J. Trop. Plant Pests Dis.
Vol. 26, No. 2, September 2026
Pages: 420–438

ISSN: 1411-7525
E-ISSN: 2461-0399
DOI : 10.23960/j.hptt.226420-438

RESEARCH PAPER

Antiviral protein genetic markers physiologically induced in cucumber by beneficial microbes against cucumber mosaic virus

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Manuscript received: 10 September 2025. Revision accepted: 10 December 2025. Available online: 08 July 2026

ABSTRACT

Plant antiviral-induced protein markers provide important indicators of the effectiveness of plant defense responses against viral infection, supporting early detection, disease monitoring, and evaluation of antiviral strategies. In this study, various microbial biotic inducers were applied to cucumber plants to evaluate their ability to trigger systemic resistance against cucumber mosaic virus (CMV-EG). Biochemical and protein-profiling analyses were subsequently performed to quantify the induction of antiviral proteins and the activities of defense-related enzymes. In addition, bioinformatic, spectroscopic, and electrophoretic approaches were employed to characterize antiviral-induced proteins in cucumber plants treated with beneficial microbial isolates under CMV-EG infection. Among the tested treatments, *Bacillus circulans* produced the highest protein content, whereas *B. subtilis* resulted in the lowest protein accumulation relative to the healthy untreated control. SDS-PAGE analysis revealed a total of 45 newly induced antiviral protein markers across all microbial treatments, with molecular weights ranging from approximately 13 to 117 kDa and varying in number among the bacterial and fungal inducers. Peroxidase (POD) and polyphenol oxidase (PPO) activities also varied among the tested microbes, with several bacterial and fungal isolates markedly enhancing these defense-related enzymes. Furthermore, all treatments generated distinct POD and PPO isoenzyme patterns, collectively producing a wide range of newly induced antiviral protein markers that confirmed the strong elicitation potential of these biotic agents. Overall, the applied microbial isolates effectively activated antiviral defense responses in cucumber plants, inducing diverse proteins and enzyme markers that highlight their strong potential as biological resistance inducers against CMV-EG.

Keywords: Antiviral induced proteins, biotic inducer, bioinformatics, CMV-EG, cucumber

INTRODUCTION

Cucumis sativus L. is one of the widely cultivated vegetable creeping vine crops commercially grown in Egypt and belongs to the family *Cucurbitaceae*. Cucumber suffers from severe losses in yield and quality under both protected and open-field conditions due to infections caused by several viral pathogens, including cucumber mosaic virus (CMV), a member of the family *Bromoviridae*, which has the ability to infect more than 1200 host species belonging to 100 families (Megahed et al., 2014; Megahed et al., 2015; Megahed et al., 2019). CMV is one of the most widespread and economically destructive viruses affecting cucurbit crops worldwide. Infection can lead to severe yield losses, reduced fruit quality, and deformities such as mosaic symptoms, leaf curling, and stunted growth. Biologically, CMV disrupts plant physiological processes, interferes with nutrient allocation, and weakens overall plant health, making crops more susceptible to secondary infections. Its broad host range and efficient transmission by aphids further exacerbate its impact, posing a major challenge for sustainable cucumber production (Ashwathappa et al., 2021). Previous studies have highlighted the importance of environmentally friendly strategies for managing plant viral diseases through biological control and induced resistance approaches, particularly in horticultural crops highly susceptible to viral infection (Akin et al., 2012; Mahfut et al., 2025).

Despite recent intensive efforts to control viral infections using alternative chemical approaches

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aimed at minimizing negative impacts on human health and the environment (Megahed et al., 2013a; Neupane & Baysal-Gurel, 2022; Masoud et al., 2023; Abdel-Kader et al., 2024), several studies have highlighted the potential of microbial biocontrol agents in inducing plant resistance. Individual strains of plant growth-promoting rhizobacteria (PGPR), such as *Pseudomonas fluorescens* 89B-27, *Bacillus pumilus* SE34, and *Serratia marcescens* 90-166, were shown to enhance resistance in cucumber, tomato, *Arabidopsis thaliana*, and melon following seed treatment applications, reducing the percentage of CMV-infected plants from 88–98% in untreated controls to 32–58% in treated plants (de Andrade et al., 2023; Mazuecos-Aguilera et al., 2025). Similarly, fungal culture filtrates from individual *Trichoderma* species effectively reduced tobacco mosaic virus (TMV) symptoms and local lesions formation on *Nicotiana glutinosa* (Imran et al., 2023; Yao et al., 2023). Additionally, crude filtrates of *Streptomyces* spp. strongly induced systemic acquired resistance (SAR) in potato cultivars (Nicola, Spunta, Diamant, and Selatar), inhibiting potato virus Y (PVYNTN) infection. Among them, *S. actuosus* SCF20 was the most effective, completely suppressing apical necrosis symptoms and fully activating the *PR-1b* gene in the leaves of potato cvs. Nicola, Diamant, and Spunta (Nasr-Eldin et al., 2019). Similar findings have also been reported in CMV-related studies, where biological and molecular protection approaches enhanced defense-associated physiological responses and reduced disease severity in virus-infected plants (Akin et al., 2012; Mahfut et al., 2025).

Induced resistance is expressed through the enhancement of plant defense mechanisms activated by different external stimuli (El-Mougy et al., 2013; Megahed et al., 2014; Wilson et al., 2023; Kessler & Mueller, 2024). Over the past decade, considerable attention has been devoted to elucidating the mechanisms of induced systemic resistance, particularly physiological alterations in induced plants, such as the synthesis of novel proteins (El-Sherif et al., 2019; Jung et al., 2024). The time required for the accumulation of novel proteins is particularly important because the buildup of pathogenesis-related (PR) proteins plays an essential role in inducing strong systemic resistance in susceptible hosts against viral infections (Megahed et al., 2019; Nasr-Eldin et al., 2019). In tobacco, SAR marker sets consist of nine gene families that are induced coordinately in the non-infected leaves of inoculated plants (Anikina et al., 2023). Some SAR gene products exhibit direct antimicrobial effects or are closely associated with antimicrobial proteins (Nasr-Eldin et al., 2019; Ajayi et al., 2024; Oliveira et al., 2024). These products include glucanases, class II and class III chitinases, thaumatin-like proteins, and several other defense-related proteins (Poveda et al., 2020; Vaghela et al., 2022). SAR-related genes include *PR-1*, *PR-2*, and *PR-5* in *Arabidopsis* plants (Anisimova et al. 2021), and are associated with increased peroxidase and class III chitinase activities in cucumber (Dos Santos & Franco, 2023), as well as elevated chitinase activity in pepper (Han & Schneiter, 2024; Zhu & Gao, 2025).

Antiviral protein markers are reliable indicators of induced resistance because they reflect the active defense responses of plants against viral infection. The accumulation of induced proteins such as pathogenesis-related (PR) proteins, peroxidases (POD), and polyphenol oxidases (PPO) signifies the activation of systemic acquired resistance and other defense pathways. Monitoring these markers helps evaluate the effectiveness of biotic or chemical resistance inducers and identify genotypes with stronger immunity. The time required for the accumulation of newly induced proteins, particularly PR proteins, is critical because their rapid accumulation plays a key role in triggering strong systemic resistance in susceptible hosts against viral pathogens (Megahed et al., 2019; Nasr-Eldin et al., 2019).

Polyphenol oxidase (PPO) catalyzes the oxygen-dependent oxidation of diphenols into o-quinones and is involved in plant senescence, wound responses, and defense against pathogens (Goel & Paul, 2015). PPO-produced quinones can modify plant proteins and reduce the nutritional availability of plant tissues to invading pathogens or herbivores. Polyphenolic polymers appear to be more toxic to prospective phytopathogens than monomeric phenols (Zagoskina et al., 2023). Peroxidases (PODs) exhibit extensive structural diversity and catalyze redox reactions between H_2O_2 and various reductants. PODs are also involved in several cellular activities, including stress tolerance, cell wall modification, salt stress responses, auxin metabolism, heavy metal detoxification, aging, and defense against pathogens. Therefore, these enzymes can serve as biomarkers indicating biotic or abiotic stress conditions (Jouili et al., 2011; Begovic et al., 2017; Twala et al., 2020; Masoud et al., 2023).

Although previous studies have demonstrated that microbial treatments can induce antiviral proteins in plants, comprehensive comparisons of the protein and isoenzyme profiles elicited by different bacterial and fungal isolates remain limited. Specifically, there is a lack of systematic analysis linking individual

microbial treatments with the number, molecular weight, and intensity of newly induced antiviral protein bands, as well as their associated defense enzyme activities in cucumber plants infected with CMV. This gap limits our understanding of which microbial agents are most effective in triggering specific antiviral defense responses (Elsharkawy et al., 2022). Therefore, this study aimed to identify and characterize antiviral-induced proteins in cucumber plants treated with selected bacterial and fungal isolates against CMV-EG infection using spectroscopic, electrophoretic, and bioinformatic analyses.

MATERIALS AND METHODS

Research Site. The greenhouse experiment was conducted in February 2024 at the Virology Laboratory, Faculty of Agriculture, Ain Shams University, Cairo, Egypt. Subsequent biochemical analyses were performed at the Molecular Biology Department and the Proteome Research Laboratory, National Research Centre, Dokki, Giza, Egypt.

Virus Isolate. An isolate of cucumber mosaic virus (CMV-EG) with accession no. EU365893 was obtained from the Virology Laboratory, Faculty of Agriculture, Ain Shams University (Megahed et al., 2012) and maintained in the propagative host *N.glutinosa.*

Chemicals. Acrylamide, bovine serum albumin (BSA), L-dihydroxyphenylalanine (L-DOPA), guaiacol, hydrogen peroxide (H_2O_2), *N,N′*-methylenebisacrylamide, and phenylmethylsulfonyl floride (PMSF) were purchased from Sigma-Aldrich Chemical Co., Darmstadt, Germany. All other chemicals used in this were of analytical grade.

Source of Biotic Inducers. Bacterial and fungal isolates were obtained from MARCIN, Faculty of Agriculture, Ain Shams University, Egypt (El-Dougdoug et al., 2013). The bacterial isolates included *B. subtilis*, *B. polymyxa, B. circulans, P. putida, P. fluorescens* 2, and *P. fluorescens* 8. Nutrient broth was used as the propagation medium for *B. subtilis*, *B. polymyxa*, *B. circulans*, and *P. putida*, whereas King's B broth was used for *P. fluorescens* 2 and *P. fluorescens* 8. Bacterial cell suspensions were adjusted at a mean density of approximately 5×10^9 CFU/mL. *Trichoderma harzianum* was propagated in potato dextrose broth, and the conidial suspension was adjusted to a mean density of approximately 1×10^{10} spores/mL (Beal et al., 2020; Megahed et al., 2023).

Greenhouse Experiment. Seven microbial liquid culture isolates (six bacterial and one fungal isolate) were individually applied to induce systemic acquired resistance (SAR) against CMV-EG by soaking cucumber seeds prior to planting. Surface-sterilized seeds of cucumber cultivar Beit Alpha (*C. sativus*) were soaked for 60 min in 50 mL of each microbial culture and then sown in sterilized soil (autoclaved at 121 ºC for 1 hour) consisting of clay, sand, and peat moss (3:1:1, v/v). Five seeds were planted per pot, and ten replicate pots were assigned to each biotic treatment.

One week after planting, the cotyledons of all treated and untreated plants (infected control) were mechanically inoculated with CMV-EG using a spatula. The inoculum consisted of filtered infectious sap obtained by grinding CMV-EG-infected *N. glutinosa* leaves in 0.1 M potassium phosphate buffer (pH 7.2) at a ratio of 1:10 (w/v), followed by filtration through two layers of cheesecloth. Healthy control plants were inoculated with sterile 0.1 M potassium phosphate buffer (pH 7.2). All treatments were maintained in a greenhouse under day/night temperatures of 29/22 ºC until symptom development (Megahed et al., 2013b).

Biochemical Assessments

Extraction and Quantitative Determination of Antiviral-Induced Proteins. The ability of the microbial isolates to induce resistance against CMV-EG was evaluated in treated cucumber leaves. Fourteen days after CMV-EG inoculation, total proteins were extracted from 1 g fresh weight (FW) of leaf tissue using 3 mL of 50 mM sodium phosphate buffer (pH 6.5) containing 1 mM PMSF (Megahed et al., 2019). The resulting protein extracts were stored at −20 ºC until further analysis. Protein concentrations were determined using the dye-binding assay with bovine serum albumin (BSA) as the standard, and absorbance was measured using a Shimadzu UV-2401 UV–VIS spectrophotometer (Abdel-Kader et al., 2024).

Qualitative Fractionation of Antiviral Induced Proteins. To qualitatively detect newly induced antiviral proteins, SDS-PAGE analysis was performed to compare protein banding patterns among treatments. Protein samples were separated on 12% SDS-PAGE gels following the method described by Joshi et al. (2025). After electrophoresis, the gels were subjected to silver nitrate staining, a highly sensitive method for detecting low-abundance proteins. This procedure enabled clear differentiation of induced protein profiles and facilitated the identification of novel proteins

associated with the defense response against CMV-EG (Rabilloud, 2013).

Enzymes Spectroscopic Assays. Peroxidase (POD) activity was determined by monitoring the increase in absorbance at 470 nm resulting from guaiacol oxidation catalyzed by the enzyme in the presence of H_2O_2. One unit (U) of POD activity was defined as the amount of enzyme required to produce a change of 1.0 in absorbance per minute (Masoud et al., 2025).

Polyphenol oxidase (PPO) activity was measured by monitoring the rate of quinone formation, indicated by the increase in absorbance at 420 nm. One unit (U) of PPO activity was defined as the amount of enzyme producing an absorbance change of 0.001 per min (Abdel-Kader et al., 2024).

Isoenzymes Patterns Analysis. POD and PPO isoenzyme profiles were analyzed using 7% native PAGE. Following electrophoresis, POD activity was visualized by immersing the gel in 50 mL of freshly prepared 0.05 M sodium acetate buffer (pH 5.6) containing 2 mM guaiacol and 266 μM H_2O_2 until distinct isoenzyme bands appeared. The reaction was subsequently stopped using 7% acetic acid. For PPO activity staining, the gel was incubated in 50 mL of 0.05 M sodium phosphate buffer (pH 8.0) supplemented with 2.5 mM L-DOPA until the isoenzyme bands developed (Megahed et al., 2019).

Bioinformatics Analyses

All obtained data and gel images were analyzed using the Syngene™ Ingenius 3 Gel Documentation System. The system was employed for high-resolution imaging, band detection, and digital enhancement of protein and isoenzyme profiles. Densitometric analysis was performed to compare band intensities, determine relative expression levels, and accurately differentiate induced and constitutive protein bands among treatments.

RESULTS AND DISCUSSION

Protein Content. The process of plant infection by viruses can be divided into two main phases: establishment and replication. The establishment phase involves successive events, including initial wounding caused by a vector or abrasion, cell penetration, and viral uncoating. The replication phase is characterized by the synthesis of viral nucleic acids and proteins, virion reassembly, and subsequent movement to adjacent cells or other plant tissues. Determining the effects of antiviral compounds on the establishment or replication phases using tissue assays is often limited by the overlap between viral uncoating and the initial synthesis of viral proteins and nucleic acids (Escalante et al., 2024). A material can be considered an effective inhibitor of viral propagation if it is applied 5–8 h after virus inoculation (Abo-Zaid et al., 2025; Elshaer et al., 2025). Four major activities have been described for antiviral-induced proteins, and some proteins may perform more than one function. These activities include viral aggregation, inhibition of viral establishment, induction of systemic resistance, and inhibition of viral replication through suppression of protein synthesis (Anikina et al., 2023).

In the present study, the application of various microbial isolates under sterilized soil conditions significantly increased the protein content of cucumber plants compared with the untreated control (1.13 mg/g FWt, Table 1). Among all treatments, *B. circulans* induced the highest protein accumulation (1.77 mg/g FW), indicating a strong stimulatory effect on host metabolism, whereas *B. subtilis* produced the lowest increase (1.305 mg/g FW). Likewise, *P. fluorescens* treatment markedly enhanced protein synthesis in both *C. sativus* and *Chenopodium amaranticolor*, exceeding the effects of the other isolates and demonstrating its superior capacity to stimulate host protein metabolism during the defense response to CMV-EG infection.

The observed increase in protein content and associated enzyme activities may be attributed to the induction of systemic resistance by rhizobacteria, which has been reported to activate host defense pathways and stimulate the accumulation of defense-related proteins (Pieterse et al., 2014). Similar observations have been reported in studies conducted in tropical plant pathosystems, where beneficial microbial treatments enhanced defense-related protein accumulation and strengthened plant resistance against pathogens. Enhanced protein synthesis following microbial induction has been associated with activation of host metabolic pathways and systemic resistance mechanisms under pathogen stress conditions (Mahfut et al., 2025). The approximately threefold increase in soluble protein content in CMV-EG-infected plants compared with healthy controls is consistent with the concept that viral infection often triggers stress-related protein synthesis, including pathogenesis-related (PR) proteins, as part of the host defense response (Ali et al., 2018). Similarly, protein content in induced *C. sativus* and *C. amaranticolor* treated with *P. fluorescens* was higher than that observed in other microbial treatments and healthy controls under CMV-EG infection (Al-

Zahrani et al., 2017). Collectively, these findings suggest that although all microbial inducers enhanced protein accumulation, *B. circulans* and *P. fluorescens* were the most effective, highlighting their potential roles in strengthening host defense and metabolic activity against viral infection.

SDS-PAGE Pattern and Induced Protein Analysis. Antiviral-induced proteins were qualitatively analyzed based on their number, molecular weight, reproducibility, and band intensity on SDS-PAGE gels. Bands with similar mobilities were considered corresponding fragments, whereas faint or smeared bands with weak intensities were excluded from the analysis. Figure 1 presents the SDS-PAGE profiles of cucumber plants treated with seven biotic inducers, revealing clear differences in the number and molecular weights of newly expressed protein bands.

The number of newly induced protein bands ranged from two to ten, with an average of six bands per inducer. Overall, 45 distinct antiviral-induced protein bands were detected (Table 2). The highest number of induced bands was observed in *T. harzianum* treatment (10 bands), followed by *P. fluorescens* 2 (8 bands), *B.*

Table 1. Effects of biotic inducers on protein content and enzyme activities

Treatment	Protein content (mg/g FW)	POD activity (U/g FW)	POD specific activity*	PPO activity (U/g FW)	PPO specific activity*
Healthy control	1.13	65.40	57.67	120.00	105.82
Infected control	2.37	173.40	73.16	468.00	197.46
B. subtilis	1.30	160.80	123.21	259.98	199.21
B. polymyxa	1.60	180.15	112.87	195.00	122.18
B. circulans	1.77	168.15	95.00	180.00	101.69
P. putida	1.58	150.75	95.35	180.00	113.85
P. fluorescens 2	1.59	134.70	84.71	204.00	128.30
P. fluorescens 8	1.60	175.65	109.64	330.00	205.99
T. harzianum	1.58	163.95	104.09	324.00	205.71

*Specific activity expressed as enzyme units/mg protein. The healthy control exhibited the lowest protein content (1.13 mg/g FW), POD activity (65.4 U/g FW), and PPO activity (120 U/g FW), indicating minimal enzymatic activity under normal conditions. In contrast, the infected control showed the highest protein content (2.37 mg/g FW), the highest PPO activity (468.00 U/g FW), and elevated POD activity (173.40 U/g FW), reflecting the plant defense response against pathogen infection. Among the biotic inducer treatments, *B. subtilis* induced the highest POD specific activity, whereas *P. fluorescens* 8 and *T. harzianum* exhibited the highest PPO specific activities, suggesting strong induction of oxidative defense enzymes. Overall, all biotic inducer treatments enhanced enzyme activities compared with the healthy control, indicating activation of plant defense mechanisms.

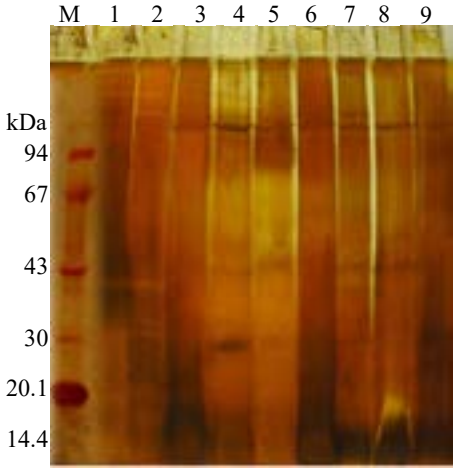

Figure 1. Electrophoretic analysis of antiviral induced proteins on SDS-PAGE of (80 µg protein each) per well: (M) molecular weight marker proteins, (1) Healthy control, (2) Infected control, (3) *B. subtilis*, (5) *B. circulans,* (4) *B. polymyxa*, (6) *P. putida*, (7) *P. fluorescens* 2, (8) *P. fluorescens* 8, and (9) *T. harzianum*.

Table 2. Numbers and molecular weights of antiviral induced proteins induced by different biotic inducers

Healthy control	Infected control	*B. subtilis*	*B. polymyxa*	*B. circulans*	*P. putida*	*P. fluorescens* 2	*P. fluorescens* 8	*T. harzianum*
-	132.26	-	-	-	-	-	-	-
131.41	-	-	-	-	-	-	-	-
-	119.01	-	-	-	-	-	-	-
-	-	117.41	-	-	-	117.41	-	-
114.25	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	112.68	-	112.68	-	-	-
-	-	-	-	110.66	-	-	-	-
-	-	109.59	-	-	-	109.59	-	-
107.80	107.80	-	-	-	-	-	107.80	107.80
-	-	104.30	-	-	-	104.30	-	-
-	100.60	-	100.60	-	100.6	-	-	100.60
-	-	-	-	-	-	-	-	93.45
-	-	-	-	89.9	-	-	-	-
-	81.34	-	-	-	-	-	-	-
77.53	77.53	-	-	-	-	-	-	-
73.21	-	-	-	-	-	-	-	
-	-	-	-	70.81	-	-	-	70.81
-	-	65.03	-	-	-	65.03	-	-
-	63.91	-	-	-	-	-	-	-
-	57.53	-	-	57.53	-	-	-	-
-	-	-	-	53.87	-	-	-	-
-	-	-	-	-	-	-	-	50.45
-	-	48.10	-	-	-	48.10	-	-
-	-	-	45.83	-	45.83	-	-	-
-	-	-	-	43.94	-	-	-	-
42.34	42.34	42.34	42.34	-	42.34	42.34	42.34	42.34
-	-	-	37.87	-	37.87	-	-	37.87
-	34.49	34.49	34.49	-	34.49	34.49	-	34.49
-	-	-	32.71	-	32.71	-	-	-
30.36	-	-	-	-	-	-	-	30.36
-	-	27.25	-	-	-	27.25	-	-
-	-	-	-	25.39	-	-	-	25.39
22.92	22.92	22.92	-	22.92	-	22.92	-	-
-	-	-	-	-	-	-	-	20.17
-	17.4	-	-	-	-	-	-	17.4
-	14.79	14.79	-	-	-	14.79	14.79	-
13.01	13.01	13.01	-	13.01	-	13.01	-	13.01
-	-	-	-	-	-	-	12.94	12.94
Total newly bands	9	8	6	7	6	8	2	10

circulans (7 bands), and six bands each for *B. subtilis*, *B. polymyxa*, and *P. putida*. In contrast, *P. fluorescens* 8 exhibited the lowest induction, with only two bands. The molecular weights of these induced proteins varied widely, ranging from 12.94 kDa (*P. fluorescens* 8 and *T. harzianum*) to 117.41 kDa (*P. fluorescens* 2), suggesting that different microbial inducers stimulate the synthesis of distinct sets of defense-related proteins.

Interestingly, the infected control plants (CMV-EG-inoculated plants without inducer treatment) also expressed nine newly induced proteins compared with the healthy control, with molecular weights ranging from 13.01 to 132.26 kDa. This finding indicates that viral infection alone can trigger de novo synthesis of stress- and defense-related proteins, consistent with previous studies reporting that pathogen attack activates pathogenesis-related (PR) proteins and enzymes associated with systemic acquired resistance (SAR) (Shoresh et al., 2010; Dos Santos & Franco, 2023).

The 45 newly detected antiviral-induced protein bands observed in treated plants may therefore be considered putative defense-related protein markers reflecting strong activation of host defense responses. These additional bands may result either from the induction of novel antiviral proteins or from the enhanced accumulation of constitutively expressed host proteins normally present at basal levels in healthy tissues. Similar findings have been reported in plants exposed to beneficial microbes, where enhanced protein synthesis was associated with improved resistance mechanisms (Pieterse et al., 2014; Ali et al., 2018). These induced proteins may contribute to restricting virus multiplication and spread within host tissues (Nasr-Eldin et al., 2019; Sabnam et al., 2023). Previous studies also demonstrated that beneficial microbes could induce distinct protein profiles and defense-associated biochemical markers in infected plants, supporting the important role of microbial elicitors in activating host resistance pathways. Variations in induced protein bands among treatments may reflect differences in signaling pathways, antimicrobial metabolite production, and elicitor specificity among microbial isolates (Septariani et al., 2014; Listihani et al., 2022).

Comparatively, *T. harzianum* and *P. fluorescens* 2 were the most effective inducers of novel protein expression, whereas *P. fluorescens* 8 had the least effect. The variation in induced protein profiles among microbial treatments suggests differences in their elicitation mechanisms and in the extent of systemic resistance they stimulate. Taken together, these results demonstrate the importance of SDS-PAGE profiling in identifying induced protein markers associated with biotic induction of resistance, which may serve as valuable tools for characterizing plant defense responses against viral pathogens.

Clusters Analysis. Cluster analysis of antiviral-induced proteins among the biotic inducers was performed based on SDS-PAGE banding patterns, and the similarity coefficient matrix was calculated accordingly. Pairwise similarity coefficient values ranged from 0.0 to 0.42, reflecting varying degrees of similarity among the biotic inducers. A dendrogram was constructed using the unweighted pair group method with arithmetic mean (UPGMA) based on the dissimilarity matrix. The seven biotic inducers were grouped into four distinct clusters (Figure 2), with the most distinct inducer separated from the remaining treatments, which were further organized into two main groups.

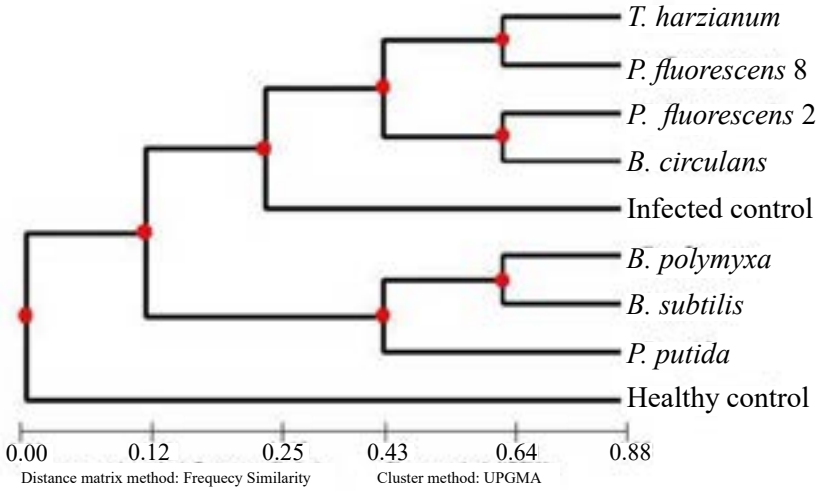

Figure 2. Dendrogram showing the relationships of antiviral-induced proteins among biotic inducer treatments.

The healthy control formed the first main group alone, whereas the second main cluster was further subdivided into five subgroups. The first subgroup contained *P. putida*; the second subgroup included *B. subtilis* and *B. polymyxa*; the fourth subgroup contained *B. circulans* and *P. fluorescens* 2; and the fifth subgroup included *P. fluorescens* 8 and *T. harzianum*, which exhibited closely related SDS-PAGE profiles and the highest antiviral-induced protein similarity (0.64). The third subgroup consisted of the infected control, which displayed the lowest antiviral-induced protein similarity (0.25).

The continuous accumulation of newly induced proteins may contribute to the localization and restriction of viral infection. However, the reverse is not necessarily true, as the presence of only a limited amount of induced proteins may still permit systemic infection. Modifications in gene expression caused by environmental and biological factors play an important role in plant adaptation to adverse conditions and promote the synthesis of specific defense-related proteins (Carfora et al., 2025; Abdel Razek et al., 2025).

Determination of Enzymes Activity

Spectrophotometric Determination of POD. The obtained results clearly indicate that all tested bioagents enhanced peroxidase (POD) activity in cucumber plants compared with the control, suggesting their important role in activating the plant defense system. Among the treatments, *B. polymyxa* was the most effective inducer, exhibiting the highest POD-specific activity (180.15 unit/g FW). This pronounced increase may reflect the strong ability of *B. polymyxa* to stimulate systemic resistance through the production of elicitors and secondary metabolites that activate defense-related enzymes.

In contrast, *P. fluorescens 2* induced the lowest POD activity (134.70 unit/g FW), although its activity remained higher than that of untreated plants, indicating a comparatively weaker defense-induction potential. The other tested strains (*P. fluorescens* 8*, B. circulans, T. harzianum, B. subtilis,* and *P. putida*) exhibited moderate induction levels ranging from 150.75 to 175.65 unit/g FW (Table 1). These differences among treatments may be attributed to variations in their interaction mechanisms with the host plant, including root colonization efficiency, secretion of signaling molecules, and the capacity to activate the host antioxidant defense system.

Overall, the increase in POD activity across all treatments emphasizes the importance of beneficial microbes in enhancing cucumber defense responses. The observed differences in induction levels suggest that certain strains, particularly *B. polymyxa*, may be promising candidates for biocontrol and induced systemic resistance strategies against plant pathogens.

The present findings, showing that all tested bioagents enhanced POD activity in cucumber plants, are consistent with previous reports highlighting the role of plant growth-promoting rhizobacteria (PGPR) and biocontrol agents in defense induction. Similar enhancement of POD activity following microbial induction has been reported in several tropical crop pathosystems, where *Bacillus*, *Pseudomonas*, and *Trichoderma* isolates stimulated antioxidant defense enzymes and contributed to pathogen suppression through induced systemic resistance mechanisms (Akin et al., 2012; Mahfut et al., 2025). These beneficial microorganisms are known to activate defense-related enzymes such as peroxidase (POD), polyphenol oxidase (PPO), and phenylalanine ammonia-lyase, while also promoting the accumulation of phenolic compounds and reactive oxygen species that strengthen cell walls and enhance plant resistance against pathogen invasion (Jakubowska et al., 2025). Similarly, *Paenibacillus polymyxa* strains have been reported to trigger antioxidant enzymes and defense responses, supporting the strong induction potential observed in the present study (Abdelkhalek et al., 2022). The variable induction levels observed among *P. fluorescens* strains also agree with previous findings demonstrating that strain-specific characteristics influence the magnitude of defense enzyme activation (Nilmat et al., 2023).

More broadly, reviews on PGPR-mediated induced systemic resistance confirm that enhanced POD activity is a common biochemical marker of primed defense states in host plants (Hamden et al., 2026). Beneficial microbes belonging to PGPR and arbuscular mycorrhizal fungi (AMF) colonize the rhizosphere of many plant species and provide protection against diverse pathogens by producing exopolysaccharides (EPS), phytohormones, and volatile compounds, as well as by inducing the accumulation of osmolytes and antioxidants. These mechanisms play important roles in increasing the activity of defense-related enzymes, including POD, PPO, and superoxide dismutase (SOD), while significantly reducing the harmful effects of pathogen-induced oxidative stress markers such as malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) (Masoud et al., 2025; Abo Saif et al., 2026; Arafa et al., 2026). Collectively, these comparisons reinforce that the elevated POD activity, particularly in plants

treated with *B. polymyxa*, reflects robust induction of host defense mechanisms and highlights its potential application in sustainable disease management.

POD Isoenzyme Pattern. The total number of POD isoenzymes induced by the different biotic inducers was seven compared with the healthy and infected controls (Figure 3 and 4). Data presented in Table 3 illustrate that all biotic inducers produced one isoenzyme band, although the band intensities varied among treatments. *B. polymyxa, B. circulans, P. putida, P. fluorescens 2,* and *P. fluorescens 8* exhibited the same RF value (0.63), with intensity values of 8055, 453, 273, 298, and 5680, respectively. In contrast, *B. subtilis* and *T. harzianum* exhibited an RF value of 0.64, with intensity values of 5657 and 252, respectively.

On the other hand, infected cucumber plants revealed three isoenzyme bands with RF values of 0.46, 0.57, and 0.66 and corresponding intensity values of 8346, 773, and 3397, respectively. Healthy plants

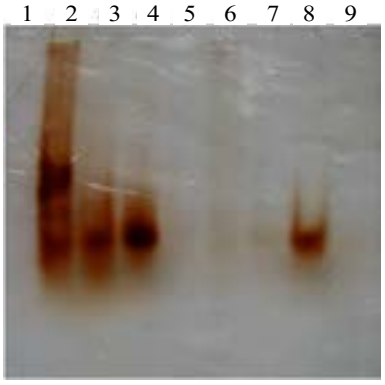

Figure 3. Electrophoretic analysis of POD isoenzyme patterns using 7% native PAGE (150 µg protein loaded per well each). 1. Healthy control; 2. Infected control; 3. *B. subtilis*; 4. *B. polymyxa*; 5. *B. circulans*; 6. *P. putida*; 7. *P. fluorescens* 2; 8. *P. fluorescens* 8; 9. *T. harzianum.*

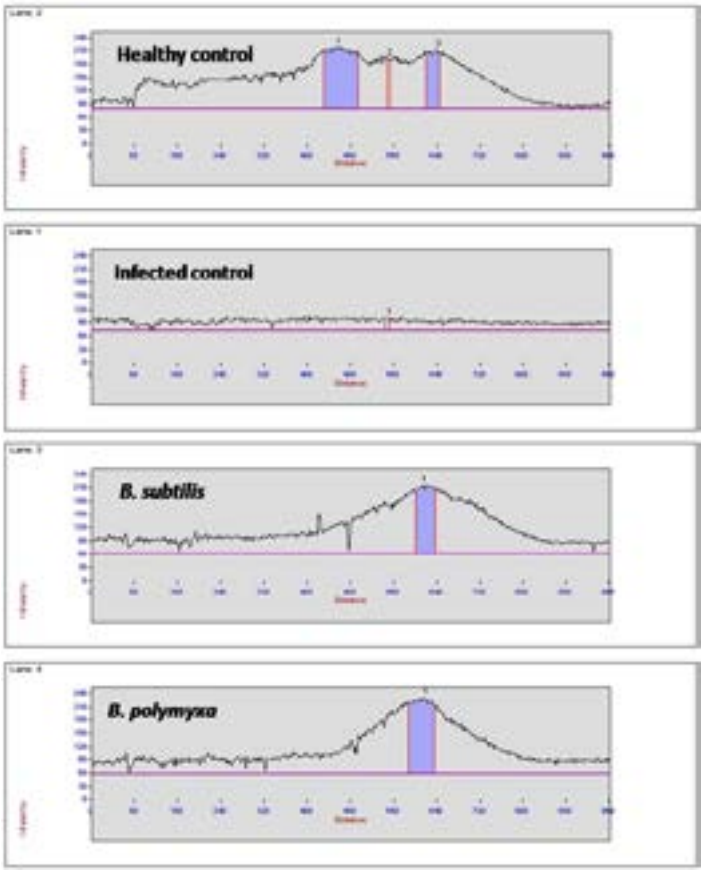

Figure 4. Gel documentation software analysis showing peroxidase isozyme patterns, band distances, and band intensities under different biotic inducer treatments.

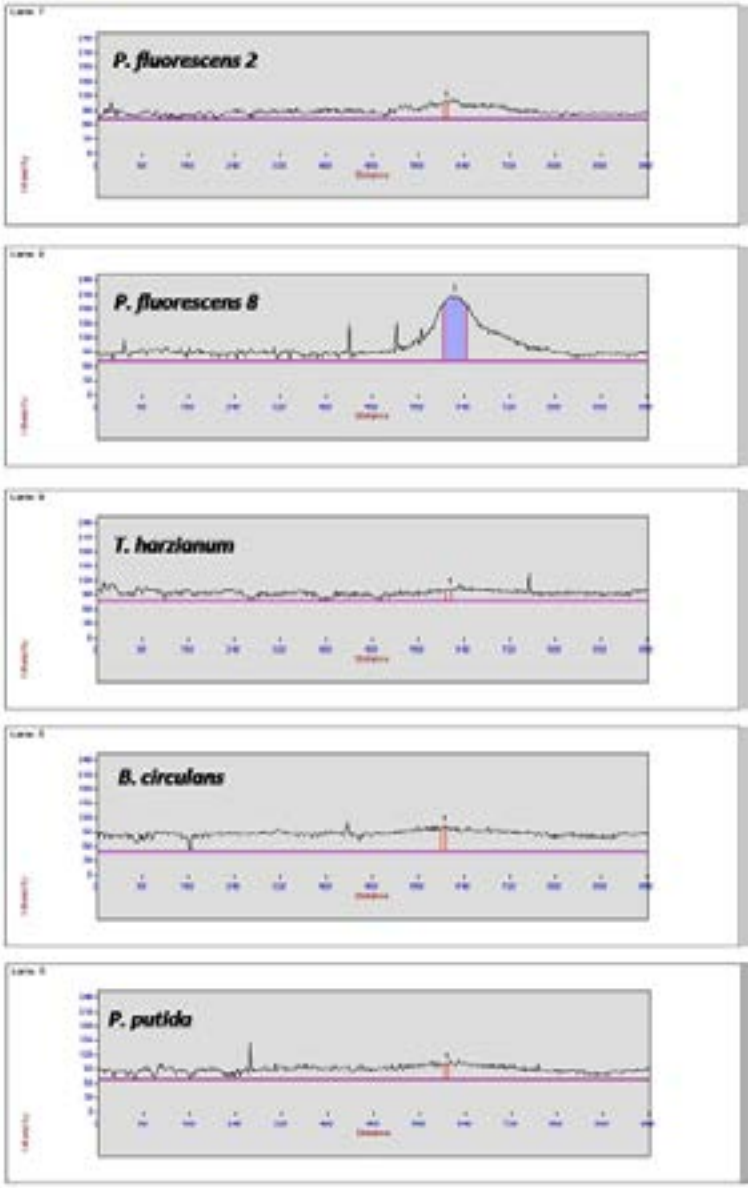

Figure 4. Continued. Gel documentation software analysis showing peroxidase isozyme patterns, band distances, and band intensities under different biotic inducer treatments.

displayed only one isoenzyme band with an RF value of 0.99 and an intensity value of 244. These findings revealed seven novel POD isoenzyme markers that differed in intensity, with one marker corresponding to each biotic inducer.

The present findings are consistent with previous studies reporting that *T. harzianum*, *B. subtilis,* and *Pseudomonas fluorescens* induced novel POD isoforms that served as biochemical markers of systemic resistance (Abdelkhalek et al., 2022). The detection of seven distinct POD isoenzymes in cucumber plants reflects a multifaceted defense system. These isoenzymes contribute to plant resistance by reinforcing cell walls through lignification and protein cross-linking, detoxifying reactive oxygen species to minimize cellular damage while maintaining defense signaling, and facilitating the synthesis of antimicrobial phenolic compounds. In addition, specific POD isoenzymes participate in stress-related signaling pathways that enhance systemic acquired resistance (SAR) and induced systemic resistance (ISR). Collectively, the diversity of POD isoenzymes equips cucumber plants with a versatile and robust mechanism to counter pathogen attack (Jakubowska et al., 2025).

Spectrophotometric Determination of PPO. The results demonstrated that cucumber plants treated with individual microbial isolates exhibited enhanced PPO activity, although the magnitude of induction varied

Table 3. Number and intensity of POD isoenzymes induced in cucumber by biotic inducers

RF	Healthy control		Infected control		*B. subtilis*		*B. polymyxa*		*B. circulans*		*P. putida*		*P. fluorescens* 2		*P. fluoresces* 8		*T. harzianum*	
	% of fraction	Area calculated	% of fraction	Area calculated	% of fraction	Area calculated	% of fraction	Area calculated	% of fraction	Area calculated	% of fraction	Area calculated	% of fraction	Area calculated	% of fraction	Area calculated	% of fraction	Area calculated
0.46	-	-	8346	66.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.57	-	-	6.2	773	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.63	-	-	-	-	-	-	100	8055	100	453	100	273	100	298	100	5680	-	-
0.64	-	-	-	-	100	5657	-	-	-	-	-	-	-	-	-	-	100	252
0.66	-	-	27	3397	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.99	100	244	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

among the inducers. Among the tested microbes, P. fluorescens 8 induced the highest PPO-specific activity (330 unit/g FW), whereas *B. circulans* elicited the lowest PPO activity (180 unit/g FW) (Table 1).

The strong induction observed with *P. fluorescens* 8 is consistent with previous findings showing that different strains of *P. fluorescens* markedly increased PPO activity and enhanced systemic resistance against pathogens (Nilmat et al., 2023). In contrast, the relatively lower response induced by *B. circulans* suggests strain-dependent variability in elicitor production and plant–microbe interactions, a phenomenon also reported for *B. subtilis* and *B. polymyxa*, which exhibited differential capacities to stimulate PPO activity in tomato and cucumber plants (Abdelkhalek et al., 2022).

These findings reinforce the importance of microbial inducers in modulating defense-related enzymes, with *P. fluorescens* 8 emerging as the most promising candidate for enhancing PPO-mediated defense in cucumber plants. PPO catalyzes the oxidation of phenolic compounds into quinones, which can directly inhibit pathogen growth, strengthen cell walls through protein–phenolic cross-linking, and generate signaling molecules that activate additional defense responses. By enhancing both chemical and structural barriers, increased PPO activity contributes significantly to the overall resistance of plants against pathogens (Zhang, 2023).

PPO Isoenzyme Pattern. All biotic inducers enhanced PPO activity compared with the healthy and infected controls, producing a total of seven isoenzymes with variable intensities (Figure 5 and 6). Data presented in Table 4 showed that the healthy control displayed only two bands (RF 0.69 and 0.61), whereas the infected control expressed three bands (RF 0.72, 0.64, and 0.54).

Several isolates shared common RF values with the controls but differed in band intensity. For example, *B. subtilis* shared an RF value with the infected control, whereas *B. polymyxa* and *P. fluorescens* 2 shared an RF value of 0.69. Other isolates induced strong but distinct isoenzymes, such as *B. circulans* and *P. putida* at RF 0.61, and *P. fluorescens* 8 together with *T. harzianum* at RF 0.59. Notably, *P. fluorescens* 8 also induced a unique isoenzyme at RF 0.74.

These results demonstrate that biotic inducers not only enhanced PPO expression but also generated diverse PPO isoenzyme patterns, with seven novel bands serving as potential biochemical markers of induced resistance. Such variations may reflect isolate-dependent differences in elicitor production, as previously reported for *Bacillus* and *Pseudomonas* species in various plant–pathogen systems (Meena et al., 2020). Previous studies also demonstrated that microbial biocontrol agents induced differential PPO isoenzyme expression patterns associated with enhanced defense responses and activation of plant defense signaling pathways under pathogen infection (Akin et al., 2012 ; Listihani et al., 2022). Similar findings further showed that *P. fluorescens* strains enhanced PPO activity and contributed to systemic resistance in cucumber and tomato plants (Serrão et al., 2024).

The tested biotic inducers exhibited different reproducibility levels of acquired resistance associated with the number of induced protein genetic markers.

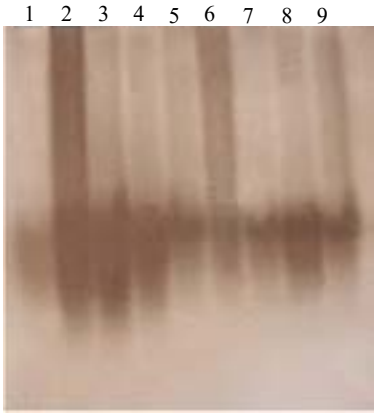

Figure 5. Electrophoretic analysis of PPO isoenzyme patterns using 7% native PAGE (150 µg protein loaded per well). 1. Healthy control; 2. Infected control; *B. subtilis*; 4. *B. polymyxa*; 5. *B. circulans*; 6. *P. putida*; 7. *P. fluorescens* 2; 8. *P. fluorescens* 8; 9. *T. harzianum*.

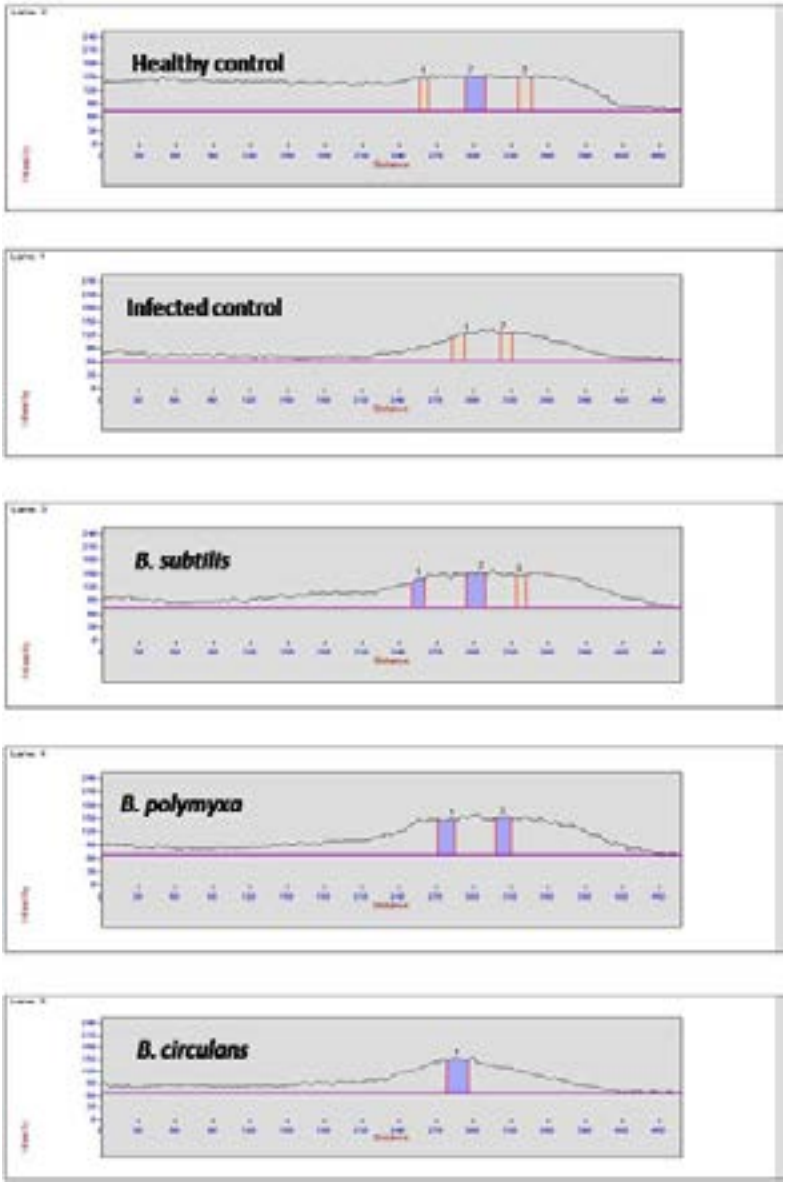

Figure 6. Gel documentation software analysis showing polyphenol oxidase isozyme patterns, band distances, and band intensities under different biotic inducer treatments.

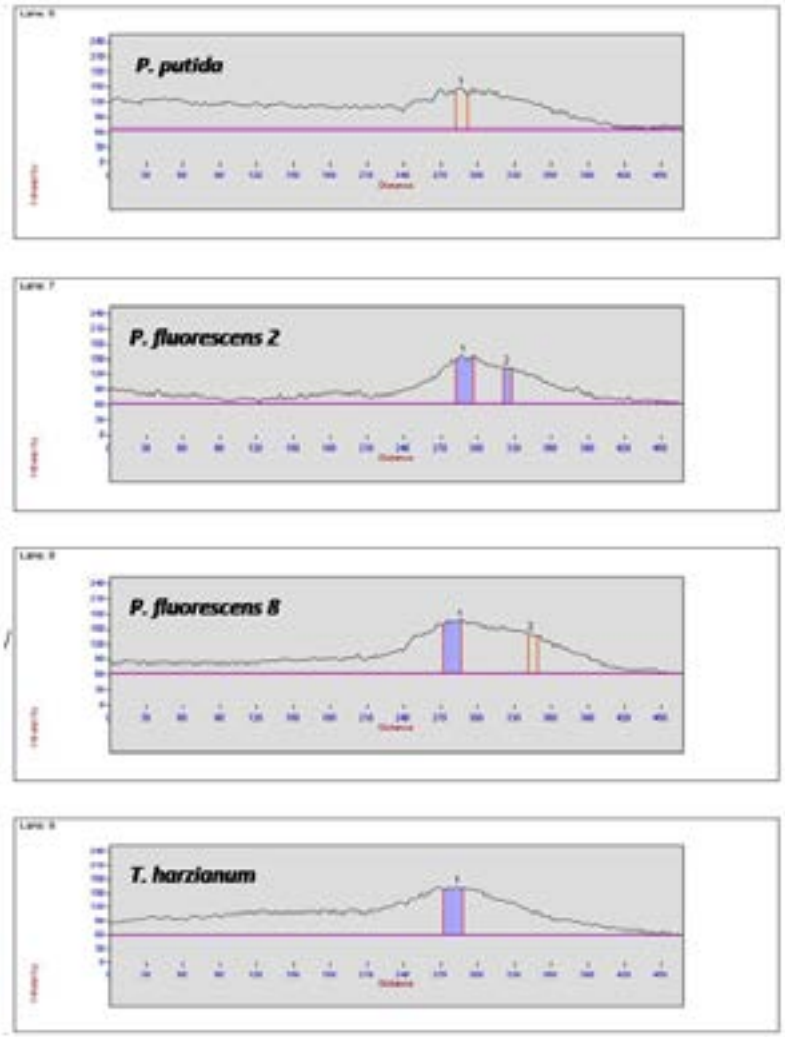

Figure 6. Continued. Gel documentation software analysis showing polyphenol oxidase isozyme patterns, band distances, and band intensities under different biotic inducer treatments.

Trichoderma harzianum produced the highest number of induced protein genetic markers (12), followed by *B. subtilis* (10), *P. fluorescens* 2 (9), *B. circulans* (8), *B. polymyxa* (8), *P. putida* (7), and *P. fluorescens* 8 (5). In contrast, the infected control exhibited 15 induced protein genetic markers (Table 5). Systemic acquired resistance in cucumber plants was positively correlated with increased POD and PPO activities in *N. glutinosa.*

In total, seven new PPO isoenzyme bands were detected in plants treated with biotic inducers, serving as potential biochemical markers for induced resistance. This finding agrees with previous studies demonstrating that novel PPO isoforms are associated with biocontrol-mediated resistance (Megahed et al., 2023; Elhalag et al., 2025; Megahed et al., 2025).

Collectively, these findings indicate that all biotic inducers enhanced PPO activity in cucumber plants and produced seven isoenzymes with variable intensities, reflecting a versatile and dynamic defense system. While certain PPO isoforms represent a conserved response to infection, microbial inducers also activated additional isolate-specific isoenzymes that strengthen cell walls through protein–phenolic cross-linking, generate antimicrobial quinones that inhibit pathogens, and trigger downstream defense signaling pathways. By limiting pathogen growth through the inactivation of microbial proteins and reducing nutrient availability, the diversity and differential expression of PPO isoenzymes enable cucumber plants to mount a flexible, broad-spectrum, and durable defense response. These findings support the use of beneficial microbes such as *Bacillus*, *Pseudomonas*, and *Trichoderma* as sustainable biological control agents (Zhang, 2023).

CONCLUSION

In conclusion, the application of biotic inducers revealed variable levels of acquired resistance, as

Table 4. Number and intensity of PPO isoenzymes induced in cucumber by biotic inducers

RF	Healthy control		Infected control		*B. subtilis*		*B. polymyxa*		*B. circulans*		*P. putida*		*P. fluorescens* 2		*P. fluoresces* 8		*T. harzianum*	
	% of fraction	Area calculated	% of fraction	Area calculated	% of fraction	Area calculated	% of fraction	Area calculated	% of fraction	Area calculated	% of fraction	Area calculated	% of fraction	Area calculated	% of fraction	Area calculated	% of fraction	Area calculated
0.74	-	-	-	-	-	-	-	-	-	-	-	-	-	-	29.7	696	-	-
0.72	-	-	32.6	940	25.3	658	-	-	-	-	-	-	-	-	-	-	-	-
0.69	50.2	649	-	-	-	-	48.1	1107	-	-	-	-	28	564	-	-	-	-
0.64	-	-	46.6	1342	48.1	1253	-	-	-	-	-	-	-	-	-	-	-	-
0.61	49.8	643	-	-	-	-	-	-	100	1616	100	842	72	1448	-	-	-	-
0.60	-	-	-	-	-	-	51.9	1196	-	-	-	-	-	-	-	-	-	-
0.59	-	-	-	-	-	-	-	-	-	-	-	-	-	-	70.3	1651	100	1699
0.54	-	-	20.8	599	26.6	694	-	-	-	-	-	-	-	-	-	-	-	-

Table 5. Protein genetic markers of cucumber plants as indicators of systemic acquired resistance against CMV-EG infection

Parameters	Infected control	*B. subtilis*	*B. polymyxa*	*B. circulans*	*P. putida*	*P. fluorescens* 2	*P. fluorescens* 8	*T. harzianum*
SDS-PAGE	9	6	6	7	6	8	2	10
POD isoenzymes	3	1	1	1	1	1	1	1
PPO isoenzymes	3	3	1	-	-	-	2	1
Total	15	10	8	8	7	9	4	12

demonstrated by the induction of novel protein genetic markers and defense-related enzymes. A total of 58 newly expressed antiviral-induced proteins were detected and considered potential genetic markers. Among the tested inducers, *T. harzianum* showed the highest induction capacity, followed *B. subtilis*, *P. fluorescens* 2, *B. circulans*, *B. polymyxa, P. putida,* and *P. fluorescens* 8. In addition, microbial treatments markedly enhanced peroxidase (POD) activity and diversified polyphenol oxidase (PPO) isoenzyme patterns, resulting in the appearance of seven novel isoforms that were absent in both healthy and infected controls. These findings indicate that microbial inducers play an important role in activating cucumber defense mechanisms against CMV-EG infection. Collectively, the results demonstrate that although all microbial isolates enhanced plant defense responses, *T. harzianum* and *P. fluorescens* 8 were the most effective in simultaneously stimulating antiviral-induced protein markers, POD activity, and PPO isoenzyme diversity. Therefore, these microbial agents may serve as promising biocontrol candidates for improving crop resistance and supporting sustainable disease management strategies.

ACKNOWLEDGMENTS

The authors acknowledge the National Research Centre and the Faculty of Agriculture, Ain Shams University, Cairo, Egypt, for their support of this study.

FUNDING

The authors declare that no funds, grants, or other financial support were received during the preparation of this manuscript.

AUTHORS' CONTRIBUTIONS

AAM provided guidance on the study design and data interpretation and contributed to manuscript writing and revision. BAO was responsible for data collection, data analysis, manuscript writing, and manuscript submission. HMMM conducted the majority of the experimental work and contributed to data collection, data analysis, and manuscript writing. MSEH contributed to conceptualization, data analysis, manuscript revision, and editing. KAE contributed to the study design and manuscript submission.

All authors commented on previous versions of the manuscript and approved the final version.

COMPETING INTEREST

The authors declare that they have no competing interests.

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