Pre-inoculation with Arbuscular Mycorrhizal Fungi and Humic Acid Enhances Tomato Growth, Nutrient Uptake, and Induced Resistance Against *Ralstonia solanacearum*

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ABSTRACT

Bacterial wilt caused by *Ralstonia solanacearum* race 1 is one of the most destructive diseases of tomato, resulting in severe yield losses worldwide. This study evaluated the individual and combined effects of humic acid (H) and arbuscular mycorrhizal fungi (AMF) with or without NPK supplementation on tomato growth, nutrient uptake, defense activation, and suppression of bacterial wilt. Disease severity was highest in inoculated controls (60.4%), while integrated treatments markedly reduced wilt incidence, with the triple combination (NPKHM$^+$) achieving the lowest severity (23.2%). Growth performance and nutrient uptake of N, P, and K were enhanced, particularly when combined with humic acid and AMF, which improved biomass, chlorophyll content, and nutrient assimilation under both healthy and infected conditions. Biochemical and molecular analyses revealed that phenol accumulation, peroxidase (POX), polyphenol oxidase (PPO), and phenylalanine ammonia-lyase (PAL) enzyme activities, as well as the expression of *POX*, *PPO*, *PAL*, and *PR-2* genes, were highly upregulated in treated plants. The NPKHM$^+$ treatment produced the highest biochemical and transcriptional responses, which coincided with maximal wilt suppression and improved plant vigor. These findings demonstrate that the integration of mineral nutrition with organic and microbial amendments synergistically enhances tomato resistance to bacterial wilt while promoting growth, providing a sustainable and effective strategy for disease management.

Keywords: Arbuscular mycorrhizal (AMF), Bacterial wilt, Humic acid, Plant defense response, *Ralstonia solanacearum*, Tomato.

INTRODUCTION

Egypt is among the world's leading tomato (*Solanum lycopersicum* L.) producers, with annual yields exceeding 7 million tons, serving both domestic consumption and export markets. However, production is constrained by pests, diseases, climate variability, postharvest losses, and market instability (Almas *et al.,* 2021; Mostafa *et al.,* 2022 and Kefas *et al.,* 2024). Among these threats, bacterial wilt caused by *Ralstonia solanacearum* race 1 is one of the most destructive vascular diseases of tomato, leading to severe yield and quality losses, long-term soil contamination, and trade restrictions (Elphinstone, 2005 and Nion & Toyota, 2015). The pathogen colonizes the xylem, causing diurnal wilting, chlorosis, necrosis, vascular browning, and bacterial oozing, ultimately resulting in plant death despite adequate soil moisture (Jiang *et al.,* 2017 and Manda *et al.,* 2020).

Conventional management strategies, including chemical control, have shown limited and inconsistent effectiveness against *R. solanacearum* due to the high genetic variability and broad host range of the pathogen. Reliance on chemicals also harms the environment, disrupts the soil microbial balance, and promotes the emergence of resistant strains, making it unsustainable in the long term (Yuliar *et al.,* 2015). Therefore, integrated and sustainable approaches that strengthen the plant's innate defense capacity have become increasingly important (Mamphogoro *et al.,* 2020). Nutrient management plays a central role in plant health and disease resistance. Balanced NPK fertilization supports primary metabolism and provides substrates for the synthesis of secondary metabolites involved in immunity, thereby maintaining plant vigor and reducing susceptibility to diseases (Tripathi *et al.,* 2022). In addition, biostimulants such as humic acid (HA) and arbuscular mycorrhizal fungi (AMF) have gained attention as eco-friendly amendments that simultaneously enhance growth and resistance.

Arbuscular mycorrhizal fungi (AMF), comprising a ubiquitous group of soil-borne microorganisms, form mutualistic symbiotic associations with the roots of most terrestrial plants, including tomato, enhancing nutrient acquisition, growth, and stress tolerance through hyphal-mediated nutrient exchange (Smith & Read, 2008 and Shi *et al.,* 2023). In tomato systems, AMF improve biomass, yield, nutrient content, and activate mycorrhiza-induced resistance (MIR), a

DOI: 10.21608/asejaiqjsae.2025.457262
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Received, August 25, 2025, Accepted, September 30, 2025.

systemic defense priming mechanism against biotic and abiotic stresses (Garcia *et al.,* 2015 and Wahab *et al.,* 2023). AMF colonization has been widely reported to activate systemic resistance by upregulating pathogenesis-related (PR) genes, enhancing phenylpropanoid pathway genes involved in lignin and phytoalexin biosynthesis, and increasing defense-related and antioxidant enzymes (Pozo & Azcón-Aguilar, 2007; Song *et al.,* 2015 and Dreischhoff *et al.,* 2020).

Humic acid (HA), a naturally occurring organic compound derived from the decomposition of plant and animal residues, plays an important role in maintaining soil structure, enhancing fertility, and improving nutrient availability. As a plant bio-stimulant, it has been shown to enhance crop productivity and quality under both optimal and stress growing conditions (Canellas *et al.,* 2015, 2024 and Abdellatif *et al.,* 2017). HA enhances tomato growth by stimulating root development, chlorophyll biosynthesis, and nutrient uptake, while mitigating biotic stresses, including bacterial wilt (Olivares *et al.,* 2017 and Ahmed *et al.,* 2022). Humic acid primes host defenses by increasing activities of enzymes such as peroxidase, β-1,3-glucanase, phenylalanine ammonia-lyase, superoxide dismutase, and catalase (Silva *et al.,* 2025). Evidence suggests the combination of AMF or HA with other amendments exerts synergistic effects on plant growth and mitigating stresses through complementary physiological mechanisms (da Silva *et al.,* 2021 and Wang *et al.,* 2025).

Due to the limited efficacy of conventional wilt management practices, AMF and HA represent promising and environmentally sustainable alternatives for integrated disease control by enhancing plant immunity, inducing systemic resistance, and suppressing pathogen proliferation. This study evaluates the individual and combined effects of NPK fertilization, HA, and AMF on tomato growth and resistance to *Ralstonia solanacearum*, to develop a biologically based strategy for sustainable bacterial wilt management.

MATERIALS AND METHODS

Greenhouse experiment and treatments:

A greenhouse experiment was carried out at the Faculty of Agriculture, Damanhour University, Egypt, during the spring season of 2024 to assess the individual and combined effects of humic acid (HA) and arbuscular mycorrhizal fungi (AMF), *Funneliformis mosseae* (formerly *Glomus mosseae*), with or without NPK supplementation, on tomato growth and induced resistance against bacterial wilt caused by *R. solanacearum*. Three-week-old tomato seedlings (cv. 023) were transplanted in 15 cm diameter plastic pots, each containing 2.5 kg of sterilized clay soil. Before planting, soil physicochemical properties were determined following the methods of Jones (2001), and results indicated an electrical conductivity (EC) of 2 dS/m, pH 8.01, total nitrogen (N) 140 ppm, phosphorus (P) 1.59 ppm, potassium (K) 40 ppm, and organic matter (OM) content 1.2%. Plants were maintained under greenhouse conditions (25–32 °C, natural daylight) and irrigated twice per week to ensure adequate soil moisture.

The AMF was obtained from the Soil, Water and Environmental Research Institute, Agricultural Research Centre (ARC), Giza, Egypt. Commercial humic acid (potassium humate gold, with 80% humic acid) and inorganic NPK fertilizer (Grow Plant, 20:20:20 + trace elements) were obtained from a certified supplier (Anthis Co. for Import and Export, Egypt). NPK was applied at 500 kg ha^{-1}, and humic acid at 5 kg ha^{-1} (Ichwan *et al.,* 2022), while AMF inoculum was applied at 5 g per pot (Toussaint *et al.,* 2008), following established protocol. All amendments were incorporated into the soil during transplantation, with irrigation applied immediately therafter.

Experimental design:

The experiment followed a randomized complete block design with 14 treatments, seven without *R. solanacearum* (−Rs) and seven with (+Rs). Non-inoculated treatments included: (1) untreated control (Ck^-), (2) NPK^-, (3) humic acid (H^-), (4) AMF (M^-), (5) NPK + humic acid ($NPKH^-$), (6) NPK + AMF ($NPKM^-$), and (7) NPK + humic acid + AMF ($NPKHM^-$). Corresponding inoculated treatments were: (8) inoculated control (Ck^+), (9) NPK^+, (10) H^+, (11) M^+, (12) $NPKH^+$, (13) $NPKM^+$, and (14) $NPKHM^+$. In combined treatments, NPK was applied at half the standard rate, while humic acid and AMF were applied at full strength. Each treatment comprised five replicates of three plants (15 plants per treatment).

Pathogen inoculation and disease evaluation:

A virulent *R. solanacearum* strain isolated from infected tomato plants and maintained at the Plant Pathology Department Lab, Faculty of Agriculture, Damanhour University, was cultured on tetrazolium chloride (TZC) agar (Kelman, 1954) at 28 °C for 48 h. Bacterial cells were suspended in sterile distilled water and adjusted to 1×10^8 CFU mL^{-1} using a spectrophotometer. Plants 14 days after transplanting were inoculated via the stem-puncture method (Prior and Steva, 1990) by inserting a dissecting needle dipped in the suspension into the plant stem 2 cm above the soil surface; controls received sterile water. Disease severity was assessed 21 days post-inoculation (dpi) using a 0–4 wilt scale (Kempe and Sequeira, 1983) whereas, 0 = no wilting symptoms; 1 = 1-25% foliar wilting; 2 = 26-

50% foliar wilting; 3 = 51-75% foliar wilting; 4 = 76-100% foliar wilting or plant death. Disease severity percentage (DS%) was calculated using the following formula:

$$DS\% = [\Sigma(n \times v)/(N \times S)] \times 100$$

where n = number of plants per category, v = category score (0–4), N = total plants assessed, and S = maximum severity score.

Assessment of leaf chlorophyll, vegetative growth, and nutrient content:

At 21 dpi with *R. solanacearum*, chlorophyll content was determined using a SPAD-portable Chlorophyll meter (Spectrum Technologies, Inc., Aurora, Illinois, USA). Vegetative growth parameters, including plant height (cm), shoot fresh and dry weight (g), and root fresh and dry weight (g), were recorded. For dry biomass determination, plants were oven-dried at 70 °C for four days to a constant weight, after which the material was homogenized in a stainless-steel electric grinder and analyzed for nitrogen (N), phosphorus (P), and potassium (K) content following Ryan *et al*. (2001).

Determination of total phenolic content:

Total phenolic content (TPC) was quantified following the Folin–Ciocalteu colorimetric method (Zieslin and Ben Zaken, 1993), and the results were expressed as micrograms of gallic acid equivalents (GAE) per gram fresh weight (FW).

Assay of defense-related enzyme activity:

The activities of defense-related enzymes, including peroxidase (POX), polyphenol oxidase (PPO), and phenylalanine ammonia-lyase (PAL), were quantified in tomato leaves at 7 days post-inoculation (dpi) with the *R. solanacearum* strain. Enzyme extraction and spectrophotometric assays were performed according to Bayoumi *et al.* (2021). POX activity was determined following Hammerschmidt and Kuc (1982) using a reaction mixture containing 1.5 mL of 0.05 M pyrogallol, 100 µL of enzyme extract, and 0.5 mL of 1% H_2O_2, and expressed as the change in absorbance at 420 nm (ΔOD_{420} min^{-1} g^{-1} FW). PPO activity was assayed as described by Mayer *et al.* (1965) in a mixture of 1.5 mL of 100 mM phosphate buffer (pH 6.5), 200 µL of 10 mM catechol, and 200 µL of enzyme extract, with activity recorded as the increase in absorbance at 495 nm (ΔOD_{495} min^{-1} g^{-1} FW). PAL activity was determined according to Dickerson *et al.* (1984) in a reaction containing 0.5 mL of 100 mM borate buffer (pH 8.8), 0.5 mL of 12 mM L-phenylalanine, and 0.4 mL of enzyme extract. The formation of trans-cinnamic acid was monitored at 290 nm and calculated using a molar extinction coefficient of 9630 M^{-1} cm^{-1}, expressed as nmol trans-cinnamic acid min^{-1} g^{-1} FW.

Analysis of gene expression by qRT-PCR:

Gene expression levels of *POX*, *PPO*, *PAL*, and *PR-2* (*β-1,3-glucanase*) were quantified in tomato leaves at 7 days post-inoculation (dpi), using *Actin* as the reference gene. Leaf tissues were collected, frozen in liquid nitrogen, and total RNA was extracted using the BioTeke RNA Isolation Kit (Maxim Biotech Inc., USA) following the manufacturer's instructions. First-strand cDNA was synthesized from the purified RNA using M-MLV reverse transcriptase (Fermentas, USA) and oligo(dT) primers according to the standard protocols. QRT-PCR was conducted on a Rotor-Gene 6000 System (Qiagen, USA) using gene-specific primers (Table 1) in 25 µL reactions containing 2× QuantiTect SYBR® Green RT Mix, primers (10 pmol/µL), and 50 ng of cDNA. Cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 10 min. Relative expression was calculated by the $2^{\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and normalized to untreated controls.

Table 1. Gene-specific primers used in gene expression analysis.

Primer name	Direction	Sequences (5′–3′)	Reference
peroxidase (*POX*)	F	GCTTTGTCAGGGGTTGTGAT	Jogaiah *et al.* (2013)
	R	TGCATCTCTAGCAACCAACG	
polyphenol oxidase (*PPO*)	F	CATGCTCTTGATGAGGCGTA	Goel *et al.* (2017)
	R	CCATCTATGGAACGGGAAGA	
phenylalanine ammonia-lyase (*PAL*)	F	CTGGGGAAGCTTTTCAGAATC	Song *et al.* (2015)
	R	TGCTGCAAGTTACAAATCCAGAG	
β-1,3-glucanase (*PR2*)	F	GGACACCCTTCCGCTACTCTT	Song *et al.* (2015)
	R	TGTTCCTGCCCCTCCTTTC	
β-Actin	F	GTGGGCCGCTCTAGGCACCAA	Adss *et al.* (2024)
	R	CTCTTTGATGTCACGCACGATTTC	

Statistical analysis:

Data on disease severity, vegetative growth, leaf chlorophyll, nutrient contents, and enzyme activity were subjected to Analysis of Variance (ANOVA) in Costat v6.4 (CoHort Software, USA). Mean separation was performed with Fisher's least significant difference (LSD) test at $P \leq 0.05$. Gene expression data were analyzed and presented as mean ± standard error (SE). Disease severity was assessed with five replicates per treatment, whereas three replicates were used for the other parameters.

RESULTS

Effect of NPK, humic acid (H), and AMF on wilt disease severity:

Data presented in Fig. (1) revealed that NPK fertilization, humic acid (H), and arbuscular mycorrhizal fungi (AMF), whether applied individually or in combination, significantly reduced tomato infection by *R. solanacearum* under greenhouse conditions compared with the inoculated control (Ck^+). The highest disease severity was observed in the Ck^+ (60.40%). Application of NPK fertilizer alone (NPK^+) reduced disease severity to 43.00%, while humic acid (H^+) and AMF (M^+) individually decreased severity to 40.00% and 32.20%, respectively. Combined applications produced higher effects, with $NPKH^+$and $NPKM^+$ reducing severity to 36.20% and 29.00%, respectively. The greatest suppression of bacterial wilt occurred in the triple combination ($NPKHM^+$), where severity was reduced to 23.20%, significantly lower than all other treatments.

Effect of NPK, Humic Acid, and AMF on Tomato Growth and Chlorophyll Content:

Tomato growth parameters were significantly affected by *R. solanacearum* inoculation and by the application of NPK fertilizer, humic acid (H), and arbuscular mycorrhizal fungi (AMF), either individually or in combination (Table 2). In general, non-inoculated plants showed pronounced effects than inoculated ones, confirming the suppressive effect of bacterial wilt on vegetative growth.

Plant height: The triple treatment (NPKHM) consistently produced the highest plants under both non-inoculated (48.83 cm) and inoculated (48.00 cm) ones. Non-inoculated plants were higher than inoculated counterparts across treatments, as shown by the control (CK^- = 33.73 cm vs. CK^+ = 30.75 cm). Among single treatments, humic acid alone (42.17 cm) promoted greater height than NPK (39.25 cm) or AMF (37.00 cm), although these differences were not statistically significant.

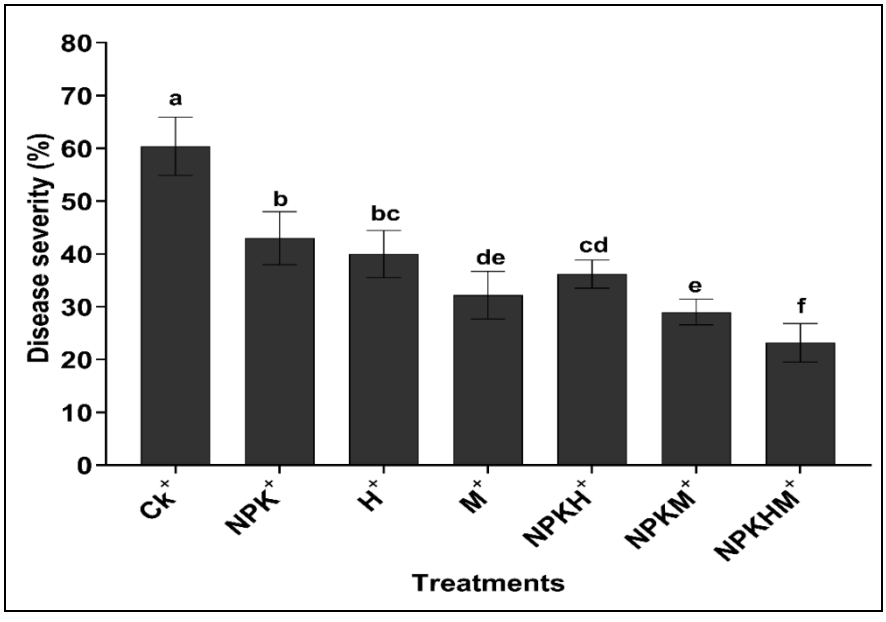

Fig. 1. Effect of NPK fertilization, humic acid (H), and arbuscular mycorrhizal fungi (AMF) on bacterial wilt severity in tomato plants under greenhouse conditions. Values represent mean ± SD (n = 5 replicates, 3 plants per replicate). Treatments: Control (Ck); mineral fertilizer (NPK); humic acid (H); arbuscular mycorrhizal fungi (M); NPK + humic acid (NPKH); NPK + AMF (NPKM); and NPK + humic acid + AMF (NPKHM). Means followed by the same letter are not significantly different according to the LSD test at $P \leq 0.05$.

Table 2. Vegetative growth parameters and chlorophyll content of tomato plants as influenced by NPK fertilization, humic acid (H), and arbuscular mycorrhizal fungi (AMF) under inoculated (+) and non-inoculated (-) conditions with *Ralstonia solanacearum*.

Treatment	Plant height (cm)	Fresh weight (g)	Dry weight (g)	Chlorophyll content (SPAD)
Non-inoculated (-) with *R. solanacearum*				
Ck-	33.73^{de}	22.31^{gh}	4.15^{ef}	37.05^{c}
NPK^{-}	39.25^{bc}	43.00^{a}	5.70^{ab}	44.87^{a}
H -	42.17^{bc}	27.22^{ef}	4.74^{de}	37.27^{c}
M-	37.00^{cde}	24.3^{fg}	4.61^{de}	37.75^{c}
NPKH -	40.00^{bc}	29.21^{de}	5.75^{c}	42.10^{ab}
NPKM-	43.75^{ab}	32.30^{bc}	5.96^{abc}	41.13^{b}
NPKHM-	48.83^{a}	32.70^{bc}	6.86^{a}	43.47^{ab}
Inoculated (+) with *R. solanacearum*				
Ck^{+}	30.75^{e}	12.99^{j}	3.48^{f}	32.40^{d}
NPK^{+}	37.25^{cd}	34.69^{b}	5.21^{cd}	35.75^{c}
H^{+}	32.00^{de}	17.60^{i}	3.56^{f}	30.75^{d}
M^{+}	32.75^{de}	20.40^{hi}	3.91^{ef}	35.67^{c}
NPKH^{+}	39.25^{bc}	26.36^{ef}	5.37^{cd}	40.97^{b}
NPKM^{+}	39.50^{bc}	25.82^{f}	5.80^{bc}	38.60^{c}
NPKHM^{+}	48.00^{a}	30.30^{cd}	5.90^{bc}	42.60^{ab}

Treatments: Control (Ck); mineral fertilizer (NPK); humic acid (H); arbuscular mycorrhizal fungi (AMF); NPK + humic acid (NPKH); NPK + AMF (NPKM); and NPK + humic acid + AMF (NPKHM). Means within a column followed by the same letter are not significantly different according to the LSD test at $P \leq 0.05$. Data represent the mean of five replicates.

Fresh weight: Fresh biomass was strongly reduced by bacterial wilt infection. The untreated control plants significantly recorded the lowest values in both non-inoculated (22.31 g) and inoculated (12.99 g). Under pathogen-free conditions, NPK^{-} produced the highest fresh biomass (43.00 g), followed by NPKHM^{-} (32.70 g) and NPKM^{-} (32.30 g). In infected plants, NPK^{+} yielded the greatest fresh weight (34.69 g), followed by NPKHM^{+} (30.30 g), showing the ability of integrated fertilization to mitigate yield loss under pathogen stress.

Dry weight: A similar trend was observed for dry matter. Wilt incidence significantly suppressed dry weight (CK^{-} = 4.15 g vs. CK^{+} = 3.48 g). Among non-inoculated plants, the highest dry biomass was obtained in NPKHM^{-} (6.86 g), followed by NPKM^{-} (5.96 g) and NPKH^{-} (5.75 g). In inoculated plants, NPKHM^{+} (5.90 g) and NPKM^{+} (5.80 g) yielded the greatest dry matter, whereas CK^{+} remained the lowest. NPK fertilization enhanced dry matter accumulation under both pathogen-free (5.70 g) and inoculated (5.21 g) conditions, while humic acid or AMF alone produced only a little increase compared with controls.

Chlorophyll content: Chlorophyll content, measured as SPAD readings, declined significantly in inoculated plants compared with pathogen-free plants (CK^{+} = 32.40 vs. CK^{-} = 37.05). Under non-inoculated conditions, NPK^{-} (44.87) and NPKHM^{-} (43.47) maintained the highest chlorophyll contents, while CK^{-}, H^{-}, and M^{-} showed lower values (37.05–37.75). In inoculated plants, combined treatments reduced chlorophyll loss, with NPKHM^{+} (42.60) and NPKH^{+} (40.97) recording the highest values. In contrast, humic acid alone in case of inoculation (H^{+} = 30.75) showed the lowest SPAD reading.

Effect of NPK, AMF, and humic acid (H) on nutrient content (N, P, and K) of tomato plants:

Leaf nutrient concentrations (N, P, and K) were significantly affected by *R. solanacearum* inoculation and by the application of NPK and/or AMF and H (Table 3). Overall, integrated treatments maintained higher nutrient accumulation in case of inoculation compared with single applications, indicating their role in sustaining nutrient metabolism during pathogen stress.

Nitrogen (N): Inoculation with *R. solanacearum* reduced foliar nitrogen content across all treatments compared with their non-inoculated ones. Among non-inoculated plants, NPKHM^{-} recorded the highest nitrogen concentration (2.82%), followed by NPKM^{-} (1.76%) and NPKH^{-} (1.66%), while the control (CK^{-}) and humic acid (H^{-}) had the lowest values (1.42%). In inoculated plants, NPKHM^{+} exhibited the greatest nitrogen content (1.75%), whereas CK+, H+, and M+ showed the lowest values (1.42, 1.42, and 1.43%,

respectively). These results confirm that integrated amendments enhance N assimilation and maintain nitrogen metabolism under pathogen stress.

Phosphorus (P%): Phosphorus content was generally low across treatments but improved with combined applications. Under non-inoculated conditions, $NPKHM^-$ achieved the highest P level (0.20%), followed by $NPKM^-$ (0.13%), compared with the control (CK^- = 0.06%). Single applications of NPK (0.11%), humic acid (0.10%), or AMF (0.11%) resulted in a moderate increase compared with the control. Similar trends were observed in inoculated plants, where $NPKHM^+$ and $NPKM^+$ exhibited higher P contents (0.14% and 0.13%, respectively) than CK^+ and H^+ (0. 10%).

Potassium (K%): Foliar potassium levels were clearly enhanced by combined treatments. In non-inoculated plants, $NPKHM^-$ produced the highest K content (2.57%), followed by $NPKM^-$ (2.52%), $NPKH^-$ (2.47%), and NPK^- (2.43%), while CK^- had the lowest (0.33%). In inoculated plants, $NPKHM^+$ also revealed the greatest K concentration (2.57%), followed by $NPKM^+$ (2.47%) and $NPKH^+$ (2.43%), whereas CK^+ (1.97%) and H^+ showed the lowest values (1.98%).

Effect of NPK, humic acid, and AMF on total phenolic content and defense-related enzyme activities:

Analysis of tomato leaves revealed that total phenols and defense-related enzymes (*POX*, *PPO*, and *PAL*) were significantly influenced by *R. solanacearum* inoculation and the applied treatments (Table 4).

Total phenols: Phenolic levels remained low in non-inoculated plants (Ck^- = 8.54 µg GAE g^{-1} FW), with little increase under H^- (1.3-fold vs. Ck^-), M^- (1.4-fold), and $NPKHM^-$ (1.6-fold). Inoculation markedly stimulated phenol accumulation with Ck^+ (16.24) ~1.9-fold higher than Ck^-. Among single treatments, NPK^+ did not significantly increase phenol levels more than Ck^+, whereas H^+ (20.36) and M^+ (25.81) induced ~1.25- and ~1.6-fold increases, respectively. Dual treatments further elevated phenols, especially $NPKM^+$ (29.35; 1.8-fold), while $NPKHM^+$ produced the maximum level (34.84; 2.15-fold more than Ck^+), corresponding with the greatest wilt suppression.

Peroxidase (POX): POX activity was low in non-inoculated Ck^- (0.424 ΔOD min^{-1} g^{-1} FW) but increased under H^- (1.4-fold), M^- (1.5-fold), and their combinations, peaking in $NPKHM^-$ (1.8-fold). Inoculation significantly elevated POX ~2.0-fold in Ck^+ (0.853) compared with Ck^-. Further increases occurred under H^+ (1.4-fold), and M^+ (1.5-fold), while $NPKHM^+$ recorded the greatest value (1.673; 2.0-fold), followed by $NPKM^+$ (1.468; 1.7-fold).

Table 3. Nutrient content (N, P, and K) of tomato plants as influenced by NPK fertilization, humic acid (H), and arbuscular mycorrhizal fungi (AMF) in case of inoculated (+) and non-inoculated (–) conditions with *Ralstonia solanacearum*.

Treatment	N% plant^{-1}	P% plant^{-1}	K% plant^{-1}
Non-inoculated (-) with *R. solanacearum*			
Ck-	1.42^{e}	0.06^{d}	0.33^{e}
NPK -	1.62^{bcd}	0.11^{bc}	2.43^{ab}
H -	1.42^{e}	0.10^{c}	2.06^{cd}
M-	1.52^{cde}	0.11^{bc}	2.42^{ab}
NPKH -	1.66^{bc}	0.11^{bc}	2.47^{ab}
NPKM-	1.76^{b}	0.13^{bc}	2.52^{ab}
NPKHM-	2.82^{a}	0.20^{a}	2.57^{a}
Inoculated (+) with *R. solanacearum*			
Ck^+	1.42^{e}	0.10^{c}	1.97^{d}
NPK^+	1.45^{e}	0.11^{bc}	2.38^{b}
H^+	1.42^{e}	0.10^{c}	1.98^{d}
M^+	1.43^{e}	0.11^{bc}	2.15^{c}
$NPKH^+$	1.47^{de}	0.12^{bc}	2.43^{ab}
$NPKM^+$	1.51^{cde}	0.13^{bc}	2.47^{ab}
$NPKHM^+$	1.75^{b}	0.14^{b}	2.57^{a}

Treatments: Control (Ck); mineral fertilizer (NPK); humic acid (H); arbuscular mycorrhizal fungi (AMF); NPK + humic acid (NPKH); NPK + AMF (NPKM); and NPK + humic acid + AMF (NPKHM). Means within a column followed by the same letter are not significantly different according to the LSD test at $P \leq 0.05$. Data represent the mean of five replicates.

Table 4. Effect of NPK, humic acid (H), and arbuscular mycorrhizal fungi (AMF) on total phenolic content and activities of peroxidase (POX), polyphenol oxidase (PPO), and phenylalanine ammonia-lyase (PAL) in tomato leaves under *Ralstonia solanacearum*–inoculated (+) and non-inoculated (−) conditions.

Treatment	Total phenolic content (µg GAE g $^{-1}$ FW)	POX (ΔOD min^{-1} g^{-1} FW)	PPO (ΔOD min^{-1} g^{-1} FW)	PAL (nmol trans-cinnamic acid min^{-1} g^{-1} FW)
Non-inoculated (-) with *R. solanacearum*				
Ck^{-}	8.54^{k}	0.424^{i}	2.000^{j}	1.465^{g}
NPK -	8.78^{jk}	0.481^{i}	2.192^{i}	1.541^{fg}
H -	11.11^{ij}	0.586^{h}	2.545^{h}	1.741^{ef}
M-	12.04^{hi}	0.629^{gh}	2.682^{gh}	1.885^{de}
NPKH -	11.22^{ij}	0.642^{gh}	2.636^{h}	1.895^{de}
NPKM-	12.88^{hi}	0.714^{fg}	2.899^{fg}	1.984^{d}
NPKHM-	13.83^{gh}	0.778^{ef}	2.989^{ef}	1.993^{d}
Inoculated (+) with *R. solanacearum*				
Ck^{+}	16.24^{fg}	0.853^{de}	3.154^{de}	1.981^{d}
NPK^{+}	16.48^{f}	0.957^{d}	3.288^{d}	1.947^{d}
H^{+}	20.36^{e}	1.211^{c}	3.851^{c}	2.280^{c}
M^{+}	25.81^{c}	1.287^{c}	4.202^{b}	2.448^{bc}
NPKH^{+}	22.89^{d}	1.292^{c}	3.902^{c}	2.378^{c}
NPKM^{+}	29.35^{b}	1.468^{b}	4.364^{b}	2.655^{b}
NPKHM^{+}	34.84^{a}	1.673^{a}	5.034^{a}	3.106^{a}

Treatments: Control (Ck); mineral fertilizer (NPK); humic acid (H); arbuscular mycorrhizal fungi (AMF); NPK + humic acid (NPKH); NPK + AMF (NPKM); and NPK + humic acid + AMF (NPKHM). Means within a column followed by the same letter are not significantly different according to the LSD test at $P \leq 0.05$. Values represent the mean of three replicates.

Polyphenol oxidase (PPO): PPO showed a similar trend. In non-inoculated plants, activity ranged from 2.00 (Ck^{-}) to 2.989 (NPKHM^{-}; 1.5-fold over Ck^{-}). Inoculation markedly increased PPO by ~1.6-fold in Ck^{+} (3.154) vs. Ck^{-}. Treated plants showed further increases, H^{+} (3.851, 1.2-fold vs. Ck^{+}), M^{+} (4.202, 1.3-fold), NPKM^{+} (4.364, 1.4-fold), and the maximum in NPKHM^{+} (5.034, 1.6-fold).

Phenylalanine ammonia-lyase (PAL): PAL activity was lowest in Ck^{-} (1.465 nmol trans-cinnamic acid min^{-1} g^{-1} FW) but increased slightly with NPKHM^{-} (1.993; ~1.4-fold). Inoculation induced PAL by ~1.4-fold in Ck^{+} (1.981 vs. Ck^{-}). Treated plants showed additional increases, with H^{+} (2.28; 1.2-fold vs. Ck^{+}), M^{+} (2.448; 1.2-fold), NPKM^{+} (2.655; 1.3-fold), and peaked in NPKHM^{+} (3.106; 1.6-fold).

Effect of NPK, Humic Acid, and AMF on Defense-Related Gene Expression:

To evaluate whether NPK fertilization, humic acid, arbuscular mycorrhizal fungi (AMF), and their combinations enhance tomato resistance against *R. solanacearum* by inducing transcription of defense-related genes, the expression patterns of the four genes (*POX*, *PPO*, *PAL*, and *PR-2*) were analyzed by real-time RT PCR at 7 days post-inoculation (dpi). The expression of these genes was significantly influenced by *R. solanacearum* inoculation and the applied treatments (Fig. 2).

***POX* gene expression**

In non-inoculated plants, *POX* transcription showed only little induction under NPK^{-} (1.2-fold), with moderate induction in H^{-} (1.4), M^{-} (1.6), NPKH (1.6), NPKM (1.8), and NPKHM^{-} (2.1) compared with the control (Ck^{-}). Inoculation with *R. solanacearum* exhibited double expression in Ck^{+} (2.3-fold compared with Ck^{-}). Single amendments further enhanced expression, particularly H^{+} (3.1) and M^{+} (3.3), while dual combinations produced higher induction, where NPKM^{+} increased (3.8). The highest transcription level was detected in NPKHM^{+} (4.5-fold compared with Ck^{-}), representing a ~2-fold increase in case of Ck^{+}.

***PPO* gene expression:**

In non-inoculated plants, *PPO* expression showed little increase in NPK^{-} (1.2-fold) compared with the control, and moderate induction in H^{-} (1.6), M^{-} (1.7), and NPKHM^{-} (2.1-fold) over Ck^{-}. Inoculation with *R. solanacearum* induced transcription in Ck^{+} (2.2-fold

compared with Ck^-). Further inductions were observed in H^+ (3.0) and M^+ (3.4), while dual applications, particularly $NPKM^+$ (3.7), amplified expression further. The highest PPO transcription level was detected in $NPKHM^+$ (4.2), twice that of Ck^+.

***PAL* gene expression:**

In non-inoculated plants, results revealed that *PAL* transcription showed low value in NPK^- (1.2-fold) and more induction in H^- (1.4), M^- (1.8), and $NPKHM^-$ (2.1) compared with Ck^-. Inoculation elevated expression to 2.3-fold in Ck^+. Treatments significantly induced expression further, with H^+ (3.2) and M^+ (4.2) showing marked effects. Dual applications, especially $NPKM^+$ (4.7), enhanced induction, while $NPKHM^+$ (5.1) achieved the maximum level, representing ~2.3-fold compared to Ck^+.

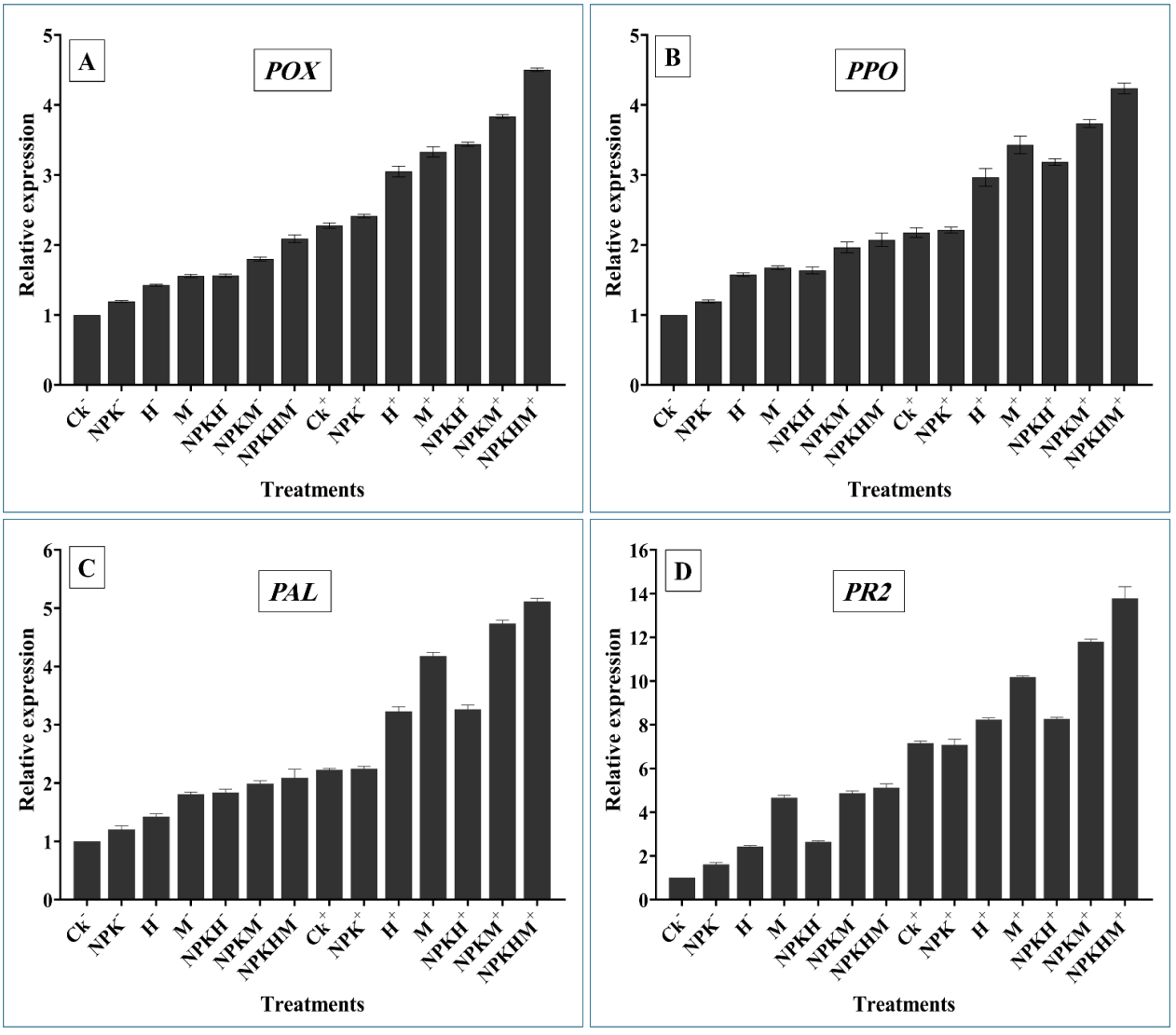

Fig. 2. Relative expression of defense-related genes in tomato leaves under different treatments. (A) *POX*, (B) *PPO*, (C) *PAL*, and (D) *PR2* (β-1,3-glucanase). Plants were treated with NPK fertilizer, humic acid (H), and/or arbuscular mycorrhizal fungi (AMF) under *Ralstonia solanacearum*-inoculated (+) and non-inoculated (−) conditions. Treatments: Control (Ck); mineral fertilizer (NPK); humic acid (H); arbuscular mycorrhizal fungi (AMF, M); NPK + humic acid (NPKH); NPK + AMF (NPKM); and NPK + humic acid + AMF (NPKHM). Expression levels were normalized to the actin gene as an internal reference. Values represent mean ± SE.

***PR-2* (β-1,3-glucanase) gene expression:**

PR-2 displayed the highest induction among the genes analyzed. In non-inoculated plants, NPK^- induced a little increase (1.6-fold), whereas H^- (2.4), NPKH (2.6), M^- (4.7), NPKM (4.9), and $NPKHM^-$ (5.1-fold) showed higher effects compared with Ck^-. Inoculation markedly upregulated expression in Ck^+ (7.2-fold). Single amendments, particularly M^+ (10.2), further enhanced levels, while $NPKM^+$ (11.8) surpassed all dual treatments. The greatest induction occurred under $NPKHM^+$ (13.7-fold), mostly a doubling of expression compared to Ck^+.

DISCUSSION

Bacterial wilt, caused by *R. solanacearum* race 1, is considered one of the most destructive tomato diseases, ranking among the top plant pathogenic bacteria due to its persistence, broad host range, and global impact (Mansfield *et al.,* 2012). Conventional control strategies, such as resistant cultivars and chemicals, often fail or pose environmental risks. Thus, integrated strategies combining cultural, organic, and biological amendments are increasingly promoted as sustainable approaches that suppress the pathogen and enhance host resistance (Yuliar *et al.,* 2015 and Chachar *et al.,* 2025).

Bacterial wilt severity was markedly influenced by nutrient and amendment treatments. The untreated control (Ck^+) showed the highest severity (60.40%), confirming the susceptibility of tomato to this pathogen under conducive conditions (Elphinstone, 2005). NPK fertilization alone reduced severity to 43.00%, confirming the importance of balanced mineral nutrition in activating defenses and supporting vigor (Dutta *et al.,* 2024). Nitrogen may regulate host–pathogen interactions and defense elicitation, phosphorus supports energy transfer and membrane stability, and potassium enhances resistance through cell wall fortification, osmotic regulation, and activation of phenolic biosynthesis and defense enzymes (Sun *et al.,* 2020 and Tripathi *et al.,* 2022). In addition to mineral nutrition, humic acid (40.00%) and AMF (32.20%) significantly suppressed wilt disease, in line with evidence that humic substances stimulate antioxidant activity and systemic resistance (Abdel-Monaim *et al.,* 2011; Canellas *et al.,* 2015 and de Moura *et al.,* 2023), while AMF improve nutrient uptake and prime defense-related gene expression (Pozo & Azcón-Aguilar, 2007 and Zou *et al.,* 2020). AMF has been widely shown to enhance tolerance against both fungal and bacterial wilt pathogens (Boutaj *et al.,* 2022). The greatest suppression occurred in the integrated treatments, particularly $NPKHM^+$ (23.20%), indicating a synergistic effect of combining mineral, organic, and microbial inputs. Such integration enhances structural and biochemical defenses, restricting pathogen spread and providing a sustainable approach for wilt management in tomato. Comparable synergisms were reported in tomato where humic acid plus PGPR reduced *Fusarium* wilt (Abdel-Monaim *et al.,* 2012) and AMF–humic acid–whey combinations suppressed *Verticillium* wilt (Demir *et al.,* 2015).

Plant growth and nutrient status were strongly influenced by infection and treatments. Wilt-inoculated controls showed reduced biomass, chlorophyll, and N, P, and K uptake, consistent with vascular disruption, photosynthetic decline, and impaired nutrient translocation caused by *R. solanacearum* (Butler *et al.,* 2012 and Rivard *et al.,* 2012). Among single amendments, NPK fertilization provided the greatest growth recovery, supporting biomass accumulation and chlorophyll synthesis under both healthy and infected conditions (Wamalwa *et al.,* 2019). Integration of NPK with humic acid and/or AMF further improved growth and nutrient assimilation, with $NPKHM^+$ maintaining the highest plants, greatest dry biomass, highest N, P, and K concentrations, and highest chlorophyll levels even under inoculation. These synergistic effects reflect complementary roles: NPK supplies essential macronutrients, humic acid improves soil structure and nutrient mobility, and AMF expand root absorptive capacity and induce systemic resistance (Tripathi *et al.,* 2022; Soussani *et al.,* 2023 and Wahab *et al.,* 2023). Nutrient uptake patterns confirmed these synergistic effects: nitrogen accumulation was highest in $NPKHM^+$, reflecting improved assimilation and sustained metabolism, phosphorus uptake was markedly enhanced in AMF-containing treatments consistent with the well-established role of mycorrhizae, and potassium peaked under $NPKHM^+$, supporting osmotic balance, enzyme activation, and defense induction (Habibzadeh & Moosavi, 2014; Dalsing *et al.,* 2015; Sardans & Peñuelas, 2021 and Tesfaye *et al.,* 2024). Pinos *et al.* (2019) observed a 12% increase in P content in maize treated with humic acid and AMF, and Cao *et al.* (2022) reported significantly higher soil organic carbon, total nitrogen, available potassium, and phosphorus in bacterial wilt–suppressive soils. Humic acid alone offered limited nutrient gains, particularly under infection, indicating primarily indirect benefits through improved soil conditions and microbial stimulation (Gent *et al.,* 2015 and Chen *et al.,* 2024). These integrated treatments enhanced nutrient assimilation, growth, and stress tolerance, mitigating wilt-induced yield losses more effectively than single amendments.

Enhanced biochemical defenses and gene expression were closely linked to wilt suppression in tomato. Treatments that increased phenolic accumulation, *POX*,

PPO, and *PAL* activities, and upregulated their genes (*POX*, *PPO*, *PAL*, *PR-2*) corresponded with lower disease severity. Inoculated controls (Ck^+) showed only moderate induction, insufficient to reduce wilt, which remained at 60.4%. Under *Ralstonia* inoculation, susceptible plants activate defense pathways but fail to induce growth because their limited resource allocation and impaired vascular function prevent them from tolerating the severe water stress induced by bacterial wilt (Meline *et al.,* 2023). By contrast, humic acid and AMF boosted phenols (1.3–1.6-fold over Ck^+), upregulated *PAL* and *POX* expression, and reduced severity to 40.0% and 32.2%, respectively. Dual integrations further enhanced responses, with $NPKM^+$ strongly activating phenols (1.8-fold), *POX* (3.8), *PAL* (4.7), and *PR-2* (11.8-fold), correlating with reduced severity (29.0%). $NPKHM^+$ triggered the strongest activation, including maximal phenolic accumulation, highest enzyme activities, and strongest gene upregulation (*POX* = 4.5; *PAL* = 5.1; *PR-2* = 13.7-fold), correlating with the lowest wilt severity (23.2%) and greatest plant vigor. These findings indicate that nutrient–biostimulant combinations both enhance defenses and reprogram transcriptional pathways, enabling stronger responses to infection. This defense activation likely explains the superior wilt suppression and growth maintenance observed under NPKHM.

These responses align with previous reports showing that phenolics function as antimicrobial compounds and lignin precursors that strengthen cell walls and restrict pathogen colonization (Lattanzio *et al.,* 2006 and Mandal *et al.,* 2010). Elevated *POX*, *PPO*, and *PAL* activities reflect the oxidative burst and secondary metabolism associated with hypersensitive responses and structural defenses (Passardi *et al.,* 2005 and Zou *et al.,* 2020). Specifically, *POD* and *PPO* oxidize phenolic compounds into quinones that are toxic to pathogens, while also participating in lignification, cell wall fortification, hypersensitive responses, systemic acquired resistance (SAR), and phytoalexin biosynthesis (Bayoumi *et al.,* 2021). *PAL*, as the key entry enzyme of the phenylpropanoid pathway, plays a pivotal role in phenolic biosynthesis and is widely regarded as a central regulator of plant defense metabolism (Mandal and Mitra, 2007). Supporting this, humic substances have been shown to upregulate *PAL*, *POX*, and *PR-proteins* in soybean (Abdel-Monaim *et al.,* 2011). Also, HA has been reported to stimulate *POX* and *PAL* activities in various plant species, including orange, coffee, sugarcane, soybeans, maize, and tomato (Silva *et al.,* 2025). Similarly, AMF colonization induces systemic resistance through activation of *POX*, *PPO*, *PAL*, *β-1,3-glucanase,* and *PR* genes (Pozo & Azcón-Aguilar, 2007; Helmy & Ibrahim, 2016 and da Silva *et al.,* 2023), and primes faster salicylic acid (SA)- and jasmonic acid (JA)-mediated defenses against *Pseudomonas syringae* and *F. oxysporum* (Fujita *et al.,* 2022 and Wang *et al.,* 2022). Notably, the strong induction of *PR-2* (a β-1,3-glucanase) gene of the SAR pathway enhances defense by hydrolyzing pathogen cell walls and generating β-1,3-glucan that acts as an elicitor of defense gene expression (Van Loon *et al.,* 2006). In tomato, AMF colonization enhanced resistance to *Alternaria solani* through upregulation of *PR1*, *PR2*, *PR3*, and other genes such as *LOX*, *AOC*, and *PAL* (Song *et al.,* 2015). Similarly, in potato, AMF inoculation triggered early induction of *PR1* and *PR2* in response to *Phytophthora infestans* (Gallou *et al.,* 2011).

CONCLUSION

Integration of mineral fertilization with humic acid and AMF provides an effective and sustainable strategy for managing bacterial wilt in tomato. While pathogen inoculation severely impaired growth, nutrient uptake, and vigor in untreated plants, combined treatments significantly reduced disease severity, with the $NPKHM^+$ integration achieving the lowest wilt incidence (23.2%) and sustaining superior growth and nutrient assimilation. These benefits were associated with enhanced phenolic metabolism, increased *POX*, *PPO*, and *PAL* activities, and strong upregulation of defense genes, including *PR-2*. Such defense responses reflect a synergistic interaction in which mineral fertilization ensures nutrient availability, humic acid improves soil fertility and metabolic activity, and AMF enhance nutrient uptake while priming systemic resistance. Collectively, these findings highlight integrated mineral–organic–microbial management as a powerful and eco-friendly alternative to chemical control, simultaneously boosting productivity and resistance against bacterial wilt in tomato.

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الملخص العربي

التلقيح المسبق بفطر الميكورايزا وحمض الهيوميك يحسن نمو الطماطم وامتصاص العناصر الغذائية ويستحث المقاومة ضد بكتيريا *Ralstonia solanacearum*

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يُعد الذبول البكتيري المتسبب عن بكتيريا race 1 *Ralstonia solanacearum* من أكثر الأمراض خطورة على الطماطم، حيث يؤدي إلى خسائر كبيرة في المحصول على مستوى العالم. تهدف هذه الدراسة إلى تقييم التأثيرات الفردية والمشتركة لحمض الهيوميك (H)، وفطر الميكوريزا (AMF)، في وجود او غياب التسميد المعدني (NPK) على نمو الطماطم، وامتصاص العناصر الغذائية، وتنشيط الاستجابات الدفاعية، والحد من مرض الذبول البكتيري. سجلت النباتات المصابة غير المعاملة ($^{+}$Ck) أعلى شدة مرضية (٦٠,٤ %)، في حين أدت المعاملات المتكاملة إلى خفض واضح في شدة الإصابة، حيث حققت المعاملة الثلاثية ($^{+}$NPKHM) أقل شدة مرضية (٢٣,٢ %). كما عزز التسميد المعدني NPK النمو وامتصاص العناصر الغذائية (N و P و K) بدرجة كبيرة، وخاصة عند دمجه مع حمض الهيوميك وفطر الميكوريزا (AMF)، مما أدى إلى تحسين الوزن ومحتوى الكلوروفيل وكفاءة التمثيل الغذائي للعناصر لكل من النباتات السليمة والمصابة. أظهرت التحليلات البيوكيميائية والجزيئية زيادة واضحة في تراكم الفينولات ونشاط إنزيمات البيروكسيداز (POX) والبوليفينول أوكسيداز (PPO)وفينيل ألانين أمونيا–لياز (PAL)، بالإضافة إلى ارتفاع التعبير الجيني لكل من *POX* و*PPO* و*PAL* و-*PR2*في النباتات المعاملة. وقد سجّلت المعاملة الثلاثية $^{+}$NPKHM أعلي مستويات من الاستجابة البيوكيمائية (فينول وانزيمات) والجزيئية (جينات)، والتي ترافقت مع أكبر انخفاض في شدة الذبول البكتيري وتحسن واضح في قوة النبات. تشير هذه النتائج إلى أن دمج التسميد المعدني مع الإضافات العضوية والميكروبية يعزز بشكل متكامل مقاومة الطماطم للذبول البكتيري، مع تحسين النمو، مما يوفر بديلاً مستداماً وفعالاً لإدارة هذا المرض.

الكلمات المفتاحية: فطر الميكوريزا– الذبول البكتيري – حمض الهيوميك – الاستجابة الدفاعية للنبات– – *Ralstonia solanacearum*– الطماطم.