (P1 strain) endophyte was separated from the sweet maize Prelude cv. grains and identified as described before [58].

Table 1. Fungi used in this research.

Species	Isolate Designation	Origin	Isolate Location	Reference
<i>Magnaporthiopsis maydis</i>	Hm2	<i>Zea mays</i> , Jubilee cv., CBS 133165	Northern Israel	[65]
<i>Magnaporthiopsis maydis</i>	Hm29	<i>Zea mays</i> , 32D99 cv.	Northern Israel	[55]
<i>Magnaporthiopsis maydis</i>	Hm30	<i>Zea mays</i> , 32D99 cv.	Northern Israel	[55]
<i>Fusarium verticillioides</i>	NRRL 26,191 [FP19] ATCC 204499	Rice (<i>Oryza sativa</i>), ‘Cypress’		
<i>Macrophomina phaseolina</i>	Mp-1	Wilted cotton plants	Northern Israel	[17]
<i>Trichoderma asperellum</i>	P1	<i>Zea mays</i> (Prelude cv.) seeds		[55,58]

The fungi were grown on solid potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) under dark conditions at an optimal temperature of 28 ± 1 °C. They were transferred to a new growth dish by extracting a 6-mm-diameter agar disk from the margins of the culture and sowing it to a new PDA Petri dish. Plates were labeled and incubated in the dark at 28 ± 1 °C for 4–6 days. For submerged cultures (in a liquid substrate), five fungal disks were sown in an Erlenmeyer bottle containing 150-mL potato dextrose broth (PDB; Difco Laboratories, Detroit, MI, USA). The bottles were plugged with a breathable stopper and incubated for six days, shaken at 150 rpm in the dark, at 28 ± 1 °C. The growth medium metabolites were extracted by filtering the liquid PDB growth medium using a Buchner funnel and Whatman filter no. 3.

2.3. *Trichoderma Asperellum* Pathogenicity Evaluation in Growth Room Sprouts

2.3.1. Inoculation Method

Two-liter pots were filled with commercial field peat soil from a location with a long record of LWD infection (Amir field, Mehogi-1 plot, coordinates: 33°09′59″ N 35°36′5″ E) [52,53]. Control was a similar peat soil from a nearby farm (Gadash experimental, coordinates: 33°10′48.6″ N 35°35′11.6″ E) with no record of LWD. If such an infestation occurred, it was supposed to be minor. The soil was blended with 30% Perlite No. 4 (for aerating). Complementary inoculation was conducted in two steps to guarantee a high and uniform

infection load: (1) pre-sowing soil infection with sterilized infected wheat grains; and (2) adding *M. maydis* colony agar disks with the seeding. The sterilized infected wheat grains were prepared as previously described [41]. Briefly, the wheat seeds were drenched in tap water overnight, filtered, and dried on paper towels in a fume hood. The seeds were sterilized in an autoclave for 30 min at 120 °C, and 150 g was incubated with 10 *M. maydis* mycelium agar disks of *M. maydis* (see Section 2.2) in sterilized plastic boxes (0.5 l volume) for 2–3 weeks at 28 ± 1 °C in the dark. The seeds were mixed every two days by shaking to ensure equal inoculation. The soil was infected by adding and mixing 12 g of sterilized and infected wheat grains to the top 10 cm of the ground in 2 L pots. The inoculated soil was kept moist until the maize was sown for a week. Additionally, three colony *M. maydis* colony agar disks were added to each seed with the sowing.

2.3.2. *Trichoderma asperellum* Biocontrol-Based Treatment

The biocontrol agent, *T. asperellum*, was grown in a liquid PDB (see Section 2.2) for six days, filtered, and chopped into small hyphae fragments, and 1 g (wet weight) of mycelia was added to each seed with the sowing.

2.3.3. Growth Room Trial Conditions

The experiment was conducted in 2019 in five biological repetitions and included two controls—infected unprotected plants and healthy plants (without any treatment). Two-liter pots were filled with field peat soil mixed with 30% Perlite, as in Section 2.3.1. Five Prelude cv. maize seeds were seeded in each pot to a depth of 4 cm. The plants were developed in a growing room for 42 days (fifth leaf appearance, V5 phenological stage) in an artificial light regime of 16 and 8 h of darkness, at 28 ± 3 °C with 45–50% humidity. Each pot was irrigated routinely with 100 mL of tap water every two days.

2.4. *Trichoderma Asperellum* Pathogenicity Evaluation in the Field

2.4.1. Overall Description of the Field Experiments

This study evaluated the *T. asperellum* biocontrol capability against *M. maydis* over a full growing season in a commercial field conditions simulation. The experiment was carried out on a trial farm (Gadash, Hula Valley, Upper Galilee, north Israel) throughout the summer and fall of 2020. The meteorological records through the growth period are listed in Table 2. The 2020 trial was conducted in autumn for pragmatic reasons (principally the field availability). Thus, due to the late season, the average meteorological parameters quantified during the experimental duration (relatively low temperature, radiation and evaporation, and high precipitation) were not optimal for the LWD burst.

Table 2. Meteorological data for the 2020 field experiments ¹.

Parameters	Value
Dates	10/9/2020–01/12/2020
Temperature (°C)	23.0 ± 7.1
Humidity (%)	64.0 ± 23.4
Soil temp. top 5 cm (°C)	25.6 ± 7.6
Radiation (W/m ²)	172.2
Precipitation (mm)	140.6
Evaporation (mm)	299.2

¹ Average data (\pm standard deviation) according to Israel Northern Research and Development, Hava 1 Meteorological Station.

The experiment field area has a history of mild LWD infestation. We infected the experimental plots (apart from the control plots) to attain a more acute disease. Therefore, the negative check plots were without pathogen enrichment. The Prelude cv. seeds were pre-treated (according to the regulations) with thiram, captan, carboxin, metalaxyl-M (Rogers/Syngenta Seeds, Boise, ID, the USA, provided by CTS, Tel Aviv, Israel). The

experiment was performed with five random blocks per treatment/control. Each block comprised five garden beds (repeats), which were 9 m long, 6 m wide, and had two rows with row spacing of 96 cm.

2.4.2. Sowing Procedure and Irrigation

The plots were seeded to a 4 cm depth with 7 plants/meter and sprouted the next day with a frontal irrigation system. Irrigation of 0.6 l h^{-1} was conducted by drip irrigation line (20 mm) for each row (Dripnet PC1613 F, Netafim USA, Fresno, CA) and was controlled using a computerized system. The watering supply was 3000 L/0.1 ha per day. The watering was conducted every two days, and the overall water supplied throughout the season was ca. 400 mm. All the plants received insecticides and fertilization at the recommended dosages (Consultation Service, SAHAM, Israel Ministry of Agriculture).

2.4.3. Complementary Inoculation Method

The plants' infection methodology and growth were according to [17,53]. The inoculation procedure includes further complementary phases to equalize and enhance the soil's pathogen infection. To this end, the trial plots (excluding the negative check) were inoculated by adding 3 *M. maydis*-infected wheat grains to each corn seed with the seeding. These grains were prepared as detailed in Section 2.3.1 with a blend of *M. maydis* isolates (20 disks/150 g seeds), *Hm2*, *Hm29*, and *Hm30* (see Table 1).

2.4.4. *Trichoderma*-Based Biocontrol Treatments

The bio-shield treatment was conducted by adding 3 sterilized wheat grains enriched with *T. asperellum* to each maize seed with the seeding. The wheat grain enrichment was conducted in a similar fashion to the pathogen enrichment described in Section 2.3.1.

2.4.5. Data Collection

Pollination occurred on 49 DAS (29 October 2020). During the season (up to the harvest), most plants had a healthy, vital appearance, typical growth, and crops (detailed in the Results) at the season's ending. Yet, close examination of the lower stalk (first internode above the ground) and cobs' spathes (the large bracts surrounding the cobs) revealed disease symptoms. Thus, the healthy (non-symptomatic) plant percentages were calculated and presented. Growth indexes (shoot height and wet weight, and the number of leaves) were determined in 5 plants (one repeat) selected randomly from the middle of every one of the experiment's garden beds. Each experimental group (controls and biological intervention) included eight repetitions. Yield evaluation was conducted on all the plants in 4–7 rows (repeats) for the biological treatment and the controls. The molecular tracking (*M. maydis* DNA diagnosis) was performed on three arbitrarily selected representative plants in those rows.

2.5. Effect of *Trichoderma Asperellum*-Secreted Metabolites in Solid Media Cultures

The impact of *T. asperellum*-secreted metabolites on *M. maydis*, *M. phaseolina*, and *F. verticillioides* growth was assessed in solid growth media (PDA) as previously described [56]. To prepare the *T. asperellum*'s secreted metabolites, five mycelial discs (6 mm) were removed from the edges of 2–4-day-old colonies and incubated for six days in 150 mL PDB in the dark at 150 rpm at a temperature of 28 ± 1 °C. The growth medium was separated by Buchner funnel filtration through Whatman filter no. 3. The medium pH was adjusted to 5.1 ± 0.2 with NaOH and filtered again for sterilization using biofilter bottles (0.22-micron filter, BIOFIL 500 mL vacuum bottle filter, Indore, India). The *Trichoderma* cultures' filtrate described above was used to prepare the PDA plates (instead of DDW). PDA powder was added to the filtrate according to the manufacturer's instructions. After autoclave sterilization, the mixture was poured into Petri plates, which were seeded with a 6-mm-diameter *M. maydis* mycelial disc (from the margin of a fungus colony, see Section 2.2). The colonies were allowed to grow for six days and photographed.

2.6. 6-pentyl- α -pyrone Evaluation in a Net House

2.6.1. Application of the Purified *T. asperellum* Antifungal Compound(s) Using Seed Dressing

The *T. asperellum* isolate activity products were tested in a net house in pots during a full growth period. The experiment included six replicates (pots). The Megaton cv. corn (sweet, LWD super-susceptible) [41] was used in the experiments. Seed coating was performed according to a standard commercial procedure by blending the seeds with the compound (0.002 mL preparation/seed) until it was ultimately absorbed in the seeds. The inspected ingredient (30 or 60 μ g 6-PP, and 100% or 50% *T. asperellum* crude, in 2 mL double-distilled water, DDW) was pipetted into a one-liter round-bottom glass flask containing 1000 seeds. The flask was rotated for ca. 10 min until the inspected ingredient was fully absorbed in the seeds. The seeds were taken out and dried in a fume hood on sterile paper towels for several hours. These seeds were kept in paper bags at 4 °C until use. The control treatment was conducted with similar seeds that underwent the addition of sterile water instead of the inspected ingredient.

2.6.2. Net House Full-Growth Season Pot Experiments Treatments and Sampling Program

The experiment was conducted in the R&D North Israel Experimental Farm located in the Hula Valley, Upper Galilee, northern Israel (33°09′08.2″ N 35°37′21.6″ E) during a full growing season. Each treatment consists of ten independent replicates (pots). The sprouts were thinned to one plant per pot towards the end of the sprouting growth stage (40 DAS). Inoculation with the *M. maydis* was carried out as described for the growth room experiment, with the following changes: in the first inoculation step, 40 g of infected sterilized wheat grains was added to the upper 20 cm of the soil of each pot. Control was peat soil from the experimental farm, having no record of LWD. If such an infestation existed, it was assumed to be very low. The evaluation of emergence (aboveground surface peek) percentages was performed 6 DAS. The male flowering was documented at 53 DAS. The growth parameters were evaluated at 40 DAS and the experiment end (96 DAS). Yield assessment and wilt determination were performed at harvest. Wilt valuation for each plant was based on 4 classifications—dead, diseased, symptoms, and healthy—as was demonstrated in [55], and the dry plants' percentages were calculated and presented. On both sampling days, a qPCR detection of the fungal DNA in the plants' roots or aboveground first internode was performed.

2.6.3. Net House Growth Conditions

The experiment's local peat soil was similar to the growth room trial soil. It had no recognized record of LWD infestation (if it existed, it was assumed to be very low). To aerate the ground, the soil was blended with coarse perlite (No. 4) at a ratio of 2:1 in 10 L pots. The pots were positioned on concrete blocks to prevent the roots from contacting the ground and to better isolate the treatments. Each pot was sown with 5 seeds previously coated with thiram, captan, carboxin and metalaxyl-M. This seed treatment is standard general pesticide care. Seed samples were tested for vitality before seeding. Computerized irrigation was conducted with drip lines and was carried out from the seeding using 2 L per pot/day. According to the plants' needs, adjustments were made in the water regime and pesticide treatments to prevent other diseases from influencing the plants during their growth (according to the Ministry of Agriculture's recommended growing protocol). During the maize growing season, the average net house temperature was 24.7 °C, with a maximum of 43.4 °C and a minimum of 12.3 °C. The average humidity was 93.6%, with a maximum of 100% and a minimum of 42.2%.

2.7. Molecular Analysis

Quantifying *M. maydis* DNA in the host plant tissues was performed using the qPCR-based technique. The plants' parts were washed vigorously with running tap water, then twice with sterile DDW and sliced into ca. 2 cm sections. The total weight of each repeat

was adjusted to 0.7 g. The pathogen DNA was isolated and extracted according to a previously published protocol [66] with slight modifications [65]. Briefly, 0.7 g of plant tissue was ground with 4 mL of cetyltrimethyl ammonium bromide (CTAB). A total of 1.2 mL was transferred to Eppendorf test tubes and heated in a bath at 65 °C for 20 min. The test tubes were concentrated at 13,000 rpm at room temperature (24 °C) for 5 min, and the upper phase (700 µL) was transferred to new test tubes with the same volume of chloroform/isoamyl-alcohol (24: 1). The last cleaning stage was repeated twice. The upper phase (300 µL) was transferred to a new Eppendorf test tube with 200 µL of cold isopropanol, and the test tubes were maintained at 20 °C for 20–60 min. The test tubes were then concentrated at 13,000 rpm at 4 °C for 20 min, and the liquid was poured into a waste vessel. The DNA precipitate was resuspended and washed with 0.5 mL of ethanol. After a further concentration and ethanol wash cycle, the DNA precipitate in the test tubes was allowed to dry in a sterile hood overnight. Finally, the DNA was resuspended in 100 µL of ultra-pure water and kept at –20 °C until used for qPCR. DNA extraction from PDA colonies (positive control) was performed using a Plant/Fungi DNA Isolation Kit (Norgen, Biotek Corporation, Thorold, Ontario, Canada) according to the manufacturer's instructions.

The molecular detection technique is based on a standard qPCR procedure to measure mRNA (converted to cDNA) levels [67]. It was adjusted here to detect *M. maydis* DNA using species-specific primers [68,69]. The A200a primers were applied for qPCR (Table 3). The housekeeping cytochrome C oxidase (COX) gene, encoding the cellular mitochondria's last enzyme in the respiratory electron transport chain, served to normalize the relative *M. maydis* DNA amount in the samples examined [70]. For the COX gene COX F/R primer set, see Table 3. The relative DNA abundance was calculated using the ΔC_t model [67,71]. All amplifications were carried out in four repetitions, and the same efficiency was assumed.

Table 3. Primers for *Magnaportheiopsis maydis* detection ¹.

Pairs	Primer	Sequence	Uses	Amplification	References
Pair 1	A200a-for A200a-rev	5'-CCGACGCCTAAAATACAGGA-3' 5'-GGGCTTTTATAGGCCTTTT-3'	Target gene	200 bp <i>M. maydis</i> species-specific fragment	[65]
Pair 3	COX-F COX-R	5'-GTATGCCACGTCGCATTCCAGA-3' 5'-CAACTACGGATATATAAGRRCCRR AACTG-3'	Control	Cytochrome C oxidase (COX) gene product	[70,72]

¹ The R symbol represents Adenine or Guanine (purine). The synthesized primers contained a mixture of primers with the two nucleotides.

All qPCR reactions were measured using the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and 384-well plates. The reaction volume was 5 µL per well. All wells contained 0.25 µL of each primer (Forward/Backward at 10 µM concentration), 2.5 µL iTaq™ Universal SYBR Green Supermix solution (Bio-Rad Laboratories Ltd., Hercules, California, USA), and 2 µL of DNA sample. Reaction conditions were 95 °C for 60 s (pre-cycle activation phase), then 40 cycles of 95 °C for 15 s (denaturation), 60 °C for 30 s (annealing and extension), and, finally, the formation of a melting curve.

2.8. Statistical Analysis

The growth room and net house experiment pots and the field plots were scattered in a fully randomized design and analyzed using the same statistical method. Statistical processing and data evaluation were achieved using JMP software, version 15 (SAS Institute Inc., Cary, NC, USA). The results were evaluated using the one-way analysis of variance (ANOVA) followed by a Student's t-test post hoc (without correction for multiple tests) at a significance level of $p < 0.05$. Due to the difficulty in creating uniform plant infection, high variability in *M. maydis*'s DNA levels and high standard error values are expected, making it challenging to obtain the alternative hypothesis.

3. Results

The current study aimed at evaluating the *Trichoderma asperellum* secreted metabolite, 6-pentyl- α -pyrone (6-PP), against the maize late wilt (LWD) pathogen, *Magnaporthe oryzae* *maydis*. To achieve this goal, a series of trials were conducted, starting by inspecting the addition of *T. asperellum* directly to seeds with the sowing (sprouts in a growth room-controlled conditions and complete season field experiment). These trials were followed by a targeted evaluation of the *T. asperellum* recently purified and identified active secreted metabolite, 6-PP (net house experiment over an entire growing season). The powerful antifungal compound was applied in seed coating and tested against the *T. asperellum* growth medium crude or by adding the fungus itself with the seeding. The results indicate a potential new control treatment to protect susceptible maize plants from LWD, as detailed below.

3.1. *Trichoderma Asperellum* Pathogenicity Evaluation in Growth Room Sprouts

Young *T. asperellum* hyphae disks were cut from the margins of culture colonies, grown on rich medium plates, and added directly to the seeds with the sowing. Inspecting this procedure's impact on seedling development (up to 42 days) and *M. maydis* establishment in their roots revealed a significant growth promotion and LWD recovery (Table 4). While the *T. asperellum* did not affect the aboveground emergence (at day 8), at the end of the sprouting phase (day 42, the fifth leaf stage), all growth indexes improved drastically ($p < 0.05$) compared to the infected, untreated plants. This improvement ranges from 1.8-fold in plant height to 2.7 and 3.3-fold in shoot and root fresh weight. These changes were accompanied by a two-fold reduction in the *M. maydis* DNA in the sprouts' roots.

Table 4. *Trichoderma asperellum* late wilt control in sprouts at 42 DAS¹.

Growth Parameter	Control –		Control + ³		P1	
	Mean	S.E.	Mean	S.E.	Mean	S.E.
Emergence (%) 8 DAS	68.60%	5.30%	64.30%	7.10%	54.30%	5.30%
Root wet weight (g)	1.17	0.44	0.6	0.14	1.98 ²	0.44
Shoot wet weight (g)	2.01	0.11	1.48	0.1	4.02 ²	0.66
Total dry weight	0.59	0.19	0.44	0.11	0.98 ²	0.2
Shoot height (cm)	7.6	0.68	5.65	0.4	10.13 ²	1.32
qPCR (Mm/Cox ratio)	0.006	0.005	0.41	0.24	0.2	0.09

¹ DAS—days after sowing. Values signify an average of five repetitions \pm standard error. ² A statistically significant ($p < 0.05$) difference was detected between the *Trichoderma asperellum* (P1) treatment and the inoculated control (+). ³ Control (–)—soil from a similar field with low levels of *M. maydis* infestation.

3.2. *Trichoderma Asperellum* Pathogenicity Evaluation in the Field

The follow-up full-season trial was conducted in an experimental field known to have a mild infection with late wilt. Since the growing season's climatic conditions were not optimal for this disease development (relatively rainy autumn season), we infected the field soil with the seeding. Still, during the growth period up to the harvest, the plants' phenological development and growth indexes were normal, and the disease symptoms were minor. These parameters did not vary statistically between the treatments (Table 5).

Only a close examination of the lower stem and cob spathes (the large bracts surrounding the cobs) revealed the disease dehydration symptoms and the impact of the *T. asperellum* treatment (Figure 2A). At the season's end (day 82, 33 days after fertilization), the improvement in these two parameters (lower stem and cob symptoms) was 1.6- and 1.3-fold, respectively, in the biological treatment compared to the non-treated control (without statistical significance). In line with these results is the *M. maydis*'s DNA in the plants' first aboveground internode (Figure 2B, qPCR results). In the *T. asperellum* treatment, the pathogen DNA dropped by five-fold compared to the control. This being said, the pathogen DNA levels in all treatments were relatively low and near the technique's lower

threshold (the qPCR method can detect variation in proportionate *M. maydis* specific DNA between 10 to 10^{−5} [7]).

Table 5. *Trichoderma asperellum* late wilt control in the field at 41 (A) and 82 (B) DAS¹.

A.		Wet Weight (g)		Number of Leaves		Plant Height (cm)		qPCR (mm/cox)	
Treatment		Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
Control−		255.7	10.6	9.87	0.2	115.4	2.1	5.50×10^{-4}	3.30×10^{-4}
Control+		240.5	11	9.79	0.2	113.7	2.3	2.50×10^{-4}	1.80×10^{-4}
P1		245.1	13.6	9	0.2	116.9	2.5	3.00×10^{-4}	1.90×10^{-4}
B.		Yield (kg/m ²)		A Class (g)		B Class (g)			
Treatment		Mean	S.E.	Mean	S.E.	Mean	S.E.		
Control−		1.2	0.08	369.2	14.2	160.3	17.3		
Control+		1.14	0.08	341.9	20.3	152.4	14.9		
P1		1.09	0.05	333.1	20.9	180.2	16.1		

¹ The experiment was carried out in late wilt-susceptible Prelude cv. Control− are plots without complementary inoculation. Control+ are plots with *M. maydis* infection. The other treatments are infected plots treated with *T. asperellum* (P1) by adding it to the seeds with sowing. The yield values were collected at 82 DAS (33 days after fertilization). The yield classified as A class had a cob weight exceeding 250 g. Values represent an average of at least four replications \pm standard error. No statistical differences could be detected between the treatments.

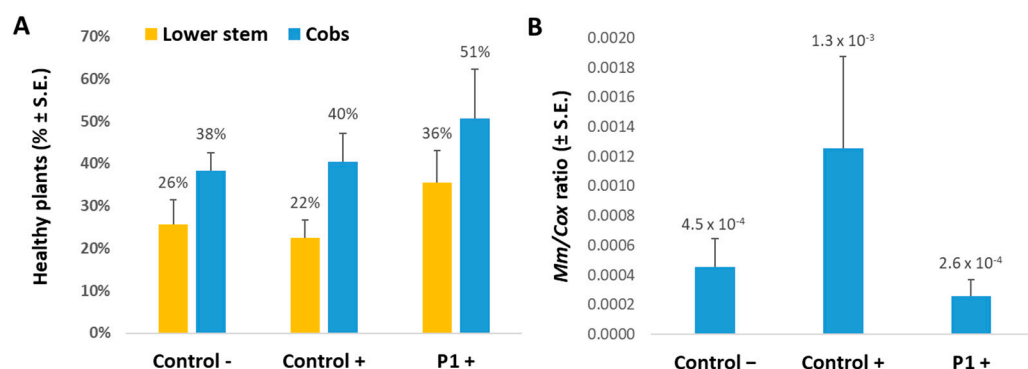


Figure 2. Plants' health and *M. maydis* infection severity in the field at day 82. Maize late-wilt disease symptoms (A) and qPCR diagnosis of *M. maydis* DNA in maize plants (B) at the end of the field experiment (33 days after fertilization) conducted in the summer and autumn of 2020. The experiment was conducted with the late-wilt-susceptible maize genotype Prelude. Control− are plots without complementary inoculation. Control+ are plots with *M. maydis* infection. The other treatments are infected plots treated with *T. asperellum* (P1) by adding it to the seeds with sowing. *M. maydis* relative DNA (*Mm*) normalized to the cytochrome C oxidase (*Cox*) DNA was evaluated in the plants' first aboveground internode. Vertical upper bars signify the standard error of the mean of 4–10 replicates.

3.3. 6-pentyl- α -pyrone Evaluation in a Net House

So far, we have evaluated the addition of *T. asperellum* directly to the seeds with sowing. The following steps were taken to inspect its secreted metabolites' bio-protective ability under late wilt stress. The potential of this *Trichoderma* species growth medium crude and its active ingredient 6-PP to restrict the LWD pathogen, *M. maydis*, in vitro was previously demonstrated [56]. It now appears that *T. asperellum* growth medium exhibits specificity activity against *M. maydis* and cannot repress two additional members of the maize post-flowering stalk rot complex [73], *Fusarium verticillioides* and *Macrophomina phaseolina* (Figure 3).

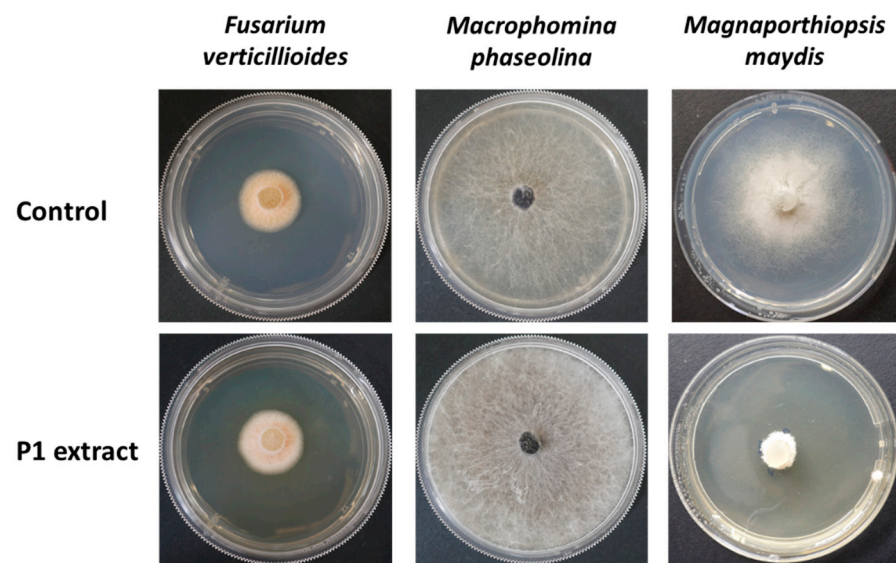


Figure 3. Bioassay of the *Trichoderma asperellum* (P1) secreted metabolites. The fungus liquid growth medium (P extract) was used to prepare a rich growth medium (PDA). The plates were used to test the maize post-flowering stalk rot complex [73]. This fungal group includes *M. maydis*, *Macrophomina phaseolina*, and *Fusarium verticillioides*. The controls are regular water-based PDAs. Representative plates' photos were taken after six days.

Until now, the *T. asperellum* secreted ingredients had never been tested as a shield against the LWD agent in plants. To accomplish this, we conducted a net house whole-season experiment. We tested the purified 6-PP metabolite against the growth medium crude and the direct addition of the *Trichoderma* to the seeds (Figure 4). The disease progression in this experiment was evident, and already at the end of the sprouting phase (day 40), significant growth reduction ($p < 0.05$) was observed in the infected, non-protected plants group (Table 6).

Table 6. 6-pentyl- α -pyrone late wilt control in the net house at the sprouting phase ¹.

Treatment	Peak	Wet Weight (g)		Number of Leaves		Plant Height (cm)		Survival (%)	
	Mean	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
Non-infected	70%	104.2 ^A	13.2	14.0 ^A	1.9	251.7 ^A	34.0	50% ^A	9%
Infected	44%	64.3 ^B	11.3	8.7 ^A	1.1	153.0 ^B	22.8	18% ^C	6%
P1 control	62%	134.3 ^A	13.8	13.1 ^A	1.0	242.7 ^{AB}	22.1	38% ^{ABC}	6%
6-PP 30 μ g	52%	122.1 ^A	19.3	14.2 ^A	3.5	247.8 ^{AB}	56.2	28% ^{BC}	10%
6-PP 60 μ g	62%	108.6 ^{AB}	16.8	19.8 ^A	7.8	200.3 ^{AB}	17.7	40% ^{AB}	4%
Ex. 50%	58%	142.6 ^{AB}	19.0	13.8 ^A	0.9	249.1 ^A	19.5	40% ^{AB}	5%
Ex. 100%	34%	103.7 ^{AB}	19.9	12.3 ^A	2.2	234.3 ^{AB}	43.8	36% ^{ABC}	9%

¹ The experiment was performed in late wilt-susceptible Megaton cv. The aboveground first emergence (peak) was recorded at 6 DAS. All other parameters were measured at 40 DAS. Non-infected are plots without inoculation. Infected are plots with *M. maydis* inoculation. The additional treatments are infected plots with *T. asperellum* (P1) secreted metabolites 6-pentyl- α -pyrone (6-PP), secreted crude (Ex. 50%—diluted to 50% and Ex. 100% undiluted), or the addition of three *T. asperellum* colony agar disks directly to the seeds with sowing (P1 control). Values represent an average of 10 replications \pm standard error per pot. Statistically significant differences ($p < 0.05$) between treatments are indicated by different letters (A–C).

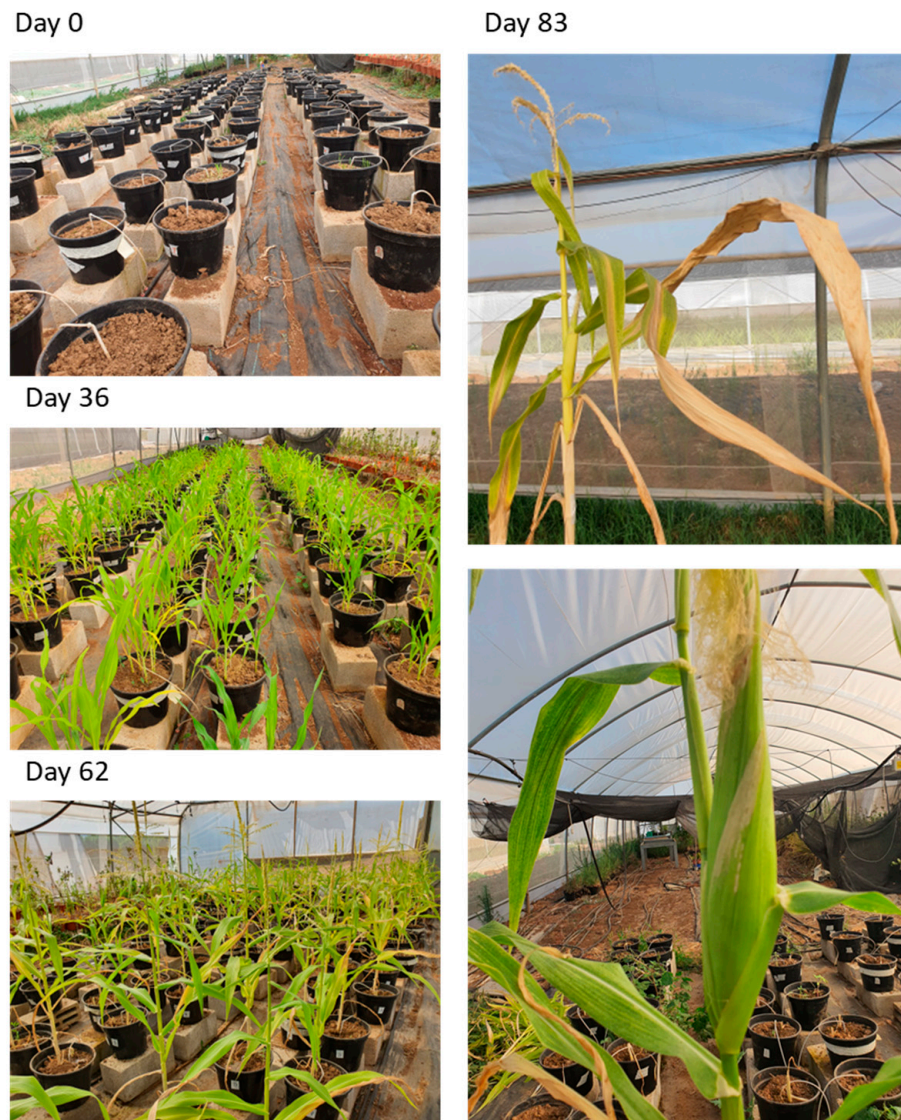


Figure 4. Photos of the net house experiment, which was performed in late wilt-susceptible Megaton cv. The male flowering was documented at 53 DAS. Disease symptoms were already clearly visible from day 62 onwards.

At the V5 (five leaves) stage, the unprotected plants suffer a 37% aboveground peak decrease (6 DAS), a 64% lower survival rate, and 38–39% growth repression (40 DAS, Table 6). All the bio-treatments led to some recovery and improvement in these parameters, especially the 6-PP (30 μ g), the crude (diluted to 50%), and the *T. asperellum* addition to the seeds. High dosages either of the clean 6-PP metabolite or the growth medium crude were apparently less effective, perhaps due to overdose, which can lead to phytotoxicity.

In a final evaluation made at the season's end (day 96, 43 days post fertilization), the tendencies that started to be revealed on day 40 were enhanced and became significant ($p < 0.05$, Figure 5). Here, the bioprotective 30 μ g 6-PP and 50% crude extract had similar (and even higher) results compared to the healthy plants' control, while the non-treated group had up to 123% and 64% lower shoot and cob weights, respectively. Parallel plant health increases accompanied these dramatic growth changes (Figure 6).

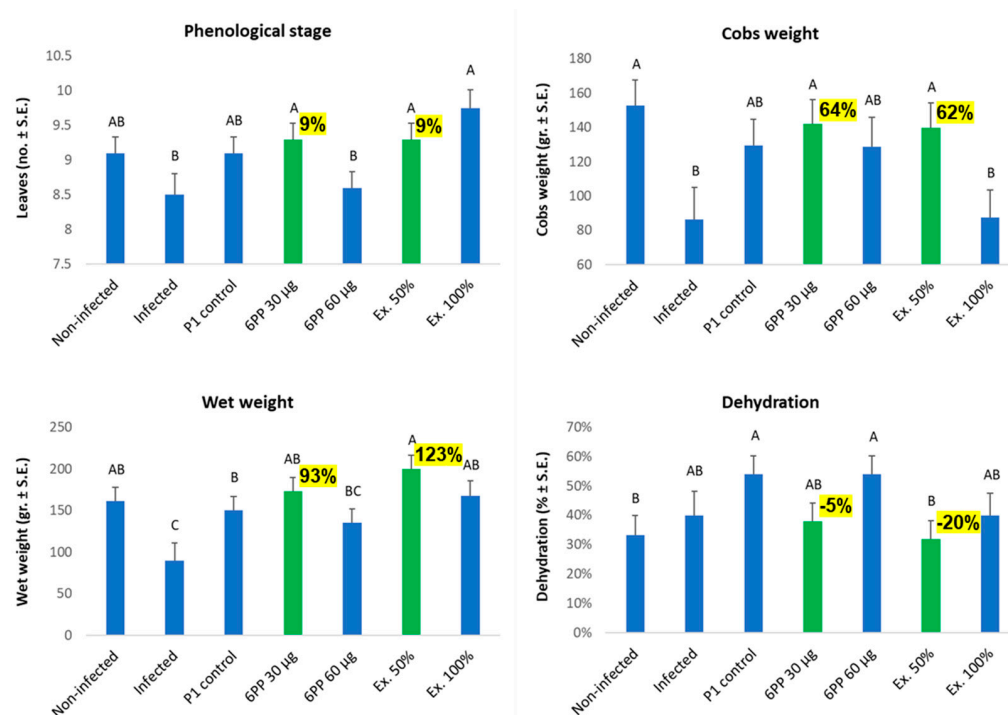


Figure 5. The *Trichoderma asperellum* secreted metabolite, 6-pentyl- α -pyrone, impacts maize development under late wilt disease stress. Net house study, day 96 data. The purified active ingredient 6-PP was tested as seed coating at 30 and 60 μ g/seed and compared to the *T. asperellum* (P1) direct addition to seeds or its crude secreted metabolites applied at 50% or 100% concentrations (Ex. 50% and Ex. 100%). The standard error of the mean of 7–10 replicates (plants) is shown. Highlighted values (in yellow) are important differences from the infected, untreated control. Statistically significant differences ($p < 0.05$) between treatments are specified by different letters (A–C).

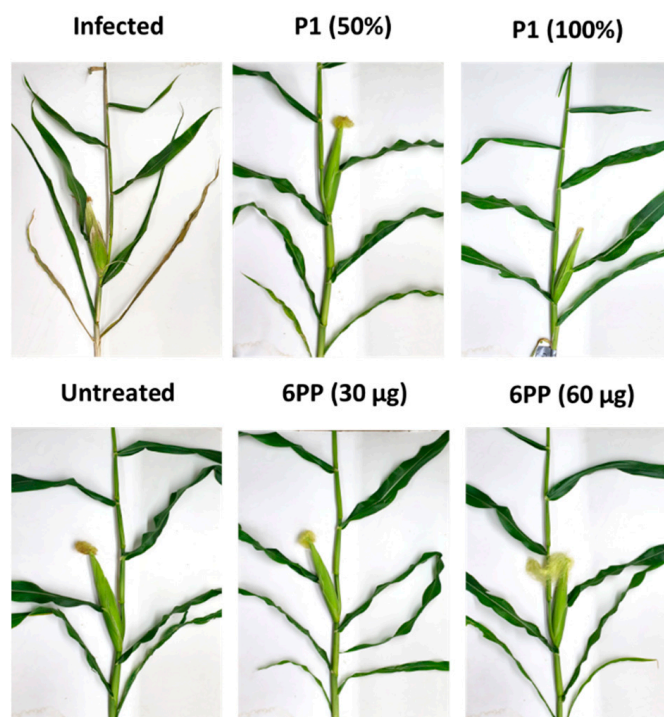


Figure 6. Net house representative plant photos on day 96. The experiment's treatments and controls are depicted in Figure 5.

The dehydration levels of the plants were attributed to variations in the *M. maydis* DNA load tracked by the molecular qPCR method. As revealed by the highly sensitive qPCR method, some very low levels of *M. maydis* DNA (near the method's lower sensitivity threshold) could be identified in the control treatment. However, such low levels were unable to evoke disease symptoms. High pathogen DNA was measured (at days 40 and 96) in the infected control plants group and the plants enriched with *T. asperellum* on the sowing day (Figure 7). This last result is unexpected since the *T. asperellum* addition to the seeds provides some protection against LWD growth reduction at the sprouting phase (40 DAS, Table 6). In contrast, the other treatments' parameters fit well with the growth and health results. At harvest (day 96), the crude extract (especially the 50% dilution) had the best influence in repressing the pathogen (43-fold). Here, too, the 6-PP diluted (30 µg) seed coating was more efficient (17-fold) than the 6-PP 60 µg treatment (7-fold *M. maydis* decrease).

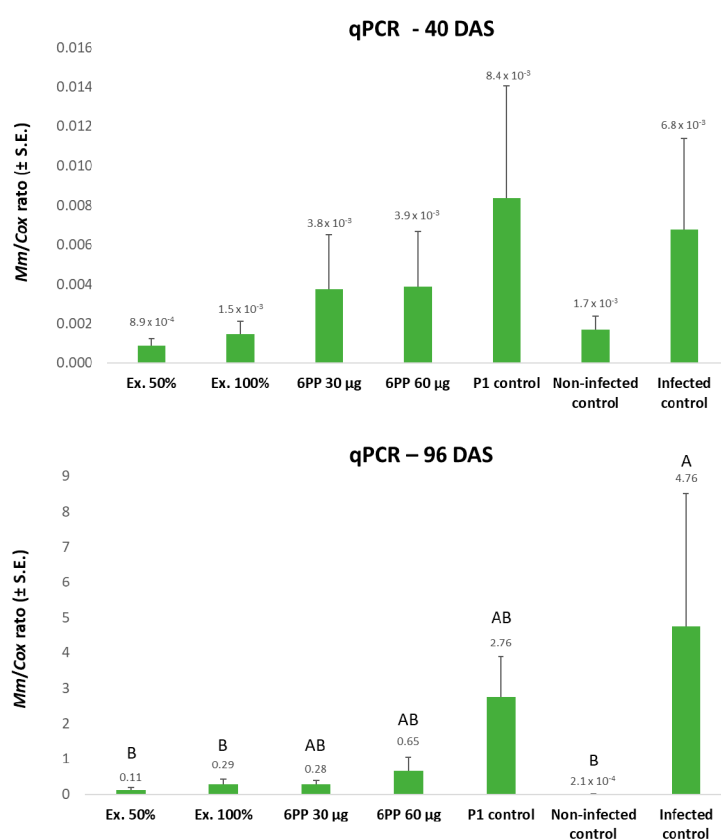


Figure 7. qPCR estimate of *M. maydis* DNA on days 40 and 96 of the net house experiment. The relative amount of *M. maydis* DNA (*Mm*) normalized to the cytochrome C oxidase (*Cox*) DNA was assessed in the plants' first aboveground internode. The experiment's treatments and controls are depicted in Figure 5. Error lines represent the standard error of an average of 7–10 repetitions (plants). If they exist, statistically significant differences ($p < 0.05$) between treatments are specified by different letters (A–B).

4. Discussion

Natural microorganisms' communities inhabiting plants include non-pathogenic members that can have shielding effects against pathogens. This work examined a new biological control agent candidate against the maize late wilt disease (LWD) pathogen, *Magnaporthiopsis maydis*. This pathogen control is challenging due to long-term soil survival, and resistant cultivars are constantly being threatened by aggressive pathogen variants [24].

In recent years, biopesticides have achieved significant consideration as an alternative to environmentally problematic chemical pesticides [54]. Understanding the roles of endophytic community assemblages and their impact on plant health would be a major contribution to sustainable integrated agriculture. *Trichoderma* species are used widely in agriculture to promote plant growth and control pathogens attacking crops, serving as valuable tools for sustainable food production [74]. Still, the action mechanism of this bio-protective species is poorly understood.

The maize-biofriendly species, *T. asperellum*, is an endophyte isolated in Israel from LWD-susceptible maize genotype seeds [58]. Here, this *Trichoderma* species showed protective ability against *Magnaportheopsis maydis* in growth room sprouts and mature plants in the field. However, it is inferred from the results that this protection was affected most at the sprouting phase and that under some field conditions, it may fail. Obvious differences exist between the growth room and the field conditions that may explain the results presented here. As a start, the field's unstable conditions may have a significant impact on the results. In our study, the field conditions were not optimal for LWD development, leading to weak dehydration symptoms appearing at the harvest. Indeed, this growing season was rainier and colder, and these circumstances were possibly the reason for lowered yields and a minor LWD outbreak. To support this, early (spring) sowing of corn in Egypt [75] and late summer planting in India [27] reduced LWD. It is possible that the full potential of the *T. asperellum*-based biological control was not manifested under these conditions.

Inspecting similar research results published recently by Estévez-Geffriaud (2021) [76] provides evidence that *T. asperellum* (strain T34 applied as seed treatment) positively affected maize productivity and health under late wilt disease stress. Regardless of the water regime, T34 improved kernel P and C content, kernel dry weight, and number. Under drought, this *Trichoderma* treatment improved leaf relative water content, water use efficiency, PSII maximum efficiency, and photosynthesis. Moreover, T34 reduced LWD incidence by up to 13% and enhanced the yield per plant in LWD moderate tolerant and resistant maize genotypes. Still, no yield improvement was measured in susceptible lines [76]. It may be inferred from Estévez-Geffriaud and other studies (for example, [33]) that the *Trichoderma* species, the specific strain, the plant's LWD susceptibility degree, and environmental conditions could all affect the success of the biological treatment.

It should also be considered that *T. asperellum* protection is mainly affected during the sprouting stage, which is regarded as the pathogen entry and establishment phase [21,23]. Later in the season, this initial protection may lose effectiveness if the pathogen finds a way to establish itself in the plant's vascular system and disrupt its development. If such a scenario is the case, integrated biological and chemical protection may assist in overcoming the pathogen's establishment and provide us with the benefit of low fungicides usage with a live protective barrier [54].

Many other factors could affect this outcome (the differences between the growth room and field assays). For example, the time lapse between pathogen and antagonist inoculation can be crucial. The preventive and curative abilities of *T. asperellum* UDEAGIEM-H01 versus *Fusarium oxysporum* were evaluated in stevia plants (*Stevia rebaudiana*) under field conditions. The occurrence of *Fusarium* wilt was 10% in the protective treatment when the antagonist was inoculated six days prior to the pathogen, whereas 70% of disease occurrence was recorded in plants treated with the antagonist six days after the infection of seedlings with the pathogen [77]. Thus, controlled conditions experiments, as demonstrated here, are difficult to replicate on a field scale where pathogens are already present [74]. Further studies are needed to optimize the maize growing time at which *T. asperellum* could present the best LWD biocontrol abilities in the field.

One of the advances in using *Trichoderma*-based protection against LWD is that these species likely maintain a symbiotic lifestyle inside the host plant organs that may provide a season-long defense. Moreover, the *Trichoderma* species can trigger the maize disease resistance response systemically through the biosynthetic induction of brassinolide, jasmonic acid, salicylic acid, defense enzymes, and reactive oxygen species [78]. *T. asperellum* substantially

impeded *F. verticillioides* growth in plate competition assay [79] and showed a biocontrol efficacy of 50% against *Fusarium graminearum* in the field [80]. These examples suggest that the field control application of *T. asperellum* may offer broad pathogen protection.

Yet, it should be considered that in severe cases, *Trichoderma* protection alone is insufficient to restrain the disease. When the pressure of infection is high, systemic fungicides must be applied. *Trichoderma*-based intervention should be part of an integrated management strategy, considering that some *Trichoderma* spp. are inefficient in combination with fungicides [54]. Compared to chemical pesticides, bio-based disease control does not generate resistance in pathogens, does not contaminate the environment, prevents the proliferation of secondary plagues, is compatible with organic production, and meets the requirements of profitable markets concerning maximum limits of chemical residues on fruits and vegetables. This work and others [55,56] suggest that *T. asperellum* bio-treatment against *M. maydis* is worth continuous future studies to maximize its potential. The findings presented in the current work imply that a major part of this antifungal activity is related to *T. asperellum* secreted metabolites in general and the 6-pentyl- α -pyrone (6-PP) active ingredient in particular.

Fungi are incredible producers of a huge range of xenobiotics, including some well-known compounds that inhibit the growth of other fungi. Secondary metabolites are pivotal defensive microorganism–pathogen interactions during host invasion and establishment, as well as the plant microbiome defense response. These include antifungals, mycotoxins, lactams/lactones, and other extracellular components. Such a compound is 6-PP [81]. This metabolite was recognized as one of the major bioactive compounds of various *Trichoderma* species [62], with a clear correlation between 6-PP biosynthesis and biocontrol capability. 6-PP inhibits spore germination, mycelial growth, and pigmentation of tested phytopathogenic fungi [63]. 6-PP also decreases the production of the mycotoxin deoxynivalenol (DON) by *F. graminearum* and fusaric acid by *F. moniliforme* [64]. Our study results support the importance of this molecule and are opening up a new research direction that could lead to a new LWD bio-pesticide product.

Significant knowledge gaps still exist regarding the action mechanism of 6-PP. The compound is oxygenated and thus was proposed to be part of the oxylipin family [82,83]. This suggests that regardless of their source, similar oxylipin-like structure compounds may provoke strong chemotropic reactions in *Trichoderma*—and possibly additional fungal species. Thus they may establish an interspecies signaling mechanism. In mycoparasitism, it was implied [84] that the plant oxylipin recruits the mycoparasite towards roots affected by fungal phytopathogens—or already as a preventive measure. At the same time, the fungal oxylipin 6-PP induces morphological adjustments in *Trichoderma* needed to launch a localized mycoparasitic attack.

The 6-PP mass release at this stage would not only have a repressive antifungal action. It might also act as a highly secreted signal in the regions where the colony contacts the prey fungus to promote hyphal redirection and hyphal growth as part of the mycoparasitism response. Other secondary metabolites produced by *T. asperellum* are likely involved in these events to refine and support the chemoattraction process and the mycoparasitism attack [84].

5. Conclusions

This research targeted the maize late wilt disease (LWD) agent, *Magnaportheiopsis maydis*, using the biocontrol *Trichoderma asperellum* and its secreted metabolites. Growth room sprouts assay and field trials that were less effective demonstrated that adding this *Trichoderma* species directly to seeds can provide some protection against the disease. The bio-friendly *Trichoderma* control is most likely attributed to this organism's secreted metabolites since applying its growth medium extract in the seed coating provided the plants with significant protection. One of these ingredients is 6-pentyl- α -pyrone (6-PP), a potent antifungal compound tested here for the first time against *M. maydis* in infected plants. The success of the 6-PP seed coating was expressed not only in a drastic reduction

in the pathogen's lower stalk infection but also in the nearly full recovery of the plant growth indices. A follow-up study should implement the 6-PP seed coating in commercial field simulations. Moreover, it would be most interesting and important to identify other active ingredients in the *T. asperellum* and other beneficial *Trichoderma* spp. (such as *T. longibrachiatum*) growing medium extract.

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Data Availability Statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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