

Biochemical Responses and Biocontrol of *Meloidogyne javanica* by *Arthrobotrys oligospora* and *Bacillus subtilis* Using the Split-Root Method on Tomato

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Abstract

Root exudates affect soil organisms through chemical reactions and nutrient supply, as well as through attraction, repulsion, or growth inhibition. This study investigated the biochemical responses of tomato roots using a split-root system after inoculation with *Meloidogyne javanica*, combined with either *Arthrobotrys oligospora*, *Bacillus subtilis*, or both. Root exudates were analyzed for H₂O₂, total phenolics, carbohydrates, and total protein, and the biocontrol potential of the two agents under nematode stress was assessed. Treatments significantly affected defensive (H₂O₂, phenolics) and nutritional (protein, carbohydrate) compounds, with strong associations among these responses. The highest concentrations of H₂O₂ (31.57 $\mu\text{mol ml}^{-1}$) and phenolics (0.56 $\mu\text{g ml}^{-1}$) were observed in the combined nematode-fungus-bacteria treatment. Protein levels peaked in both the nematode-fungus-bacteria and nematode-bacteria treatments, reaching 33.65 and 33.59 $\mu\text{g ml}^{-1}$, respectively. Carbohydrate levels were highest in the fungus treatment, measuring 33.32 mg ml^{-1} , and lowest in the nematode-only treatment, which recorded 9.76 mg ml^{-1} . These findings underscore the significance of root exudates in the interactions among plants, microbes, and nematodes. They also suggest that biological control could be a sustainable pest management strategy for improving crop productivity.

Keywords: Biological control, Rhizosphere interactions, Root exudates, Root-knot nematode.

Introduction

The root-knot nematode *M. javanica* is a major constraint on tomato (*Solanum lycopersicum* L.) production, causing root galling and severe histological alterations by establishing giant cells as

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permanent feeding sites for second-stage juveniles (J2). This parasitic interaction typically alters host metabolic allocation and often triggers localized host defenses, which are frequently characterized by a transient accumulation of reactive oxygen species such as H_2O_2 and the mobilization of defense-related secondary metabolites, particularly phenolic compounds (Asadi-Sardari *et al.*, 2022; Ghahremani *et al.*, 2020). Given the environmental and toxicological constraints associated with synthetic nematicides, evaluating biological control agents within integrated pest management frameworks has become increasingly important (Abd-Elgawad, 2021; Forghani and Hajihassani, 2020). Beneficial rhizosphere microorganisms, including the plant growth-promoting rhizobacterium *B. subtilis* and the nematophagous fungus *A. oligospora*, represent viable candidates for the suppression of plant-parasitic nematodes (Topalovi and Geisen, 2023; Zhang *et al.*, 2020).

B. subtilis colonizes host roots and mitigates nematode infection through direct antagonism mediated by the secretion of antimicrobial metabolites and lytic enzymes, as well as by triggering systemic defense responses (Majeed *et al.*, 2023; Gao *et al.*, 2016). Concurrently, the predatory fungus *A. oligospora* develops specialized morphological trapping devices predominantly adhesive three-dimensional networks to capture and digest second-stage juveniles (Yang *et al.*, 2020). Trap morphogenesis in *A. oligospora* is modulated by nematode-derived signals, specific root exudate constituents, and bacterial metabolites (Wang *et al.*, 2014; Hsueh *et al.*, 2017). Although co-inoculation of rhizobacteria and predatory fungi can yield synergistic pathogen suppression (El-Ashry *et al.*, 2021), the inter-organismal chemical communication and physiological dynamics underpinning this tripartite plant–microbe system warrant further clarification (Backer *et al.*, 2018). Root exudates function as dynamic chemical interfaces within the rhizosphere, encompassing carbohydrates, amino acids, and defense-related secondary metabolites that modulate microbial recruitment and influence nematode chemotaxis and egg hatching (Pantigoso *et al.*, 2022). In addition to soluble exudates, the detachment of border cells and mucilage secretion from the root cap contribute to protecting meristematic tissues against soil-borne pathogens (Yang *et al.*, 2016; Driouich *et al.*, 2021; Fortier *et al.*, 2023). Despite the well-documented role of root exudates in mediating rhizosphere interactions, qualitative and quantitative changes in root exudation during concurrent exposure to *M. javanica* and biocontrol agents remain poorly understood. Furthermore, the inhibitory effects of host defense compounds,

particularly H₂O₂ and phenolic compounds, on the mycelial growth of *A. oligospora* warrant detailed investigation.

The present study evaluated the interactions among tomato plants, the beneficial microbes *B. subtilis* and *A. oligospora*, and *M. javanica*, with a focus on root exudation patterns. Using a split-root system together with in vitro assays, three specific hypotheses were tested: (1) inoculation with *B. subtilis* and *A. oligospora* elicits systemic defense responses against *M. javanica* and alters biochemical profiles in exudates collected from the non-inoculated root compartment; (2) *A. oligospora* colonizes and establishes within nematode-infected root tissues; and (3) host defense compounds, specifically H₂O₂ and phenolic compounds, exert concentration- and time-dependent inhibitory effects on *A. oligospora* mycelial growth.

Materials and Methods

Nematode inoculum preparation

Tomato plants infected with *M. javanica* were collected from the research greenhouse of the Agricultural College of Aburairhan, University of Tehran. Infected roots were thoroughly washed, and nematode eggs were extracted from the root tissues using 10% sodium hypochlorite followed by sieving, and were subsequently used for downstream experiments (Hussey and Barker, 1976).

Preparation of Bacteria and Fungal inoculum

Stock cultures of *B. subtilis* BS-AB101 and *A. oligospora* AO-AF45 were obtained from the culture collection of the Department of Plant Pathology, Abureihan Faculty, University of Tehran, Iran. *B. subtilis* was cultured on potato dextrose agar (PDA) at 28 °C for 2 days. Following incubation, the cells were scraped from the agar surface and transferred to 100 mL of sterile distilled water. The suspension was stirred thoroughly to obtain a homogeneous cell suspension, and its concentration was determined and adjusted to 1×10^8 cells mL⁻¹ (Sun *et al.*, 2021).

Fungal (*A. oligospora*) inoculum for experiments was cultured on potato dextrose agar (PDA) in 100 × 15 mm Petri dishes at 25°C in the dark. Fungal spores were harvested at maturity by flooding the dishes with sterile water and scraping the plate with a sterile razor blade. Spores were filtered through autoclaved cheesecloth. Spore concentration was quantified using a hemacytometer (Thomas Scientific, Philadelphia, PA, USA), and finalized to 10⁶ spores ml⁻¹ for each treatment.

Plant material

Tomato seeds (*Solanum lycopersicum* L.) cultivar Early Urbana were planted in the seed starter trays (96 wells), a mixture of peat moss and perlite (1:1). Ten days after planting, they were watered with fertilizer (NPK 20-20-20) every three days. Then, in the four-leaf stage they were transferred to plastic pots with a diameter of 15 cm filled with 1 kg of sterile soil (mixture of sand, field soil, humus 1:1:1 v:v:v).

Experiment one: Split root system

Biochemical analyses were performed on two distinct fractions: (1) root exudates collected in test tubes containing sterile distilled water and (2) root tissue extracts prepared by homogenizing roots grown in soil. The split-root system was established in three successive steps (Figure 1).

Plant establishment

Two weeks after transplanting, the root system of each plant was carefully divided into two approximately equal portions. One portion remained in the pot, while the other was placed in a test tube. Before inoculation, the separated root portion was transferred to a test tube containing sterile distilled water. The tube opening was tightly sealed to prevent evaporation, and the test tube was positioned downward through a small hole drilled in the bottom of the pot.

Inoculation:

Simultaneous inoculation was performed to facilitate concurrent rhizosphere interactions, following the protocols described by Oka *et al.* (1999), with minor modifications. The soil-grown root portion was inoculated by making three to four small holes (2–3 cm deep) in the soil surrounding the crown and root zone. Subsequently, 25 mL of *B. subtilis* suspension (1×10^8 CFU mL⁻¹), 25 mL of *A. oligospora* suspension (1×10^6 CFU mL⁻¹), and 15 mL of a nematode suspension containing approximately 3,000 *M. javanica* eggs (approximately 200 eggs mL⁻¹; mean hatching rate $\approx 82\%$) were evenly dispensed into the holes using a micropipette.

Sampling at 3 days post-inoculation

Three days post-inoculation (dpi), two distinct sample types were collected. Root exudates were collected from the liquid in the test tubes containing the separated root portions, which had no physical contact with the soil or the inoculated microorganisms. Concurrently, soil-grown root

tissues in direct contact with the inoculated microorganisms were harvested for biochemical analyses, immediately snap-frozen in liquid nitrogen, and stored at -70°C until further assays. For root exudate collection, the separated root portions were submerged in 10 mL of sterile distilled water for 72 h (3 days) post-inoculation. Plants in Treatment C, in which the separated root portions were maintained in sterile distilled water without soil inoculation, served as blank controls. Although root biomass within individual collection tubes was not quantified, all treatments were subjected to identical collection volumes (10 mL), collection periods (72 h), and experimental conditions.

The experiment was arranged in a completely randomized design (CRD) with four replications per treatment, with each replicate consisting of a single pot (four pots per treatment). Greenhouse conditions were maintained at $20\text{--}28^{\circ}\text{C}$ and approximately 80% relative humidity. For clarity, the treatment codes and their corresponding treatments are defined below:

- N: *Meloidogyne javanica* (nematodes)
- Ba: *Bacillus subtilis*
- Ar: *Arthrobotrys oligospora*
- BA: *B. subtilis* + *A. oligospora*
- NA: *M. javanica* + *A. oligospora*
- NB: *M. javanica* + *B. subtilis*
- NBA: *M. javanica* + *B. subtilis* + *A. oligospora*
- C: Control.

Experiment two: Fungus and root interaction

In completely sterile laboratory conditions, after removing the soil and washing a portion of the root of a tomato plant previously inoculated with *M. javanica*, a root with fewer galls, preferably containing a single gall, was selected. This root was first surface-sterilized with 5% ethanol. Using sterile forceps, the root was transferred to a Petri dish containing water agar. Before this, holes had been made on both sides of the Petri dish to allow the root to pass through and lay flat on the culture medium. The other end of the root, which remained attached to the plant, was covered with sterile cotton to prevent dehydration and was regularly sprayed with sterile distilled water. The Petri dish was then completely sealed with parafilm, and its outer surface was covered with aluminum foil to provide a dark environment for the root and fungus (Figure 2). The remaining

part of the root, along with the plant, was returned to the pot and kept under conditions that minimized pressure and movement on the root inside the Petri dish. After three days, and following the release of root exudates onto the culture medium, 1 μ L of an *A. oligospora* spore suspension at a concentration of 1×10^5 spores/mL was applied vertically opposite the root using a sampler, under sterile laboratory conditions. In another sample, a bacteria suspension was first sprayed onto the root surface. To maintain sterility of the culture medium, sterile Whatman paper was placed over the medium during spraying and removed afterward. Then, similarly to the previous sample, 1 microliter of fungal spores was applied vertically opposite the root on the medium. After seven days, transverse sections of the roots were prepared using a microtome, and the colonization and fungal penetration patterns of *A. oligospora* within gall and non-gall root tissues were examined using light microscopy. This sampling procedure was specifically designed to obtain high-quality, intact root tissue sections from co-inoculated roots in the NBA treatment. In contrast, root tissue samples from the remaining treatments (N, Ar, and C) were collected 14 days after inoculation of the plant roots with the respective microbial agents.

Histological Preparation of Root Gall Samples

Transverse sections of tomato root galls and intergall tissues were prepared using a modified method described by Hood and Shew (1996). On the day of harvest, fresh root systems from different treatments were carefully sampled, gently washed under tap water to remove adhering soil particles, and surface-sterilized with 0.5% (v/v) sodium hypochlorite for 2 min, followed by thorough rinsing with sterile distilled water. Fresh root segments containing galls and adjacent healthy tissues were immediately mounted on the freezing stage of a freezing microtome (Reichert-Jung, Vienna, Austria), and transverse sections were prepared without chemical fixation or paraffin embedding, thereby preserving the native structural integrity of the root tissues and fungal structures. The obtained sections were cleared by incubation in a 1 M KOH aqueous solution for 15 min at room temperature to eliminate cytoplasmic background and subsequently rinsed three times with deionized water. The cleared sections were mounted on glass slides with several drops of staining solution containing 0.05% (w/v) aniline blue dissolved in 0.067 M K_2HPO_4 at pH 9.0), prepared at least 2 h prior to use. This alkaline aniline blue formulation specifically binds to β -1,3-glucans abundant in fungal cell walls and host callose deposits, providing high-contrast differential staining of fungal hyphae within the cleared root tissues. Microscopic observations and

measurements were performed using a light microscope equipped with an image analysis system (Leica Microsystems QWIN 550IW, Cambridge, UK) at magnifications ranging from $\times 100$ to $\times 400$.

Experiment three: Effect of H₂O₂ and Phenol on the Growth of *A. oligospora*

To evaluate the effect of H₂O₂ and phenol, the disk diffusion method was employed. PDA medium was prepared, and a plug approximately 6 mm in diameter was taken from an actively growing fungal colony and placed in the center of each plate. Sterile paper disks with a diameter of 6 mm were prepared, and each disk was impregnated with 10 μ L of H₂O₂ and phenol solutions at different concentrations (10, 15, 20, 25, 30, 35 mM). Disks impregnated with 10 μ L of sterile distilled water served as the negative control. The disks were placed on the solid surface of the culture medium at an appropriate distance (about 2–3 cm) from the fungal plug. Each concentration was tested in triplicate. Plates were incubated at 28°C under semi-dark conditions until complete fungal growth. After incubation, the diameter of each fungal colony and the inhibition zone around each disk were measured using a ruler (Figure 3). The percentage of mycelial growth inhibition (I%) was calculated using the following formula:

$$I\% = \frac{C-T}{C} \times 100$$

where I (%) represents the percentage of mycelial growth inhibition, C represents the colony diameter in the control treatment, and T represents the colony diameter in the treatment group. To account for actual fungal growth, the initial diameter of the fungal plug (6 mm) was subtracted from each measured colony diameter before calculating the percentage of mycelial growth inhibition.

Evaluation of some plant defense compounds

Preparation of extracts

The tissue extract was prepared by the method proposed by Ganguly and Dasgupta (1979) with some changes. 1 gram of plant material (root) was weighed and homogenized with 10 ml of 80% methanol. The mixture was centrifuged at 4025g and the supernatant was transferred into a test tube and stored at -4°C.

Total phenolic content (TPC)

According to the method described by Skerget *et al.* (2005), total phenolic content was determined using the Folin–Ciocalteu method. Briefly, samples were reacted with Folin–Ciocalteu reagent and sodium carbonate, and the absorbance was measured at 765 nm. Total phenolic content was quantified using a calibration curve prepared with gallic acid as the standard.

Hydrogen peroxide (H₂O₂)

According to the method of Velikova *et al.* (2000), hydrogen peroxide content was determined using a one-step extraction with 0.1% TCA and 1 M KI at 4 °C. After centrifugation (12,000 × g for 15 min), the absorbance of the supernatant was measured at 390 nm, and the concentration was calculated using an H₂O₂ standard curve. Samples were analyzed in triplicate under dark conditions.

Phenylalanine ammonia lyase (PAL) activity

Phenylalanine ammonia-lyase (PAL) activity was determined according to the method described by Reuveni (1995) by measuring the formation of trans-cinnamic acid at 290 nm. The enzyme reaction was performed in Tris-HCl buffer containing L-phenylalanine (pH 8.8) and incubated at 37 °C for 30 min. The reaction was then terminated by adding HCl. PAL activity was quantified using a calibration curve prepared with trans-cinnamic acid as the standard, and protein concentration was determined using the Bradford assay.

Total Carbohydrate

Total carbohydrate content was determined using the anthrone method described by Yemm and Willis (1954). Fresh root tissues were extracted with ethanol, and the resulting extracts were used for carbohydrate determination. The extracts were reacted with freshly prepared anthrone reagent in sulfuric acid and incubated in a water bath. Absorbance was subsequently measured at 630 nm. Total carbohydrate content was quantified using a calibration curve prepared with glucose as the standard.

Total proteins

Total protein content was determined using the Bradford method (Bradford, 1976). After extraction and centrifugation, aliquots of the supernatant were reacted with Bradford reagent, and

absorbance was measured at 595 nm. Protein concentration was calculated from a standard curve using known concentrations of standard protein.

Statistical analysis

All experiments were conducted using a completely randomized design (CRD). Prior to parametric analyses, the normality of the data and homogeneity of variances were assessed using the Shapiro–Wilk and Levene’s tests, respectively, supplemented by residual diagnostic plots in R software (version 4.6.1). For single-factor experiments, differences among treatment means were analyzed using one-way analysis of variance (ANOVA), followed by Duncan’s multiple range test at $p \leq 0.05$. For the inhibition assays, a two-way ANOVA was performed with concentration and time as fixed factors, including their interaction (concentration $\times$ time). Pearson’s correlation analysis was performed using pooled data across all treatments to assess the relationships among biochemical traits and enzyme activities.

Results

Experiment 1: Biochemical evaluations of root exudates:

Analysis of root exudates (Table 1) revealed significant differences among treatments in the measured biochemical parameters ($p \leq 0.05$). For defensive compounds, H_2O_2 was highest in the NB and NBA treatments (group a), followed by NA (ab) and the intermediate Ar and Ba treatments (abc); BA (bcd) and N (bcd/cd) showed lower values, while C had the lowest concentration (d). Phenolics were highest in the NBA treatment (group a), with NA (ab) and NB (b) also high; Ar was intermediate (bc), Ba and N lower (cd), and C and BA lowest (d). For nutritional compounds, protein was highest in NBA and NB, lowest in BA and C, with Ar and Ba intermediate. Carbohydrates peaked in the Ar treatment (significantly higher than all others), followed by similar levels in Ba and BA; the N, NA, NB, and NBA treatments showed progressively lower carbohydrate contents, with N lowest (Figure 4).

Biochemical evaluation of inoculated root

ANOVA revealed that the applied treatments significantly affected the biochemical parameters of tomato root exudates (Table 2). Phenol, H_2O_2 , and PAL activity differed significantly among treatments at the 5% level, while protein and carbohydrate contents were significantly affected at the 5% and 1% levels, respectively, indicating that the applied treatments strongly altered both

defense-related and nutritional traits. Consistent with this, Figure 5 showed clear treatment-dependent variation in all measured parameters: PAL activity, H₂O₂, and phenolic content were highest in the nematode treatment (N), with BA and Ar also showing elevated PAL or phenolic levels, whereas the control (C) generally had the lowest values. In contrast, protein and carbohydrate contents were highest in BA (with Ba also high for protein), while N and NA showed the lowest levels.

Comparison of biochemical compounds in root exudates and Root tissue extracts

Pearson correlation analysis ($P \leq 0.01$; Figure 6) revealed several significant relationships among the studied traits. The strongest correlations were between cellular H₂O₂ and cellular phenolics ($r = 0.96$) and between root protein and root phenolics ($r = 0.96$). Additional strong correlations included root H₂O₂–root phenolics ($r = 0.91$), root protein–root H₂O₂ ($r = 0.91$), root carbohydrate–cellular protein ($r = 0.85$), and cellular phenolics–cellular PAL ($r = 0.79$), with cellular H₂O₂–cellular PAL slightly lower ($r = 0.78$). In contrast, root carbohydrate showed only weak correlations with cellular H₂O₂ ($r = 0.11$), cellular phenolics ($r = -0.03$), and cellular PAL ($r = -0.20$).

Experiment two- Assessment of Pathogenicity Parameters

Analysis of variance of nematode-related parameters revealed significant differences among the biological treatments (Figure 7). The N treatment (nematodes only) showed the highest levels of gall formation (129) and total egg production (350). In contrast, the NA (Nematodes + *A. oligospora*) and NB (Nematodes + *B. subtilis*) treatments significantly reduced these parameters compared with the N treatment. Among all nematode-infected treatments, the lowest gall formation (56) and total egg number (163) were recorded in the NBA treatment (*A. oligospora* + *B. subtilis* + Nematodes).

Histopathology analysis

Microscopic observations of galls formed on tomato roots following infection by root-knot nematode. Histopathological analysis of plant roots after 14 days in different treatments showed no differences in fungal structures, which were similar for each plant-fungus combination. Additionally, fungal hyphal growth toward galled roots was examined using agar plate assays after 7 days (Figure 8). Root necrosis due to infiltration of second-stage juveniles, galling, and nematode

egg production and variations in the shape and density of the root system were observed. The onset of necrosis was always associated with the production of the gelatinous matrix by the root-knot nematode female (Figure 8). Microscopic analysis of transverse sections of tomato root galls infected with eggs, second-stage larvae and the female nematode body at the edges of giant cells inside the necrotic root knot ring (Figure 8A, B). Egg masses within the galls were often surrounded by cork cells formed through periclinal division (Figure 8C). In examining the interaction between root cells and *A. oligospora*, the fungus was extensively branched within epidermal cells (Figure 8D). In contrast, it was not observed in root cells of the control group (Figure 8E). Occasionally, root hairs were colonized by the fungus, and when the fungus spread in the epidermis, an onion-like infection expansion was observed (Figure 8F). Necrotic root areas were found near the inoculation site, with hyphae always associated with these necroses (Figure 8G). Conversely, root cells from adjacent non-necrotic areas did not show these features (Figure 8H). These were also absent in the root cells of the control group.

Experiment three: Effects of H₂O₂, and phenol on the Growth and Morphology of *A. oligospora*

The disk diffusion assay showed that *A. oligospora* growth was inhibited around the disk in a concentration-dependent manner: higher H₂O₂ and phenol concentrations produced a wider zone of inhibition, with almost no fungal growth near the disk and growth only resuming at a greater distance and after a delay. Over time, gradual degradation of H₂O₂ and phenol (due to light and temperature) lowered their concentrations, allowing the fungus to advance from the colony edge into previously inhibited areas. This recovered growth was slower and accompanied by hyphal morphological changes, mainly thickening and altered branching, which were more pronounced and clearly visible under the H₂O₂ treatment than under phenol (Figure 3). Additionally, direct medium supplementation with 35 mM H₂O₂ reduced mycelial growth and visibly altered hyphal architecture relative to the control, confirming the effect of oxidative stress on *A. oligospora* (Figure 9). Quantitative image analysis demonstrated an approximately 80% reduction in hyphal branching density under oxidative stress ($p < 0.001$), with 285 ± 42 junction points per field of view compared with $1,365 \pm 180$ junction points per field of view in the control group. In addition, H₂O₂-treated hyphae exhibited abnormal swelling and increased structural thickness, with an

approximately 1.8-fold increase in hyphal caliber compared with the slender, relatively uniform hyphae observed in the control group.

Concentration and Time effects on H₂O₂ and Phenol Inhibition

ANOVA revealed significant effects of concentration, time, and their interaction on growth inhibition of *A. oligospora* under both phenol and H₂O₂ treatments ($P < 0.05$; Table 3). In the disk diffusion assay, both compounds inhibited fungal growth, with inhibition increasing from 10 to 35 mM (Figure 10). For phenol, the highest inhibition occurred at 35 mM after 72 and 120 h, and the lowest at 10 mM; inhibition peaked at 72 h, declined relatively at 96 h, then rose again at 120 h to near the previous peak. A similar pattern was observed for H₂O₂: the highest inhibition was recorded at 35 mM and the lowest at 10 mM at the end of incubation. Over time, inhibition fluctuated, with peaks at 48 and 96 h at 35 mM, and at 72 and 120 h at 30 mM. Overall, phenol showed greater inhibitory efficacy than H₂O₂ at all time points. Concentration, time, and their interaction all significantly influenced fungal growth inhibition ($P < 0.05$).

Discussion

Infection of tomato plants with *M. javanica* was associated with biochemical alterations in root tissues and oxidative changes in root exudate profiles, whereas inoculation with *B. subtilis* and *A. oligospora* modulated these physiological responses. The split-root experimental system enabled simultaneous assessment of biochemical parameters in spatially separated root compartments, facilitating the evaluation of localized and systemic physiological responses. Nematode infection was associated with increased phenylalanine ammonia-lyase (PAL) activity and hydrogen peroxide (H₂O₂) accumulation in the nematode-only treatment (N), consistent with the early activation of reactive oxygen species (ROS)-associated responses and the phenylpropanoid pathway during biotic stress (Mittler *et al.*, 2022). Increased PAL activity is closely associated with the subsequent accumulation of phenolic compounds in plant tissues (Dong and Lin, 2021). Concurrently, analysis of root exudates revealed higher H₂O₂ levels in the co-inoculation treatments, particularly NB and NBA, than in the control (C). The detection of these oxidative changes in the root compartment spatially separated from nematode inoculation suggests the involvement of systemic biochemical signaling, consistent with coordinated whole-plant

physiological responses and root-mediated systemic signaling reported in rhizobacteria-inoculated plants (Martinez-Medina *et al.*, 2017; Pieterse *et al.*, 2014).

Root exudates serve as critical chemical interfaces in the rhizosphere and undergo substantial compositional changes during multitrophic plant–microbe interactions (Araujo *et al.*, 2012). Fluctuations in total carbohydrate and soluble protein contents further reflected metabolic adjustments across treatments. The lowest carbohydrate level was recorded in the nematode-only treatment (N), consistent with the sustained sink activity of nematode feeding sites and the diversion of host photoassimilates toward nematode-induced feeding structures (Kyndt *et al.*, 2013). In contrast, the elevated total protein levels observed in the combined treatments, particularly NB and NBA, indicate pronounced changes in host metabolic activity and may reflect alterations in host protein synthesis as well as contributions from rhizosphere microbial biomass or activity. Correlation analysis revealed strong positive associations among several stress-related variables, including total protein with total phenolics ($r = 0.96$), total protein with H_2O_2 ($r = 0.91$), and H_2O_2 with total phenolics ($r = 0.91$). Furthermore, PAL activity was positively correlated with total phenolics ($r = 0.79$) and H_2O_2 ($r = 0.78$), supporting the concurrent association of oxidative responses with phenylpropanoid metabolism (Khanna *et al.*, 2019; Labudda and Rozanska, 2020). In contrast, total root carbohydrate content showed weak correlations with H_2O_2 ($r = 0.11$), total phenolics ($r = -0.03$), and PAL activity ($r = -0.20$). These weak associations suggest that, within the conditions examined, variation in carbohydrate content was not closely associated with the defense-related biochemical parameters evaluated.

Histological observations corroborated these biochemical patterns and provided structural context for the tripartite interaction. Microscopic examination confirmed successful nematode establishment and the development of typical giant cells within gall tissues of infected roots, whereas biocontrol treatments were associated with reduced feeding-site development. Localized cellular necrosis, cortical suberization, and the presence of *A. oligospora* hyphae within root tissues provided evidence of fungal colonization and localized host structural responses, consistent with general patterns of endo-epiphytic fungal interactions described by Topalovic *et al.* (2020). These histological and biochemical responses were accompanied by significant reductions in nematode infection parameters. The highest gall number (129) and egg mass count (350) were recorded in the nematode-only treatment (N), whereas the combined treatment (NBA) reduced these parameters by more than 50%, to 56 galls and 163 egg masses, respectively. This suppression

is consistent with the findings of El-Ashry *et al.* (2021), who reported enhanced biocontrol efficacy under co-inoculation conditions. The greater suppression observed in the NBA treatment suggests potential complementary effects of *B. subtilis* and *A. oligospora*, potentially involving both modulation of host physiological responses and direct antagonistic interactions within the rhizosphere.

Overall, these findings highlight the potential of co-applying bacterial and nematophagous fungal agents to suppress *M. javanica* infection under controlled conditions. Further studies are needed to elucidate the underlying molecular signaling mechanisms and to evaluate the efficacy and consistency of this combined approach under field conditions.

Conclusions

The present findings indicate that the co-application of *A. oligospora* and *B. subtilis* was associated with reductions in *M. javanica* infection parameters, including root gall formation. This biological suppression coincided with successful root colonization, fungal trap formation, and modulation of plant biochemical parameters, including defense-related metabolites and antioxidant activities, which may contribute to mitigating nematode-induced stress. Overall, the combined use of these microorganisms may represent an effective component in integrated root-knot nematode management strategies. However, further studies are required to evaluate their practical efficacy under field conditions and to clarify the biochemical and molecular mechanisms involved.

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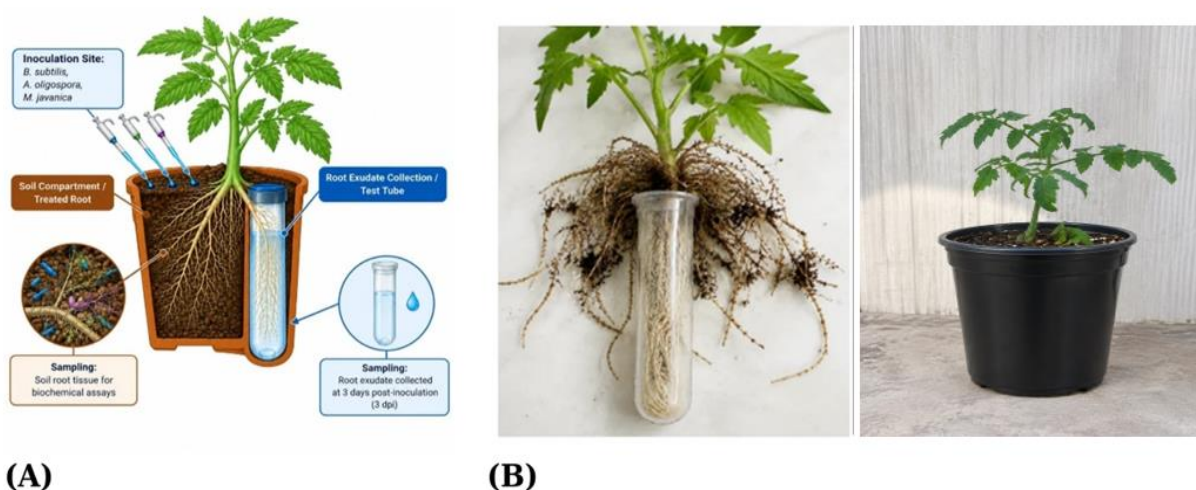


Figure 1. Split-root system for analysis of tomato root exudates. (A) Schematic representation of the split-root design, showing the inoculation sites for *B. subtilis*, *A. oligospora*, and *M. javanica* and the collection of root exudates in a sterile glass tube. (B) Photograph of the experimental setup, showing the separated root portion maintained in the tube for soil-free collection of root exudates and the remaining root system maintained in the pot.

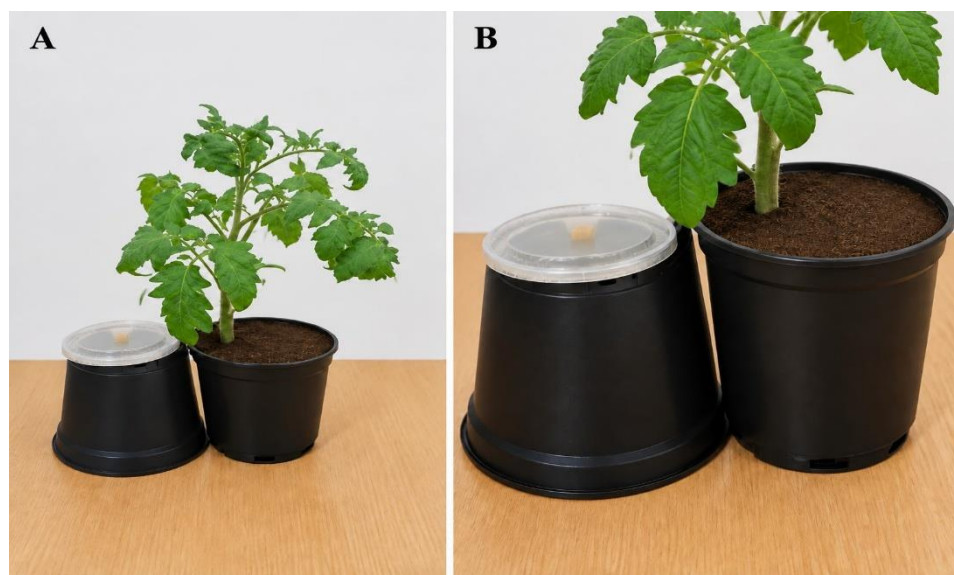


Figure 2. Experimental setup for in vivo fungal colonization of tomato roots in the NBA treatment. The setup maintained a living, plant-attached root on water agar under sterile conditions to facilitate fungal inoculation and subsequent collection of high-quality root sections. (A) Overall view of the tomato plant grown in soil, with one root portion extended into an adjacent Petri dish supported on an inverted pot. (B) Close-up view of the root placement and sealed Petri dish interface used to maintain sterile conditions during fungal inoculation and subsequent microscopic sampling of gall and adjacent root tissues.

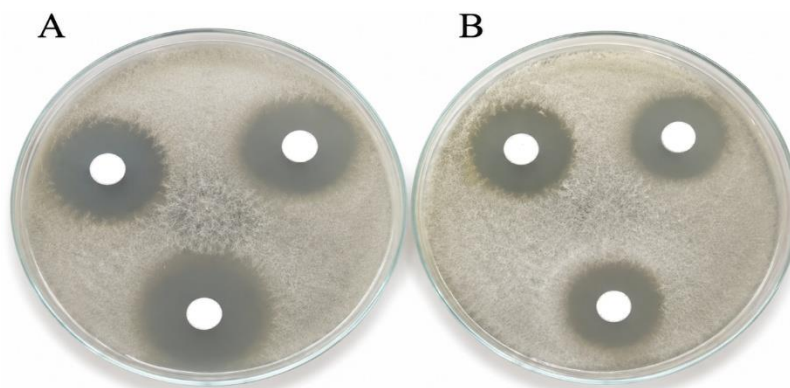


Figure 3. Disk diffusion assay illustrating inhibition zones around phenol disk (A) and H₂O₂ disk (B) on PDA medium. The clear halo surrounding each disk indicates the extent of microbial growth inhibition in response to the applied compounds.

Table 1. Analysis of variance (mean squares) of defense compounds and nutrients in tomato root exudates under different treatments.

Source of variation	df	Total phenolics	H ₂ O ₂	Total protein	Total carbohydrates
Treatment	7	0.07763**	158.10*	321.6**	276.64**
Error	24	0.00350	14.18	24.8	18.25
CV(%)	-	18.44	16.09	19.24	18.20

*Significant at the 5% probability level ($p \leq 0.05$); **significant at the 1% probability level ($p \leq 0.01$).

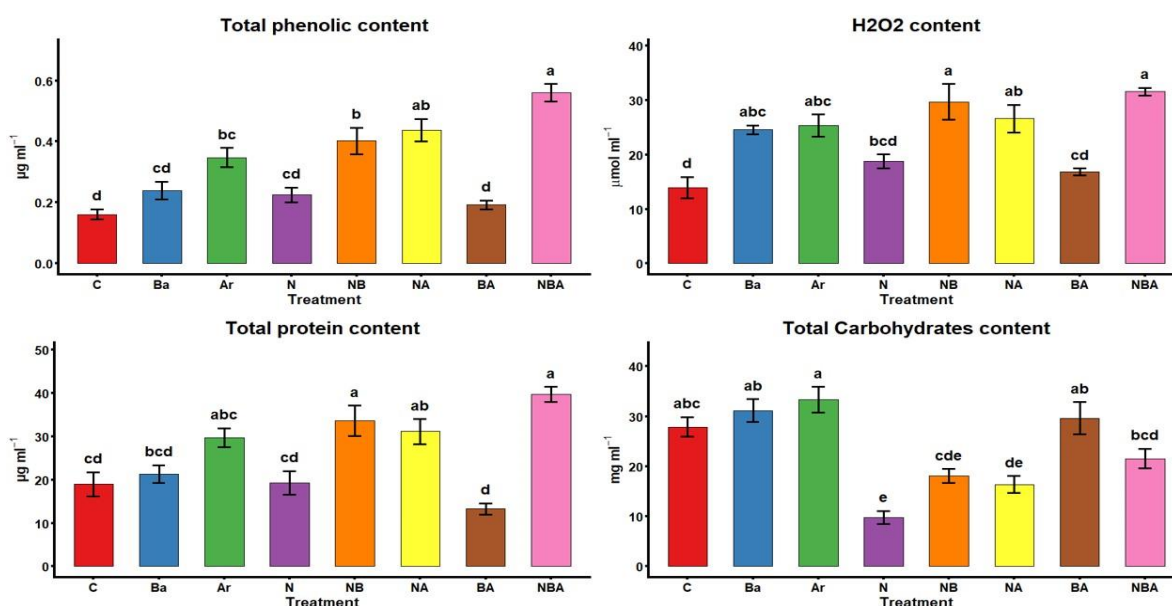


Figure 4. Effects of different treatments on H₂O₂ content, total phenolic content, total protein content, and total carbohydrate content in tomato root exudates. Values represent the accumulated concentrations measured in the root exudate collection solution (mean ± standard error of four replicates). Significant differences were observed at $p < 0.01$. C, control; N, *M. javanica* (nematodes); Ba, *B. subtilis*; Ar, *A. oligospora*; BA, *B. subtilis* + *A. oligospora*; NA, *M. javanica* + *A. oligospora*; NB, *M. javanica* + *B. subtilis*; NBA, *M. javanica* + *B. subtilis* + *A. oligospora*.

Table 2. Analysis of variance (mean squares) for defense-related compounds (total phenolic content, H₂O₂, and PAL activity) and nutrient-related parameters (total protein and total carbohydrate content) in tomato root tissue extracts under different treatments.

Source of variation	df	Total phenolics	H ₂ O ₂	PAL	Total protein	Total carbohydrates
Treatment	7	0.028037**	254.26*	266.65*	265.85*	557.2**
Error	24	0.000318	10.91	12.67	13.97	29.6
CV(%)	-	7.76	14.67	11.54	16.37	13.05

*Significant at the 5% probability level ($p \leq 0.05$); **significant at the 1% probability level ($p \leq 0.01$).

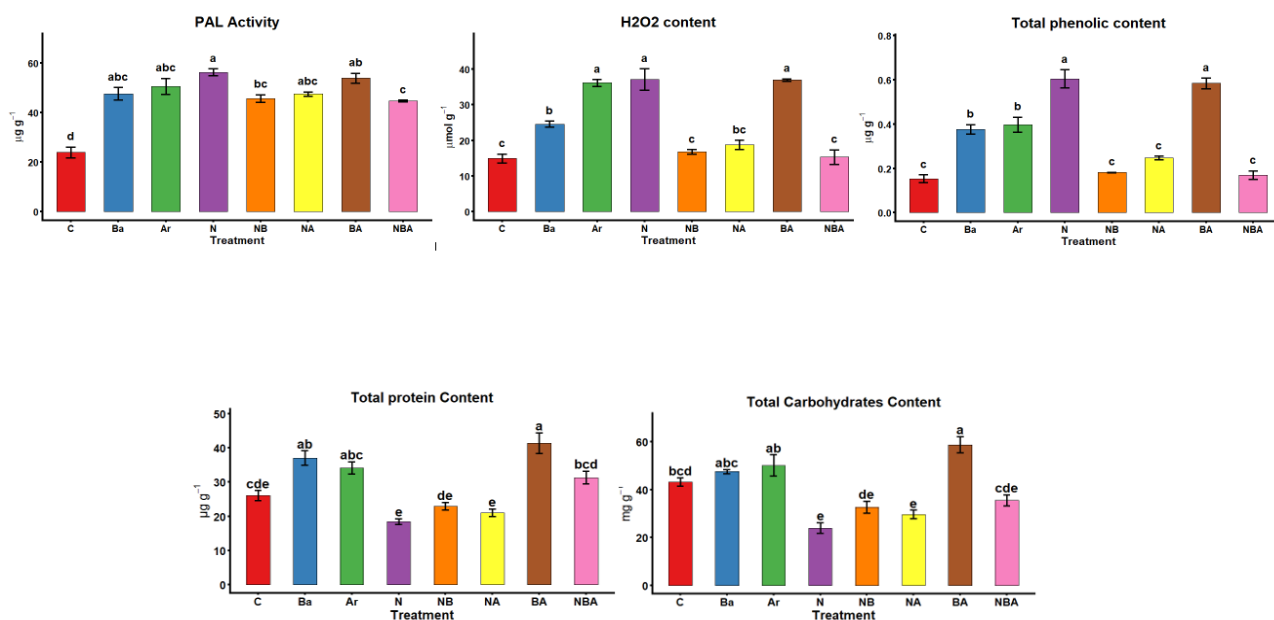


Figure 5. Effects of different treatments on PAL activity, H₂O₂ content, total phenolics, total proteins, and total carbohydrates in tomato root tissue extracts. Values represent the mean of four replicates, with significant differences indicated at $p < 0.01$. C, control; N, Nematodes (*M. javanica*); Ba, *B. subtilis*; Ar, *A. oligospora*; BA, *B. subtilis* + *A. oligospora*; NA, Nematodes + *A. oligospora*; NB, Nematodes + *B. subtilis*; NBA, Nematodes + *B. subtilis* + *A. oligospora*.

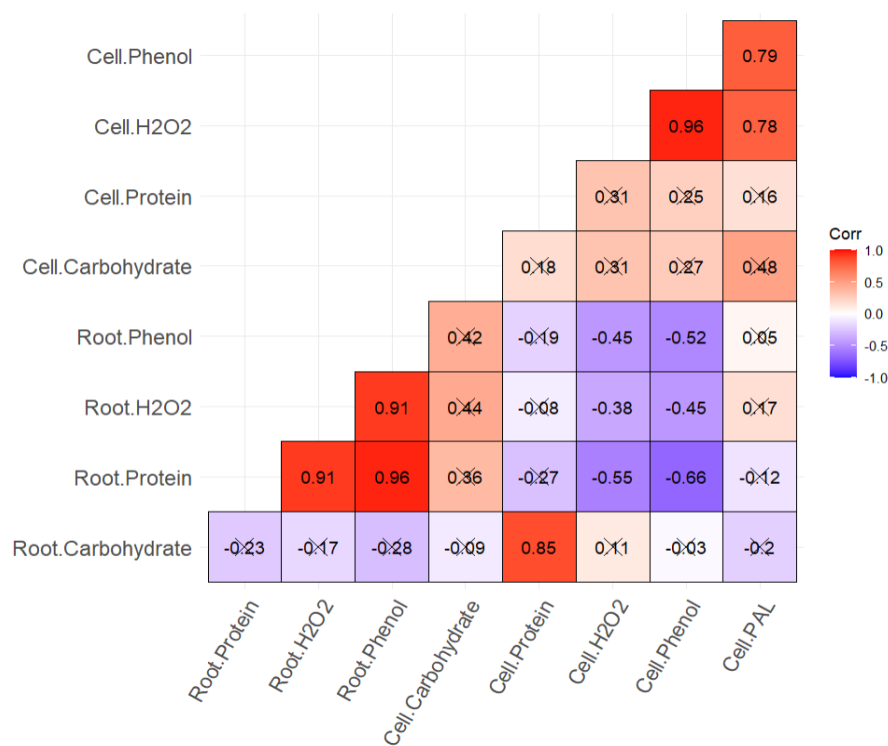


Figure 6. Correlation matrix of total protein, cell wall components (Cellulose, H₂O₂, PAL, Cellulose, and Cellulose), and root phenolics (Root) across different treatments.

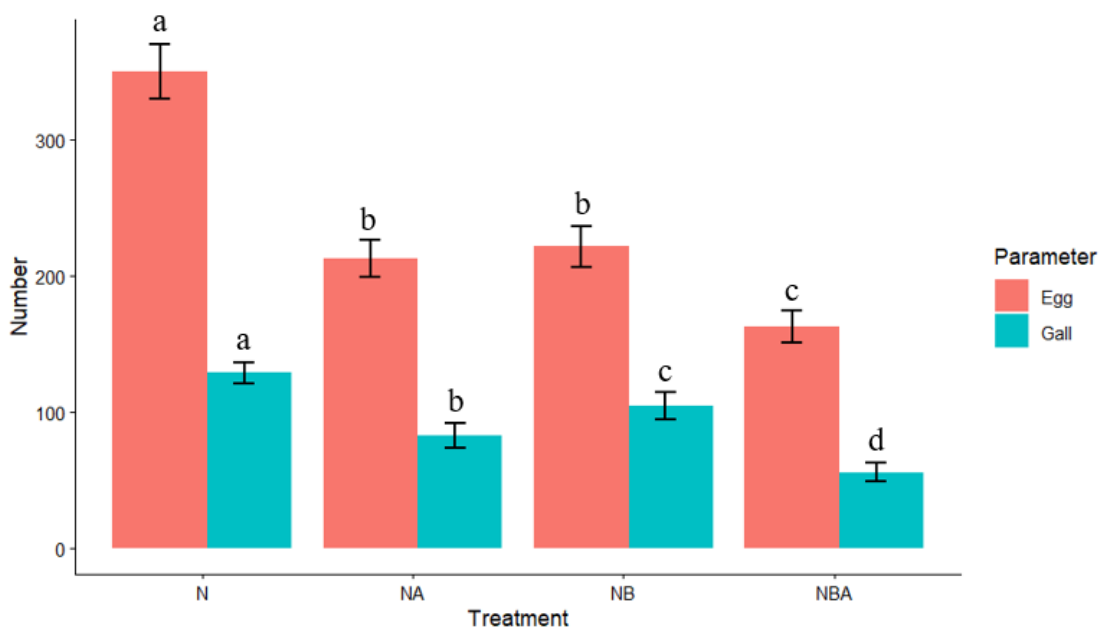


Figure 7. Mean numbers of galls and eggs in tomato roots treated with *B. subtilis* and *A. oligospora*. Data are presented as mean $\pm$ standard deviation (SD) (n = 4). Different letters indicate significant differences (p < 0.05) according to Duncan's test.

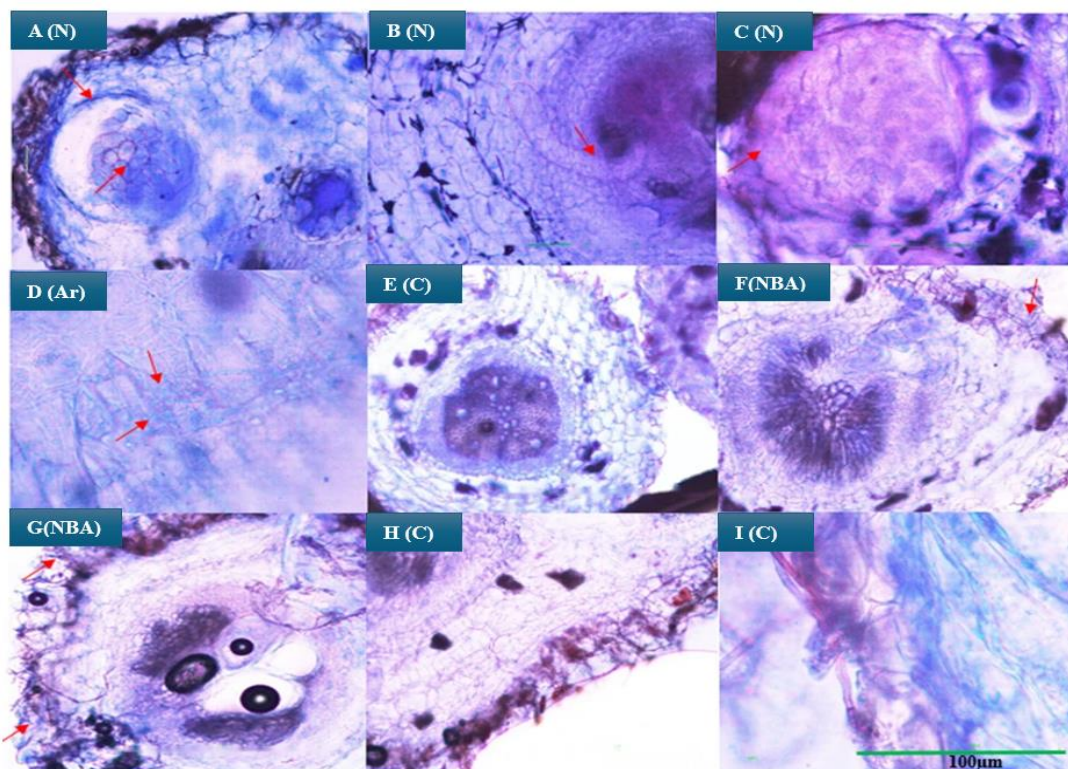


Figure 8. Histological analysis of transverse sections of tomato roots showing cellular and structural changes in gall and intergall regions under different experimental treatments. Panels (A–C) represent the nematode-only treatment (N); (D) represents the *A. oligospora* treatment (Ar); (E, H, and I) represent the control treatment (C); and (F and G) represent the combined *M. javanica* + *B. subtilis* + *A. oligospora* treatment (NBA). Red arrows indicate histological features associated with nematode penetration, cellular structural changes, and fungal colonization. Scale bars = 100 µm in all panels; images were captured using a 100× objective lens.

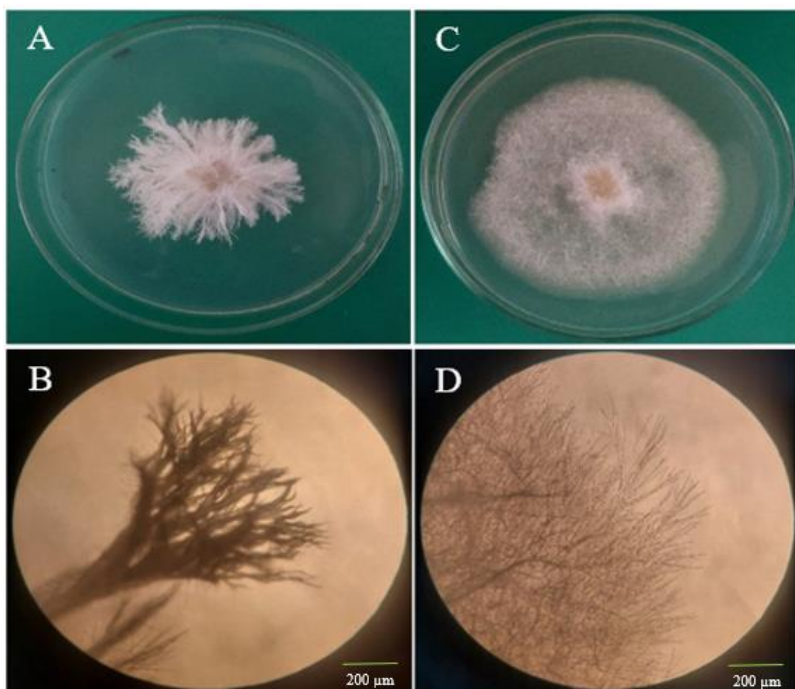


Figure 9. Effect of H_2O_2 on the growth and hyphal morphology of *A. oligospora*. (A) Hyphal growth inhibition on PDA medium under H_2O_2 treatment compared with (C) the untreated control. (B) Microscopic view showing sparse, swollen hyphae with an approximately 80% reduction in branching frequency under H_2O_2 -induced oxidative stress. (D) Microscopic view of the untreated control showing dense, delicate, and highly branched mycelial networks. Microscopic images (B, D) were captured using a light microscope with a 10× objective lens; scale bars = 200 μm .

Table 3. Analysis of variance (ANOVA; mean squares) showing the effects of concentration, time, and their interaction on the percentage inhibition of *A. oligospora* colony growth under phenol and H_2O_2 treatments.

Source of variation	df	Phenol	H_2O_2
Concentration (Con)	5	3594**	5441**
Time	5	78*	1890**
Con $\times$ Time	25	36*	87*
Error	72	19	19
CV(%)	-	9.35	11.67

*Significant at $p < 0.05$; **significant at $p < 0.01$.

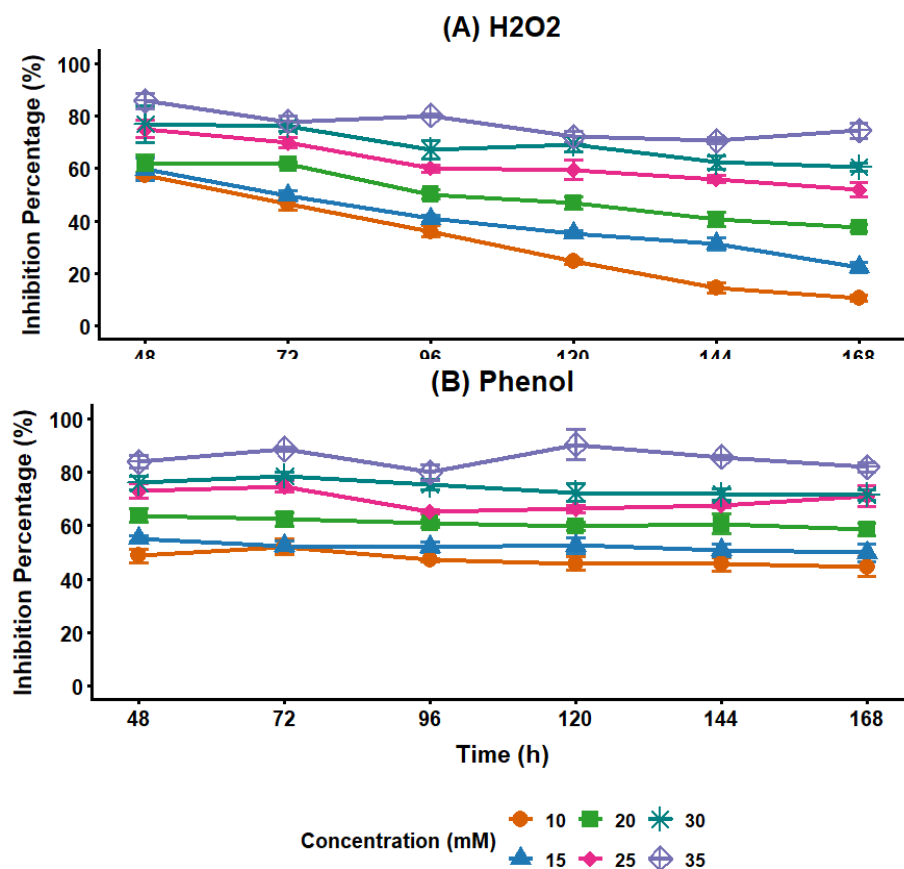


Figure 10. Interaction plots showing the combined effects of concentration and time on the inhibition percentage of *A. oligospora* colony development under (A) H₂O₂ and (B) phenol treatments. Data are presented as mean $\pm$ SE of three independent replicates (n=3).

پاسخ‌های بیوشیمیایی و کنترل زیستی نماتد *Meloidogyne javanica* توسط *Arthrobotrys*
Bacillus subtilis و *oligospora* با استفاده از روش ریشه تقسیم‌شده در گوجه‌فرنگی

مهسا مفتاحی، نوازاله صاحبانی، حسین رامشینی، محمودرضا روزبان

چکیده

ترشحات ریشه از طریق واکنش‌های شیمیایی، تأمین مواد مغذی، و جلب، دفع یا بازدارندگی از رشد، بر جانداران خاکزی تأثیر می‌گذارند. در این پژوهش، پاسخ‌های بیوشیمیایی در ریشه گوجه‌فرنگی با استفاده از یک سیستم ریشه تقسیم‌شده پس از مایه‌زنی با *Meloidogyne javanica* در ترکیب با *Arthrobotrys oligospora*، *Bacillus subtilis* یا هر دو مورد بررسی قرار گرفت. ترشحات ریشه از نظر میزان H_2O_2 ، ترکیبات فنلی کل، کربوهیدرات‌ها و پروتئین کل آنالیز شدند و پتانسیل مهار زیستی این دو عامل تحت تنش نماتد مورد ارزیابی قرار گرفت. تیمارها تأثیر معنی‌داری بر غلظت H_2O_2 ، ترکیبات فنلی، پروتئین و کربوهیدرات داشتند و همبستگی‌های قوی میان این متغیرها مشاهده شد. بالاترین غلظت‌های H_2O_2 $31/57 \mu\text{mol ml}^{-1}$ و فنل کل $0/56 \mu\text{g ml}^{-1}$ در تیمار ترکیبی نماتد-قارچ-باکتری مشاهده گردید. میزان پروتئین در هر دو تیمار نماتد-قارچ-باکتری و نماتد-باکتری به اوج خود رسید و به ترتیب به $33/65 \text{ mg/ml}^{-1}$ و $33/59$ رسید. میزان کربوهیدرات‌ها در تیمار قارچ بالاترین مقدار را با $33/32 \text{ mg/ml}^{-1}$ و در تیمار نماتد تنها کمترین مقدار را با $9/76 \text{ mg/ml}^{-1}$ نشان داد. این یافته‌ها اهمیت ترشحات ریشه را در برهم‌کنش‌های میان گیاه، میکروب‌ها و نماتدها برجسته می‌سازد. همچنین این نتایج نشان می‌دهند که مهار زیستی می‌تواند به عنوان یک راهبرد پایدار در مدیریت آفات جهت بهبود بهره‌وری محصول مطرح باشد.