Hungarian University of Agriculture and Life Sciences

# Pathogenesis and biological control of fungal pathogens in garlic (*Allium sativum* L.), with a comparative analysis of host defense responses in garlic and okra (*Abelmoschus esculentus* (L.) Moench)

**Thesis of PhD Dissertation**

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# 1. INTRODUCTION AND OBJECTIVES

Garlic (*Allium sativum* L.) is a globally significant vegetable and spice crop, valued for its culinary applications and medicinal properties attributed to bioactive compounds such as allicin (Amarakoon and Jayasekara, 2017). In 2021, global production exceeded 28 million tons, with China and India as leading producers (FAOSTAT, 2021). Beyond food use, garlic exhibits antioxidant, antimicrobial, and anticancer properties (Kovarovič et al., 2019) and has been investigated for therapeutic potential against infectious, metabolic, and genetic disorders (Amarakoon and Jayasekara, 2017). Garlic-derived allelochemicals also act as biostimulants, improving crop resilience (Hayat et al., 2018).

Okra (*Abelmoschus esculentus* L.) is another crop of high nutritional and economic value, particularly in tropical and subtropical regions. Global production rose from 1.8 million tonnes in 1972 to 11.2 million tonnes in 2022, with India accounting for over 60% (FAOSTAT, 2022). Okra is rich in vitamins, fibre, and minerals, while its seeds and leaves show antidiabetic, antioxidant, and antimicrobial properties (Ardestani et al., 2020).

Soil-borne fungi are particularly destructive in both crops, compromising yield and postharvest quality (Ounis et al., 2025). In garlic, *Stromatinia cepivora* (anamorph *Sclerotium cepivorum*) causes white rot, among the most severe *Allium* diseases (Hanci, 2018). First reported in Hungary in 2011 (Bakonyi et al., 2011), it produces long-lived sclerotia that make rotation ineffective (Wu et al., 2010). Germination, triggered by root exudates, leads to bulb rot and plant collapse (Sammour et al., 2011). *Fusarium proliferatum* also causes major losses, inducing basal plate and bulb rot in fields and storage (Leyronas et al., 2018). Its wide host range and adaptability heighten its threat, with symptoms including necrotic lesions, water-soaked decay, and latent storage infections (Medina et al., 2017).

In okra, major fungi include *Sclerotinia sclerotiorum* and *Rhizoctonia solani*. *Sclerotinia sclerotiorum*, a necrotrophic pathogen with a broad host range, produces long-lived sclerotia and causes white mold or pod rot, with water-soaked lesions, cottony mycelium, and black sclerotia that reduce marketability (Prova et al., 2017). By contrast, *R. solani* induces damping-off, root rot, and stem necrosis in seedlings, often leading to plant death (Anees et al., 2016). Its persistence and interactions with nematodes like *Meloidogyne incognita* exacerbate yield loss (Sharma, 2015).

Pathogen management remains difficult. Fungicides are limited by resistance, environmental risks, and high costs (Coşkuntuna and Özer, 2008). Biological control agents (BCAs) offer sustainable alternatives. Notably, *Trichoderma harzianum* and *Bacillus subtilis* reduced white rot and basal rot in garlic (Poromarto et al., 2022). Nonetheless, the breeding of resistant cultivars is regarded as

the most durable long-term strategy (Fang et al., 2019). Advancing these strategies requires clearer insights into host defense. Oxidative enzymes such as guaiacol peroxidase (POX) and polyphenol oxidase (PPO) are established immune markers (Zafar et al., 2020). POX contributes to lignification and $H_2O_2$ metabolism (Santos et al., 2023), while PPO oxidises phenolics to antimicrobial quinones and mediates hypersensitive cell death (Mayer et al., 2017).

Although single host–pathogen systems are better understood, major gaps remain. Multipathogen infections are frequent in fields and intensify disease severity (Abdullah et al., 2017), yet interactions such as those between *F. proliferatum* and *S. cepivora* in garlic are still insufficiently studied. Moreover, BCAs often show antagonism *in vitro* but inconsistent protection *in planta*, with mechanisms—from mycoparasitism to induced systemic resistance—not fully clarified (Poromarto et al., 2022). The role of oxidative enzymes under co-infection or BCA application is little explored, and cross-crop comparisons remain scarce. While *Bacillus–Trichoderma* combinations have shown efficacy in onion (Shalaby et al., 2013), their potential in garlic–okra multipathogen systems is largely unknown.

This study addresses these gaps with the following objectives:

- Investigate the dynamics of the co-occurrence of the pathogenic fungi responsible for garlic bulb rot and white rot and pathogen–pathogen interactions.
- Evaluate the resistance of eleven garlic cultivars under single and simultaneous infections.
- Assess the effectiveness of *Trichoderma asperellum* and *Bacillus amyloliquefaciens* as biocontrol agents against garlic and okra pathogens.
- Analyse POX and PPO enzyme activities in garlic and okra to elucidate biochemical defense responses under biotic stress and to assess their modulation under biocontrol treatments across both crops.

# 2. MATERIALS AND METHODS

## 2.1. *In vitro* investigation of pathogen–pathogen interactions between *Fusarium proliferatum* and *Stromatinia cepivora* on garlic

### 2.1.1. Isolation and identification of the phytopathogens

Garlic plants displaying characteristic symptoms of fungal infection, including foliar yellowing, wilting, and localized decline patches, were collected during the 2022 growing season from fields in the Makó region, Csongrád-Csanád County, South-East Hungary (46.2185°N, 20.5299°E). The symptomatic areas were confirmed through field inspection and aerial surveys using drones, which allowed visualization of irregular yellowing spots associated with possible white rot and basal rot infections.

Diseased bulbs were placed in a humid chamber for 24 hours to stimulate fungal sporulation and mycelial growth. Clove fragments were aseptically excised and inoculated onto Potato Dextrose Agar (PDA) plates under sterile conditions. Plates were incubated at 25 °C for five days, after which emerging colonies were purified using hyphal-tip isolation.

Preliminary identification was performed based on colony morphology on different media and microscopic features of hyphae and reproductive structures. Molecular identification was achieved by sequencing the internal transcribed spacer (ITS1–2) region of ribosomal DNA. DNA extraction, amplification, and sequencing were carried out according to SOP-115-11 protocols. Sequences were compared against the NCBI GenBank database using the BLAST algorithm, and results were confirmed by Eurofins BIOMI laboratory. Pure cultures were preserved on PDA slants at 5 °C for use in subsequent assays.

### 2.1.2. Selective media screening

To investigate selective media that promote the fungal growth of isolated pathogens, pure fungal colonies were subcultured on potato dextrose agar, Sabouraud Dextrose agar, Czapek-Dox Agar and Malt Extract Agar. All plates were incubated at 25°C and monitored for the following 10 days.

### 2.1.3. *In vitro F. proliferatum* and *S. cepivora* interaction test

The interaction between *F. proliferatum* and *S. cepivora* was assessed using the dual culture method. Opposed 8 mm plugs (from 8-day cultures, taken from actively growing margins) were placed on Petri dish peripheries; single-pathogen plates served as growth controls under identical conditions (25°C). Three independent experiments were performed, each including four replicates per treatment. Colony growth was measured daily as the mean of two perpendicular diameters. Interaction strength was evaluated both at the point of colony contact and when control cultures reached full plate coverage.

Growth inhibition was calculated using the following equations:

$$\textit{F. proliferatum}\ \textbf{growth inhibition (\%)} = (Cf - Df) / Cf \times 100,$$

where **Df** is the distance the *Fusarium* colonies grow towards the *Stromatinia* colonies and **Cf** is the mean of the radial growth of the *Fusarium* control plates.

$$\textit{S. cepivora}\ \textbf{growth inhibition (\%)} = (Cs - Ds) / Cs \times 100,$$

where **Ds** is the distance the *Stromatinia* colonies grow towards the *Fusarium* colonies, and **Cs** is the mean of the radial growth of the *Stromatinia* control plates.

### 2.2. *In planta* screening for resistance against single infections of *F. proliferatum* and *S. cepivora* of garlic cultivars

#### 2.2.1. Plant material preparation

Eleven garlic cultivars were evaluated to capture a wide range of genetic diversity. The spring cultivars included Flavor, Arno, and the Hungarian local variety Makói Tavaszi. Autumn cultivars comprised Thermidrome, Messidor, Sabadrome, Sabagold, Aulxito, Garcua, and Elephant garlic. The latter, though marketed as garlic, belongs to *Allium ampeloprasum* and is genetically closer to leeks. Its inclusion allowed assessment of resistance in a species of commercial importance but distinct genetic background.

#### 2.2.2. *In planta* inoculation of the pathogens

Following Esler and Coley-Smith (1984), 8 mm disks from 10-day cultures of each pathogen (grown on selective media) were placed on wounds made on the basal plates of individual cloves and sealed with parafilm. Cloves were incubated at room temperature in humid chambers. Three independent experiments were conducted. Each experiment consisted of five replicates per treatment group, and each replicate contained five cloves. Cloves were carefully selected to standardize size and weight, thereby minimizing variability in physiological state. Inoculated cloves were incubated in humid chambers at room temperature, conditions favourable to pathogen colonisation.

#### 2.2.3. Inspection and analysis

Disease incidence (DI) was calculated using the formula described by Manandhar et al. (2016):

**Disease incidence (DI (%)) = Number of diseased samples/Total number of samples × 100**

Disease severity was scored according to Mondani et al. (2021) using five classes:

- **Class 0:** asymptomatic cloves (0%).
- **Class 1:** small brown spot at basal plate (10%).

- **Class 2:** moderate basal infection (35%).
- **Class 3:** extensive basal infection with occasional mycelium or sclerotia (65%).
- **Class 4:** severe infection extending to bulb with visible mycelium and sclerotia (90%).

### 2.3. *Resistance against simultaneous infections of F. proliferatum and S. cepivora*

To simulate co-infection scenarios, cloves were inoculated with both pathogens simultaneously. Agar plugs (8 mm) of *F. proliferatum* and *S. cepivora* were placed side by side in wounds at the basal plate and sealed with parafilm. Incubation conditions, replication design, and disease assessment methods were identical to those described in Section 1.2. Disease incidence and severity were quantified with the same indices as in single infections.

### 2.4. *In vitro* study of the antagonistic activity of *T. asperellum and B. amyloliquefaciens against garlic pathogens F. proliferatum and S. cepivora*

#### 2.4.1. Isolation and identification of *T. asperellum* and use of commercial *B. amyloliquefaciens*

During the initial isolation of pathogens, colonies showing green mycelial growth characteristic of *Trichoderma* spp. were obtained. Microscopic analysis of conidiophores and conidia confirmed the species as *T. asperellum*. Pure cultures were stored on PDA slants at 5 °C.

*Bacillus amyloliquefaciens* was isolated from the commercial biopesticide Serenade® ASO (Bayer, strain QST 713, $1\times10^9$ CFU/mL). A measured amount of the product was suspended in sterile distilled water and subjected to serial dilution to obtain well-isolated colonies. A loopful of the diluted suspension was streaked onto NA plates and incubated at 25 ° C for 24 to 48 hours to facilitate bacterial growth. Colonies displaying characteristic morphology were purified through streaking.

#### 2.4.2. Dual culture assays

To assay direct antagonism, target pathogens were grown in their optimal media (*S. cepivora* on SDA; *F. proliferatum* on PDA). In each Petri plate, an 8 mm disk of *T. asperellum* and a 13 mm disk of *B. amyloliquefaciens*, both taken from pure cultures, were placed on opposite sides of the dish to allow direct interaction and observation of inhibition effects. The control plates, fungal disks containing only pathogens were placed peripherally to ensure consistency. The plates were then incubated at a constant temperature of 25°C, and colony growth was measured daily.

#### 2.4.3. Inspection and analysis

The inhibitory impact of *T. asperellum* and *B. amyloliquefaciens* on plant pathogenic fungi was quantified using the equation provided by Hernandez Castillo et al. (2011), which is as follows:

**Inhibition (%) = [(D1-D2) / D1] ×100,**

where D1 is the growth of the pathogen in the absence of the antagonist and D2 is the growth of the pathogen in the presence of the antagonist.

The extent of antagonistic activity of the BCAs was assessed using the following the classification system proposed by Bell et al. (1982):

- **Class 1:** Complete overgrowth of the pathogen by the antagonist (100% coverage).
- **Class 2:** The antagonist overgrown at least 3/4th of pathogen surface (75% coverage).
- **Class 3:** The antagonist colonizes half of the pathogen's growth area (50% coverage).
- **Class 4:** The pathogen and the antagonist locked at the point of contact.
- **Class 5:** Dominance of the pathogen, overgrowing the antagonistic agent.

### 2.5. *In planta* study of the effectiveness of *T. asperellum and B. amyloliquefaciens* against garlic pathogens *F. proliferatum* and *S. cepivora* under controlled phytotron conditions

#### 2.5.1. Preparation of *T. asperellum* and *B. amyloliquefaciens* suspensions and application

Antagonist suspensions were prepared from pure cultures. *T. asperellum* was grown on PDA (10 days, 25 °C) and *B. amyloliquefaciens* on NA (2 days, 25 °C). Colonies were scraped in 20 mL sterile distilled water, and suspensions were adjusted to $1 \times 10^7$ conidia $mL^{-1}$ (*T. asperellum*) and $1 \times 10^8$ CFU $mL^{-1}$ (*B. amyloliquefaciens*) using Neubauer chamber counts. Immediately after planting, cloves were drenched with 50 mL of suspension; for combined treatments, 25 mL of each was applied sequentially. Controls received equal volumes of sterile water to standardize moisture.

#### 2.5.2. Experimental setup

The experiment was carried out in a phytotron at 22 °C, 70% relative humidity, under a 12 h light/12 h dark regime. Uniform garlic cloves were surface-sterilized (1% NaOCl, 2 min) and rinsed three times with sterile distilled water before planting in perlite-filled pots. Perlite was selected as an inert, sterile substrate ensuring aeration and minimizing microbial interference; a balanced nutrient solution was applied at planting. Pathogen inoculation involved placing 1 cm agar plugs from actively growing 8-day cultures of *F. proliferatum* (PDA) or *S. cepivora* (SDA) directly beneath the clove basal plate, mycelial side facing the tissue, to ensure immediate root contact and simulate natural soilborne infection. Five treatments were established: (i) *F. proliferatum* inoculated (untreated), (ii) *S. cepivora* inoculated (untreated), (iii) *F. proliferatum* plus antagonist(s), (iv) *S. cepivora* plus antagonist(s), and (v) uninoculated controls. Each treatment included five biological replicates, and the experiment was repeated twice independently, yielding 50 experimental units per group.

### 2.5.3. Plant height measurements and inspection for symptoms

Plant performance was monitored for 22 days to capture both growth dynamics and potential disease progression. Plant height was measured weekly from the stem base to the tip of the tallest fully extended leaf. This parameter was used as an indirect indicator of both pathogen-induced growth suppression and the capacity of antagonists to mitigate such effects. In parallel, visual inspections were carried out throughout the trial, with systematic scoring of disease symptoms including foliar stunting, wilting, and chlorosis.

## 2.6. Enzymatic analysis of oxidative responses in garlic and okra seedlings under pathogen and biocontrol treatments

### 2.6.1. Plant material and seedling preparation

To investigate defense responses, seedlings of garlic (cv. Makoi) and okra were evaluated for oxidative enzyme activity following pathogen infection and biocontrol treatments. Garlic cloves and okra seeds were first surface-sterilized in 1% sodium hypochlorite solution for 2 minutes and rinsed three times with sterile distilled water to eliminate external contaminants. Germination was carried out in humid chambers maintained at 24 °C under ambient light. Garlic seedlings were grown for 10 days and okra for 4 days, corresponding to developmental stages with well-formed roots and shoots suitable for experimental inoculation.

### 2.6.2. Pathogen and biocontrol inoculation

Garlic seedlings were inoculated with *F. proliferatum* and *S. cepivora*, while okra seedlings were inoculated with *R. solani* and *S. sclerotiorum*. For pathogen inoculations, mycelial disks (1 cm for garlic and 0.7 cm for okra) were placed directly at the basal root–hypocotyl interface to ensure immediate pathogen contact. Biocontrol treatments involved *T. asperellum* and *B. amyloliquefaciens*. *Trichoderma asperellum* was cultured on PDA for 7 days, and conidial suspensions were prepared in sterile distilled water containing 0.01% Tween 20, adjusted to $1 \times 10^7$ conidia $mL^{-1}$ using a hemocytometer. *Bacillus amyloliquefaciens* was cultured on NA for 24 h, and suspensions were standardized to $1 \times 10^8$ CFU $mL^{-1}$. For each seedling, 100 µL of the antagonist suspension was carefully applied at the root zone to promote early colonization. Control groups comprised uninoculated seedlings maintained under identical growth conditions to distinguish pathogen-induced oxidative responses from basal enzyme activity. All treatments were carried out with three independent biological replicates to ensure reproducibility and account for biological variation.

### 2.6.3. Enzyme extraction

Forty-eight hours after inoculation for okra and five days for garlic—corresponding to the onset of visible symptoms—500 mg of fresh seedling tissue was collected per treatment. Samples were ground in a pre-chilled mortar with liquid nitrogen to ensure rapid freezing and prevent enzymatic degradation. The resulting powder was transferred into 2 mL of ice-cold Tris extraction buffer (0.05 M Tris-HCl, pH 7.8; 1 mM EDTA-$Na_2$; 7.5% PVP K25), formulated to stabilize proteins and inhibit phenolic oxidation. Homogenates were centrifuged at 12,000 × g for 15 min at 4 °C, and the clear supernatant maintained on ice for immediate use in guaiacol peroxidase (POX) and polyphenol oxidase (PPO) assays.

#### 2.6.3.1. Guaiacol peroxidase (POX)

POX activity was determined by guaiacol oxidation following Chance and Maehly (1955) with minor modifications. Absorbance was measured at 470 nm using a reaction mixture containing 2.2 mL of 0.1 M sodium phosphate buffer (pH 6.0), 100 µL of 50 mM guaiacol, 100 µL of 32.5 mM hydrogen peroxide, and 50 µL of enzyme extract. Results were expressed as $\Delta A \cdot min^{-1} \cdot g^{-1}$ FW using a conversion factor of 11.05.

#### 2.6.3.2. Polyphenol oxidase (PPO)

PPO activity was assessed via catechol oxidation following Kar and Mishra (1976) with minor modifications. Absorbance was measured at 400 nm using a mixture of 1.6 mL of 0.1 M sodium phosphate buffer (pH 6.0), 200 µL of 0.2 M catechol, and 200 µL of enzyme extract. Results were calculated with a conversion factor of 63.16 and expressed as $\Delta A \cdot min^{-1} \cdot g^{-1}$ FW.

### 2.6.4. Enzyme activity assays

Enzyme activity was recorded spectrophotometrically at 0, 30, and 60 seconds after mixing. Values at 1 minute were used for graphical presentation, while all timepoints were incorporated into the statistical analysis. Each treatment was performed with three independent biological replicates.

## 2.7. Statistical analysis

All analyses were performed using IBM SPSS (version 27.0) and Python (version 3.11.6). Normality of residuals was tested using the Shapiro–Wilk test and homogeneity of variance with Levene's test. Parametric data were analysed by one- or two-way ANOVA, with post hoc comparisons by Tukey's Honestly Significant Difference (HSD). Non-parametric datasets were analysed using the Kruskal–Wallis test followed by Dunn's post hoc analysis with Bonferroni correction.

Effect sizes were reported alongside p-values. For ANOVA, eta squared ($\eta^2$) was used to indicate the proportion of variance explained, while $\eta^2$ values for non-parametric tests were derived from the H statistic.

# 3. RESULTS AND DISCUSSION

## 3.1. *In vitro* investigation of pathogen–pathogen interactions of *F. proliferatum* and *S. cepivora* on garlic

### 3.1.1. Isolation and identification of the phytopathogens

Inoculation of symptomatic garlic tissues onto growth media yielded two distinct pathogens. On PDA, *F. proliferatum* produced dense white aerial mycelia with violet pigmentation, while *S. cepivora* was characterized by abundant black microsclerotia. Microscopic examination of *F. proliferatum* revealed straight, thin-walled macroconidia with 3–5 septa, curved apical cells, together with abundant microconidia in chains from monophialides and polyphialides. No chlamydospores were detected, a defining trait of the species, corroborating earlier descriptions (Leslie and Summerell, 2006). The *S. cepivora* isolate showed 100% similarity to ITS1-2 sequence KP257580.1, while the second isolate clustered within the *Fusarium fujikuroi* species complex (FFSC) with 100% similarity. Considering morphology and prior reports identifying *F. proliferatum* as the principal causal agent of garlic dry rot (Gálvez and Palmero, 2022), the isolate was conclusively identified as *F. proliferatum*.

### 3.1.2. Selective media analysis

Growth of *F. proliferatum* was supported by all tested media. On MEA, colonies were white with progressively dark red pigmentation. On SDA, white aerial colonies with yellow to light brown pigmentation developed. On PDA, white mycelia with dark violet pigmentation formed, while CDA supported cotton-like colonies with white pigmentation. For *S. cepivora*, abundant growth and microsclerotia production were supported only by MEA and SDA, producing yellowish and white pigmentations, respectively. CDA and PDA were unsuitable, as growth was limited.

### 3.1.3. *In vitro F. proliferatum* and *S. cepivora* interaction assay

The dual culture assay on SDA revealed that both pathogens expanded normally until contact, where growth ceased and a stable inhibition zone formed. Neither fungus overgrew the other, indicating competitive coexistence. Colonies of *S. cepivora* at the interaction front exhibited pronounced darkening.

Daily radial growth measurements confirmed these dynamics. At Day 8, radial growth was reduced compared to controls: *F. proliferatum* (5.60 cm vs. 6.10 cm) and *S. cepivora* (5.35 cm vs. 5.72 cm), with growth inhibition of 8.27% and 6.40%, respectively. ANOVA confirmed significant differences ($p < 0.001$, $\eta^2 = 0.57$), with inhibition effects moderately significant ($p = 0.01$, $\eta^2 = 0.23$). By Day 14, single cultures reached full radial growth (7.5 cm), while dual cultures remained restricted (5.60 cm and 5.35 cm), corresponding to growth inhibition of 25.39% and 28.61%.

Statistical analysis confirmed significant effects ($p < 0.001$, $\eta^2 = 0.60$), demonstrating the cumulative impact of prolonged interaction. These findings provide evidence of competitive coexistence between *F. proliferatum* and *S. cepivora*. By Day 8, growth ceased at the interaction fronts, reflecting rapid establishment of inhibitory effects and resource competition, consistent with the "competition" interaction type described by Abdullah et al. (2017). The persistence of the inhibition zone through Day 14 further supports a stable equilibrium, where neither pathogen displaced the other.

The darkening of *S. cepivora* colonies near *F. proliferatum* suggests an intensified stress response, likely involving enhanced sclerotia melanization. Similar physiological adaptations have been described in fungi exposed to competition or oxidative stress (Lin et al., 2023). Comparable responses were noted by Yuan and Chen (2021), where *Monascus* spp. increased pigment production when co-cultured with *Aspergillus niger*. Such parallels indicate that *S. cepivora* responds biochemically to competitive stress, producing more sclerotia as a survival mechanism.

### 3.1.4. Resistance against single infections of *F. proliferatum* and *S. cepivora*

The *in planta* inoculation of eleven garlic cultivars with single fungal isolates revealed significant differences in incidence and severity. Symptoms first appeared 5 days post-inoculation. *F. proliferatum* caused Class 4 infections in 'Garcua' (96%), 'Thermidrome' (93.33%), 'Topadrome' (93.33%), and 'Aulxito' (93.33%), characterized by basal plate rot, extensive brown spotting, and abundant mycelium and sclerotia. 'Messidor' (68%), 'Arno' (92%), and 'Sabagold' (82.67%) displayed Class 3 severity with half-basal plate lesions. 'Flavor' (89.33%) exhibited mild Class 1 symptoms with isolated brown spots, while 'Makói Tavaszi' (29.33%) showed low incidence but severe Class 4 infections in affected cloves. 'Elephant' remained completely symptomless (0%).

For *S. cepivora*, severe Class 4 infections were recorded in 'Flavor' (65.33%), 'Messidor' (85.33%), 'Arno' (90.67%), and 'Sabadrome' (97.33%). 'Makói Tavaszi' (98.67%) and 'Aulxito' (93.33%) were also highly susceptible. 'Topadrome' (97.33%) showed Class 3 symptoms with localized mycelial growth and browning, while 'Garcua' (84%) and 'Thermidrome' (96%) presented moderate infections. Again, 'Elephant' showed complete resistance. ANOVA confirmed significant differences among cultivars under single inoculations ($p < 0.001$, $\eta^2 = 0.48$).

Cultivar 'Elephant' was uniquely resistant, corroborating earlier reports of rare resistance in *Allium* species (Coley-Smith and Entwistle, 1988). Akter et al. (2021) also reported significant variation across cultivars, consistent with the complete resistance of 'Elephant' and partial tolerance in 'Sabagold' and 'Thermidrome' found in our study. Esler and Coley-Smith (1984) suggested that

resistance may arise from failure to stimulate sclerotial germination, providing a plausible explanation for the robust resistance of 'Elephant'.

Responses to *F. proliferatum* were similarly variable. 'Makói Tavaszi' demonstrated reduced incidence but severe symptoms when infection occurred, suggesting initial resistance without post-infection containment (Jannatun et al., 2020). The partial resistance of 'Flavor' and the complete resistance of 'Elephant' may reflect underlying tolerance or genetic factors. Filyushin et al. (2021) identified higher expression of chitinase genes in resistant cultivars, supporting the possibility that 'Elephant' carries unique genetic traits that could be used in breeding programs.

#### 3.1.5. Resistance against simultaneous infections of *F. proliferatum* and *S. cepivora*

Dual inoculation accelerated symptom development compared to single infections. Advanced browning and mycelial growth appeared within 2 days, followed by sclerotia formation by Day 4. Co-inoculated cultivars, except 'Elephant', developed Class 4 symptoms with incidences above 96%. These were characterized by extensive basal rot, browning extending into cloves, and heavy mycelium and sclerotia formation. 'Elephant' again remained asymptomatic (0%). ANOVA confirmed significant differences under dual inoculation ($p < 0.001$, $\eta^2 = 0.78$), with stronger effects than single infections. The consistent resistance of 'Elephant' even under co-inoculation underscores its exceptional genetic defense capacity, in contrast to other cultivars that were uniformly susceptible, warranting further study.

Despite the competitive coexistence observed *in vitro*, the *in planta* dual inoculations revealed a synergistic interaction that increased disease severity. Symptoms appeared earlier and were more aggressive, showing that host factors can shift pathogen–pathogen interactions. Fang et al. (2021) similarly found that co-infection of alfalfa by *Fusarium oxysporum f. sp. medicaginis* and *Rhizoctonia solani* increased disease severity compared to single infections.

### 3.2. *In vitro* study of the effectiveness of *T. asperellum* and *B. amyloliquefaciens* against garlic pathogens *F. proliferatum* and *S. cepivora*

#### 3.2.1. *T. asperellum* vs. *F. proliferatum*

When co-cultured on PDA, *T. asperellum* exhibited dense green mycelium that advanced into the *F. proliferatum* colony, while the pathogen developed a darkened reaction zone at the interface. The reverse side showed a distinct brown–black band, consistent with melanin deposition.

Two-way ANOVA confirmed significant effects of treatment and time. Inhibition of *F. proliferatum* began on Day 3 (6.66%) and rose steadily, plateauing at 45.60% by Day 13. Control colonies reached 7.5 cm, while dual cultures stabilized at ~4.1 cm. These observations highlight

the strong antagonistic capacity of *T. asperellum*. Comparable findings of hyphal melanization as a stress response to *Trichoderma* spp. metabolites have been reported by Atanasova et al. (2013).

#### *3.2.2. B. amyloliquefaciens* vs. *F. proliferatum*

In PDA dual cultures, *F. proliferatum* formed dense colonies separated from *B. amyloliquefaciens* by a clear inhibition zone, with compressed fungal margins and suppressed pigmentation near the bacterial colony.

Kruskal–Wallis analysis confirmed significant inhibitory effects from Day 5 onward, with inhibition peaking at 26.06% on Day 10. Control colonies reached 7.5 cm, while dual cultures plateaued at ~5.6 cm. While inhibition was moderate compared to *T. asperellum*, results align with previous reports of *Bacillus* spp. providing consistent but less aggressive suppression of *Fusarium* spp. (El Barnossi et al., 2024).

#### *3.2.3. T. asperellum* vs. *S. cepivora*

On SDA, *T. asperellum* rapidly expanded, producing a clear inhibition arc around *S. cepivora*. The pathogen displayed compressed margins and reddish-brown pigmentation at the interaction zone, consistent with stress-induced metabolite accumulation.

Two-way ANOVA showed treatment effects accounting for nearly all variance ($\eta^2 = 0.99$). Inhibition increased from 16.82% on Day 4 to 77.4% by Day 14. Control colonies reached 7.5 cm, while dual cultures plateaued at ~1.7 cm by Day 8. The overgrowth and complete encirclement of *S. cepivora* confirmed potent antagonism, consistent with mycoparasitic suppression and secretion of antifungal metabolites. These findings support earlier reports of *Trichoderma* spp. effectively controlling garlic white rot (Elshahawy et al., 2019).

#### *3.2.4. B. amyloliquefaciens* vs. *S. cepivora*

Dual culture on SDA revealed a persistent inhibition zone between *B. amyloliquefaciens* and *S. cepivora*, accompanied by melanization of the fungal margin facing the bacterium.

Two-way ANOVA confirmed significant effects ($\eta^2 = 0.95$). Inhibition rose from 9.75% on Day 4 to 26.0% by Day 10. Control colonies expanded to 7.5 cm, while dual cultures stabilized at ~5.6 cm. These results indicate progressive antibiosis via bacterial metabolites, reflected in reduced growth and stress pigmentation.

Across all *in vitro* assays, *T. asperellum* consistently outperformed *B. amyloliquefaciens*. Against *F. proliferatum*, *T. asperellum* achieved >45% inhibition through direct overgrowth and metabolite secretion, while *B. amyloliquefaciens* achieved ~26% inhibition through antibiosis alone. Against *S. cepivora*, *T. asperellum* reached 77.4% inhibition, whereas *B. amyloliquefaciens* achieved 26%.

These findings confirm that *Trichoderma* spp. exerts stronger direct antagonism, supported by prior reports (Elshahawy et al., 2019). Nevertheless, *Bacillus* spp.-based antibiosis contributes additional, though moderate, inhibitory effects and is relevant in integrated biocontrol strategies (Cawoy et al., 2015).

### 3.3. *In planta* study of the effectiveness of *T. asperellum* and *B. amyloliquefaciens* under phytotron conditions

Under phytotron conditions, garlic plants inoculated with pathogens showed significant growth suppression. Controls (uninoculated) grew normally to 17.93 cm by Week 3, with healthy morphology.

Inoculation with *F. proliferatum* reduced height severely (5.05 cm by Week 3), with symptoms of wilting, curling, and chlorosis. *T. asperellum* treatment (TF) restored growth to 10.30 cm by Week 3, reducing symptoms markedly. *B. amyloliquefaciens* (BF) provided partial restoration (8.12 cm), while combined treatment (TBF) reached 8.35 cm, suggesting additive but not superior effects.

Inoculation with *S. cepivora* prevented shoot emergence entirely (0 cm at Week 3). Application of *T. asperellum* (TS) or *B. amyloliquefaciens* (BS) enabled limited emergence, with mean heights of 5.57 cm and 4.68 cm, respectively. Combined treatment (TBS) achieved the greatest improvement (6.83 cm), though still far below controls.

*In planta* results corroborate *in vitro* assays, showing *T. asperellum* as the more effective antagonist. Against *F. proliferatum*, it significantly reduced growth suppression, consistent with reports of *Trichoderma* spp. enhancing plant defenses and root colonization (Elshahawy and Marrez, 2024). *Bacillus amyloliquefaciens* provided moderate benefits via antibiosis and possible ISR effects (Cawoy et al., 2015). Against *S. cepivora*, no treatment fully prevented losses, though combined use of *T. asperellum* and *B. amyloliquefaciens* offered partial protection, reflecting complementary modes of action. Such synergistic effects have been documented in microbial consortia (Guetsky et al., 2002).

### 3.4. Enzymatic defense responses in garlic under pathogen infections and biocontrol treatments

The activity of guaiacol peroxidase (POX) and polyphenol oxidase (PPO) was evaluated in garlic under pathogen challenge and biocontrol treatments. Plants were inoculated with *F. proliferatum* (F) or *S. cepivora* (S), dual pathogen treatment (FS), and co-inoculations with *T. asperellum* (T) or *B. amyloliquefaciens* (B). Measurements were recorded at 0, 30, and 60 seconds, with 1-minute values used for comparison.

Kruskal–Wallis tests revealed significant treatment effects for both POX ($H = 42.85$, $df = 7$, $p < 0.001$, $\eta^2 = 0.51$) and PPO ($H = 43.12$, $df = 7$, $p < 0.001$, $\eta^2 = 0.51$), confirming treatment-driven variation in oxidative enzyme responses. For POX, the highest activity occurred in BF (0.0847 $\Delta A \cdot min^{-1} \cdot g^{-1}$ FW), followed by TF (0.0743) and TS (0.0693). Intermediate levels were recorded in FS (0.0647), BS (0.0627), and control (0.0617), while the lowest were in S (0.0583) and F (0.0563). Thus, POX induction was most pronounced when biocontrol agents were applied with pathogens, particularly *B. amyloliquefaciens* or *T. asperellum* in the presence of *F. proliferatum*.

For PPO, the highest activities were recorded in TS (0.0893), S (0.0847), and FS (0.0793). Intermediate values were observed in F (0.0747), TF (0.0697), and BF (0.0647). The lowest occurred in the control (0.0597) and BS (0.0547). Notably, the BS treatment reduced PPO, relative to pathogen-only infection.

The enzymatic profiling indicates that *T. asperellum* consistently elevated oxidative defenses, aligning with previous evidence of *Trichoderma* spp. priming host peroxidase activity and strengthening structural defenses such as lignification (Shalaby et al., 2013). By contrast, *B. amyloliquefaciens* enhanced POX in garlic but reduced PPO in the presence of *S. cepivora*. Such variability reflects *Bacillus* spp.-induced systemic resistance, which may selectively prime certain pathways while dampening others (Saleh et al., 2024).

### 3.5. Enzymatic defense responses in okra under pathogen infections and biocontrol treatments

To assess defense responses in okra, POX and PPO were measured following inoculation with *Rhizoctonia solani* (R), *Sclerotinia sclerotiorum* (Sc), dual infection (ScR), and co-inoculations with *T. asperellum* (T) or *B. amyloliquefaciens* (B). Enzyme activity was expressed as $\Delta A \cdot min^{-1} \cdot g^{-1}$ FW, with final values taken at 1 minute.

Kruskal–Wallis tests revealed significant treatment effects for POX ($H = 15.76$, $df = 7$, $p = 0.027$, $\eta^2 = 0.55$). POX was highest in R (0.867), followed by TR (0.694). Intermediate levels were recorded in ScR (0.433), TSc (0.434), BSc (0.385), and control (0.450). The lowest were in BR (0.215) and Sc (0.189). These results suggest that *R. solani* strongly induces peroxidase activity, while *B. amyloliquefaciens* can suppress POX when combined with R.

PPO activity showed a different profile. The highest activity was recorded in ScR (0.754), followed by TSc (0.716), BSc (0.678), and R (0.634). Intermediate levels were observed in TR (0.456) and BR (0.445), while Sc (0.093) and control (0.123) showed the lowest activity. These data indicate that PPO is strongly induced by dual-pathogen infections and *T. asperellum*, while *S. sclerotiorum* alone suppresses PPO activity.

### 3.6. Cross-crop analysis of biocontrol-induced defence pathways in garlic and okra

The enzymatic defense responses of garlic and okra revealed both crop-specific and treatment-dependent patterns. In garlic, POX was strongly enhanced by co-inoculation with *B. amyloliquefaciens* + *F. proliferatum* and by *T. asperellum* combinations, confirming that *Trichoderma* spp. can stimulate peroxidase-linked lignification defenses. PPO was highest under *T. asperellum* + *S. cepivora* and in single *S. cepivora* infections, while *B. amyloliquefaciens* reduced PPO activity when paired with *S. cepivora*, reflecting the selective modulation of oxidative pathways by *Bacillus* spp. (Saleh et al., 2024).

In okra, POX was most strongly induced by *R. solani* alone, consistent with necrotroph-triggered lignification (Yang et al., 2024). The BR treatment suppressed POX, suggesting interference of Bacillus metabolites with host peroxidase responses (Alexis et al., 2021). PPO activity, in contrast, peaked under dual stress (*S. sclerotiorum* + *R. solani*) and with *T. asperellum*, whereas *S. sclerotiorum* alone produced minimal PPO.

Overall, *T. asperellum* consistently enhanced oxidative defenses in both crops, while *B. amyloliquefaciens* exerted variable effects depending on host and pathogen context. These results highlight the advantage of integrating fungal and bacterial BCAs to activate complementary defense pathways, especially where single agents are insufficient (Miljaković et al., 2020).

# 4. NEW SCIENTIFIC FINDINGS

- **Co-infection of *Fusarium proliferatum* and *Stromatinia cepivora* in garlic synergistically intensified disease *in planta* despite competitive coexistence *in vitro*.** Dual culture assays showed stable inhibition without dominance, with mutual suppression of 25.39% (*F. proliferatum*) and 28.61% (*S. cepivora*) by Day 14.
- **Garlic variety 'Elephant' was completely resistant to both pathogens under single and dual inoculation**, marking the first report of broad-spectrum resistance to *Fusarium* bulb rot and white rot in garlic under controlled conditions.
- ***Trichoderma asperellum* exhibited stronger antagonism than *Bacillus amyloliquefaciens*.** *T. asperellum* fully overgrew *S. cepivora* (77.4% inhibition) and suppressed *F. proliferatum* (45.6%), while *B. amyloliquefaciens* acted mainly through antibiosis, achieving 26.06% inhibition of *F. proliferatum* and 26.0% against *S. cepivora*.
- ***In planta* assays showed *S. cepivora* caused complete emergence failure, whereas *F. proliferatum* severely stunted growth.** *T. asperellum* restored seedling height most effectively, and combined *T. asperellum* + *B. amyloliquefaciens* treatments showed synergistic improvement over single applications.
- **Oxidative enzyme responses were pathogen- and treatment-specific.** In garlic, POX was maximally induced by *B. amyloliquefaciens* + *F. proliferatum*, while PPO peaked with *T. asperellum* + *S. cepivora*. In okra, *R. solani* strongly triggered POX, *S. sclerotiorum* suppressed it, and PPO peaked under dual stress, with *T. asperellum* restoring enzyme activity and *B. amyloliquefaciens* showing variable effects.
- **Combined biocontrol treatments provided superior suppression**, particularly against *S. cepivora*, improving emergence, reducing severity, and enhancing enzymatic defense more effectively than single agents.

# 5. CONCLUSIONS AND RECOMMENDATIONS

This dissertation investigated the interactions between soil-borne fungal pathogens, garlic cultivars, and microbial biocontrol agents, with okra included as a comparative host. Through integrated morphological, molecular, and physiological approaches, *Fusarium proliferatum* and *Stromatinia cepivora* were identified as the principal causal agents of garlic bulb rot and white rot. While dual culture assays revealed mutual inhibition between the two fungi *in vitro*, co-inoculation *in planta* produced synergistic pathogenicity, with earlier symptom onset and markedly greater severity compared to single infections. This contrast demonstrates the limitations of *in vitro* models and underlines the necessity of host-based experimentation when evaluating pathogen behavior.

Resistance screening across eleven garlic cultivars showed that susceptibility was generally high under dual infection, with incidences surpassing 96%. The single exception was the cultivar 'Elephant', which remained entirely symptomless even under simultaneous infection. This unique resistance represents a significant advance, pointing to the presence of durable genetic traits that could be harnessed for breeding programmes. Such resistance requires field validation and molecular characterisation, yet it already provides a clear direction for reducing reliance on chemical control measures through varietal improvement.

The performance of biocontrol agents constituted the second major axis of this study. *Trichoderma asperellum* and *Bacillus amyloliquefaciens* were confirmed and evaluated both *in vitro* and in controlled phytotron experiments. *In vitro*, *T. asperellum* exhibited aggressive mycoparasitic overgrowth and strong suppression of pathogen growth, while *B. amyloliquefaciens* produced stable inhibition zones characteristic of antibiosis. *In planta*, *T. asperellum* consistently mitigated the effects of *F. proliferatum* and restored seedling vigour, while *B. amyloliquefaciens* provided additional though more moderate protection. Against *S. cepivora*, the most aggressive of the pathogens, individual treatments were insufficient to prevent seedling death, but their combination achieved partial protection, demonstrating the value of microbial consortia.

Enzymatic profiling provided further mechanistic insight into these interactions. In garlic, peroxidase (POX) activity was most strongly induced in treatments combining *F. proliferatum* with biocontrol agents, particularly *B. amyloliquefaciens*, while polyphenol oxidase (PPO) activity was elevated under *S. cepivora* stress, especially when paired with *T. asperellum*. In okra, POX was primarily activated by *Rhizoctonia solani*, whereas PPO activity was enhanced under dual infection and in treatments involving *T. asperellum*. These results confirm that plant oxidative defences are shaped by both pathogen identity and biocontrol presence, and that the two agents studied contribute not only by direct antagonism but also by activating host defence pathways.

These findings advance our understanding of how host plants, pathogens, and biocontrol agents interact under conditions of co-infection. They show that cultivar resistance, as exemplified by ‘Elephant’, provides a promising foundation for breeding resistant garlic lines, while *T. asperellum* emerges as a consistently effective biocontrol agent with *B. amyloliquefaciens* providing complementary effects. At the same time, the incomplete protection achieved against *S. cepivora* illustrates that biocontrol alone cannot ensure reliable disease management under severe infection pressure. Future work should therefore aim to validate these findings in field conditions, to elucidate the molecular basis of resistance in ‘Elephant’, and to expand the search for additional microbial consortia capable of enhancing suppression. Broader system-level studies, integrating enzymatic, metabolomic, and transcriptomic profiling, will be required to clarify how plants balance structural and biochemical defences under pathogen pressure.

# RELATED PUBLICATIONS

## Articles in peer-reviewed scientific journals

- **Ounis, S**., Turóczi, G., & Kiss, J. (2024). Arthropod pests, nematodes, and microbial pathogens of okra (*Abelmoschus esculentus*) and their management—A review. Agronomy, 14(12), 2841.
- **Ounis, S**., Turóczi, G., & Kiss, J. (2025). Co-Occurrence of *Stromatinia cepivora* and *Fusarium proliferatum* Fungi on Garlic: *In Vitro* Investigation of Pathogen–Pathogen Interactions and *In Planta* Screening for Resistance of Garlic Cultivars. Plants, 14(3), 440.
- Alabbasi M.H., **Ounis, S**., Turóczi, G., Veres A., & Juhász A. Gyapjú a növényvédelem szolgálatában: kölcsönhatások vizsgálata talajlakó kórokozókkal *in vitro* körülmények között. Submitted in Növényvédelem Journal on 1st September 2025, under review.

## Posters

- Presented a poster on "Pathogenic fungi on garlic in Makó region and the possibility of the biological control" in the 70th Plant Protection Scientific Days, Budapest, Hungary.
- Presented a poster on the "Resistance of garlic cultivars to plant pathogens *Fusarium proliferatum* and *Stromatinia cepivora*" the 70th Plant Protection Scientific Days, Budapest, Hungary.

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