PLANT SCIENCE TODAY
Vol x(x): xx–xx
https://doi.org/10.14719/pst.11831

eISSN 2348-1900

RESEARCH ARTICLE

Eco-friendly control of zucchini leaf blight disease in the greenhouse

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Received: 17 September 2025; Accepted: 17 December 2025; Available online: Version 1.0: 28 January 2026

Cite this article: Hussein SN. Eco-friendly control of zucchini leaf blight disease in the greenhouse. Plant Science Today (Early Access). https:/doi.org/10.14719/pst.11831

Abstract

Leaf blight is one of the diseases prevalent in zucchini fields in Iraq. This study investigated the etiological agent of zucchini leaf blight in agricultural fields across the Erbil, Diyala and Salah Al-Din governorates of Iraq. The fungus *Alternaria cucumerina* was found to be the predominant cause, with a percentage of appearance reaching 70.7 % in the samples and a frequency rate of 55 %. The Eac-12 strain showed the highest virulence among the 44 fungal isolates, with disease severity reaching 80.5 % under greenhouse conditions. Copper sulphate and neem oil showed 100 % efficacy in inhibiting pathogenic fungal growth *in vitro*, followed by plant growth-promoting bacteria (PGB), *Azotobacter vinelandii* and *Azospirillum brasilense*. In the greenhouse, the dual inoculum treatment of *A. brasilense* and *A. vinelandii* isolates significantly reduced disease incidence and severity. The quadruple combination treatment (copper sulphate, neem oil, *A. brasilense* and *A. vinelandii*) achieved the highest disease control rate, with disease incidence and severity recorded at 9 % and 4.3 % respectively. This study demonstrated that the rhizobacterial isolates of *A. brasilense* and *A. vinelandii* induced disease resistance in zucchini plants through elevated peroxidase levels, as well as the effectiveness of neem oil and copper sulphate in controlling the pathogenic fungus *A. cucumerina*.

Keywords: *Alternaria cucumerina*; *Azospirillum brasilense*; *Azotobacter vinelandii*; copper sulphate; neem oil

Introduction

Zucchini (*Cucurbita pepo* var. *cylindrical*) is a popular vegetable crop in Iraq. The total cultivated area is 39836 hectares, with an average yield of 167576 t (1). Zucchini is grown in all Iraqi governorates, especially in the central and northern regions, including cultivation in greenhouses, glass buildings and low tunnels (2). Zucchini is susceptible to several pathogens that can infect the root system and cause diseases such as Fusarium wilt caused by *Fusarium oxysporum*, seed rot, seedling dieback and root and stem rot caused by several common fungi such as *F. solani*, *Rhizoctonia solani*, *Phytophthora* spp. and *Pythium* spp., as well as diseases that affect the foliage, such as powdery mildew caused by *Erysiphe cichoracearum* and scab caused by *Cladosporium cucumerinum* (3). Zucchini leaf blight, caused by *A. cucumerina*, is a frequent disease of Cucurbitaceae plants worldwide. The disease is also known as leaf blight, ring leaf spot, Alternaria leaf blight and Macrosporium blight (4). The upper leaf surface of plants is attacked by the fungus *A. cucumerina*. Symptoms emerge as pale brown patches measuring 1–2 mm in size. As the condition progresses, the spots grow and form concentric rings that are light brown in the middle and darker near the borders. In the latter stages of the disease, the spots spread to cover the entire leaf, resulting in plant death (3). *A. cucumerina* has been found in numerous Asian and European countries (5). In Florida, USA, the microscopic morphology of *A. cucumerina* was examined and compared with other species of the same genus after the fungus was found on cucurbits (4). Up to 30 % of economic losses are caused by the leaf blight disease (5). It is now clear that plant diseases can be increasingly managed in an environmental friendly manner. The copper chemical product known as liquid copper sulphate ($C_{16}H_{30}CuO_4$) which is made from derivatives of caprylic acid $C_8H_{16}O_2$ and Bordeaux mixture, created by Millardet in 1882, is the most famous of the many copper combinations that have been used over the years (6). Since 1997, it has been utilized as a fungicide in the US (6). Parts of the neem tree (*Azadirachta indica* A. Juss) contain chemical ingredients that give the resulting oil antibacterial characteristics. Many studies have shown that neem oil (NO) is efficient in controlling insect pests, fungi and nematodes (7). Plant growth-promoting bacteria (PGBs) are significant biological agents used in biological control. *Azotobacter*, which is a biocontrol agent, is a free-living biofertilizer that fixes nitrogen and is typically found in neutral and alkaline soils (8). *Azospirillum* spp. also increase the availability of phosphorus to plants by releasing organic acids and absorbing phosphorus from inaccessible forms, resulting in the release of significant amounts of soluble phosphate into the soil solution. These organisms are known as phosphate solubilizers and they function by a variety of processes, including the release of organic acids and protons (9). Although rhizobacteria and species belonging to the genera *Bacillus* and *Pseudomonas* have been used in several studies to control seed and root rot diseases in Iraq, there are no previous studies on controlling zucchini leaf blight disease in Iraq. Furthermore, there are no studies on the use of NO

or copper sulphate, either alone or in combination with antagonistic bacteria, in controlling zucchini leaf blight. The purpose of this study was to evaluate the efficacy of eco-friendly treatments, including copper sulphate, NO and PGBs, in controlling zucchini leaf blight caused by *A. cucumerina* under greenhouse conditions in Iraq.

Materials and Methods

Isolation and identification of the causative agent of leaf blight disease

Leaf samples exhibiting indications of Alternaria blight were collected from 9 fields across the Erbil, Diyala and Salah Al-Din governorates, with ten leaves gathered from each affected area during 2024. Following a 15 min rinse under running water, the leaf samples were cut into 0.5 cm pieces and surface-sterilized for 2 min in a 1 % sodium hypochlorite solution (10). After that, the pieces were dried on sterile filter paper and rinsed with sterile distilled water. Four plates were inoculated with autoclaved potato dextrose agar (PDA) medium containing 200 mg/L amoxicillin and incubated at 25 ± 2 °C for 7 days. Following analysis of the plates, the single-spore technique was used to purify the fungal isolates grown on PDA (11). For diagnostic purposes, the isolates were cultivated on V4 medium composed of 150 mL of beetroot, celery, carrot and tomato juice extracts in the ratios of 1:2:3:4, augmented with 20 g of agar and 200 mg/L amoxicillin and incubated at 24 °C under a 12/12 hr light/dark cycle for 7 days (12). The average dimensions of conidia produced on each medium were computed individually. Following the established taxonomic keys, the cultivated isolates were examined under a light microscope and categorized at the genus and species levels based on cultural and morphological traits (13, 14). The following formula was used to calculate the percentage of fungal appearance and frequency (15).

Appearance (%) = (Number of samples with occurrence/ Total number of samples) × 100

Frequency (%) = (Number of plant segments with species occurrence/ Total number of segments used) × 100

Pathogenicity assessment of *A. cucumerina* isolates

The pathogenicity of 44 *A. cucumerina* isolates was assessed individually. A 5 mm mycelial disc from a 5-day-old culture was transferred to fresh PDA and incubated for 10 days. Each plate was then flooded with sterile distilled water and passed through 3 layers of sterile gauze to separate spores from the mycelium. The spore suspension was diluted with sterile distilled water containing 0.01 % (V/V) Tween 20 and the concentration of the spore suspension was modified to 10^5 CFU/mL using a haemocytometer. The soil was autoclaved at 121 °C for 60 min and repeated after 48 hr. Sterile soil was placed into 1 kg pots (16). Five zucchini seeds (Dahlia F2 cultivar) were planted in each pot and surface-sterilized with a 1 % sodium hypochlorite solution. Upon the seedlings attaining the age of four true leaves, both sides of the leaves were treated with a spore suspension from each of the 44 fungal isolates, applied separately using a mechanical hand sprayer. The control treatment consisted of sterile distilled water supplemented with Tween 20. The pots were sealed with pristine polyethene bags for 48 hr. The pots were allocated based on a complete randomized design (CRD), with 4 replicates for each treatment, at a temperature of 25 °C. The pots were irrigated regularly. The plants were treated with the fungal suspension a total of 5 times, with an interval of 3 days between each application. Disease severity was determined using a 5-score disease index: 0 = no lesion, 1 = 1–20 % area affected, 2 = 21–40 % area affected, 3 = 41–60 % area affected, 4 = 61-80 % area affected, 5 = 81–100 % area affected (17, 18).

$$DSI\ (\%) = [\Sigma\ (F \times V) / (N \times M)] \times 100$$

Where,

F = frequencies of infection categories; V = number of leaves within infection categories; N = total number of observed leaves; M = maximum value of the infection categories.

Antagonism assay *in vitro*

The effectiveness of some biological and chemical control agents against the pathogenic fungal isolate (Eac-12), which belongs to the fungus *A. cucumerina*, was tested *in vitro*. Two isolates of PGBs, *Azospirillum brasilense* (Ab) and *Azotobacter vinelandii* (Av), were used. These isolates were obtained from Mustansiriyah University, Laboratory of Microbiology. The double culture technique was used in Petri dishes of 9 cm diameter containing PDA culture medium. The culture medium was inoculated by the striping method with Ab and Av bacterial isolates at a concentration of 10^9 CFU/mL, each grown separately in nutrient broth medium for 48 hr, at a distance of 2 cm from the edge of the dish. The centre of the remaining area of the dish (3.5 cm from the edge opposite the bacterial line) was inoculated with the Eac-12 fungal isolate by placing a 0.5 cm diameter disk of the growing fungus on the PDA culture medium. The disk was taken from the edge of a 7-day-old fungal colony using a sterile cork piercer. Each treatment was repeated four times and the dishes were incubated at a temperature of 25 ± 2 °C until the fungal growth reached the edge of the plate in the control treatment (19). The inhibitory capacity of copper sulphate ($CuSO_4.5H_2O$) and NO was tested using the culture medium poisoning technique. Sterilized PDA medium was prepared and cooled to 50 °C. A series of copper sulphate concentrations (10, 20, 30, 40, 50, 60 and 70 mg/L) was added. The flasks were placed on a shaker. NO (produced by Bonide, USA) was prepared at a concentration of 0.66 mg/L. 1 mL of each concentration of copper sulphate solution and NO was added to sterile Petri dishes and 15–20 mL of sterile PDA culture medium was poured into the plates. The centre of each plate was inoculated with the Eac-12 fungal isolate by placing a 0.5 cm diameter disc taken from the edge of a 7-day-old growing fungal colony on PDA culture medium (15).

Each treatment was repeated four times and the control treatment included a disk of *Alternaria* fungus. Plates were incubated at 25 ± 1 °C. The percentage inhibition of fungal growth was calculated after the fungal growth in the control treatment reached the edge of the plates, according to the following equation (20):

Inhibition (%) = [(fungal growth rate in the control treatment – fungal growth rate in the treatment) / fungal growth rate in the control treatment] × 100

Management of leaf blight disease in greenhouse

Five surface-sterilized zucchini seeds (Dahlia F2 cultivar) were planted in pots containing 1 kg of sterile soil. 10 mL of Ab and Av inoculum, each at a concentration of 10^9 CFU/mL, were introduced

into the soil of each pot concurrently with seedling planting. When the seedlings reached the four-true-leaf stage, both sides of the leaf surfaces were treated with an Eac-12 fungal spore suspension at a concentration of 10^5 CFU/mL using a mechanical hand sprayer. A total of five sprays were applied at 3-day intervals. In alternative treatments, plants were treated with copper sulphate (Cu) at a dosage of 50 mg/L and NO at a concentration of 0.66 mg/L, in accordance with the manufacturer's guidelines, 24 hr after the application of the fungal spore suspension. The spraying procedure was conducted every 5 days for a duration of 60 days. Pots were allocated according to a CRD design, with four replicates for each treatment. The pots were meticulously irrigated. Seed germination rate was assessed after 14 days, while the disease incidence was evaluated 60 days after planting according to the formula below (21). Disease severity percentage was determined using the same disease index outlined in a previous pathogenicity test experiment (17).

Disease incidence (%) = (Number of infected plants/ Total number of plants assessed) × 100

Enzyme activity assessment

Peroxidase (PO) activity was measured by taking 0.5 g of plant leaves, which were crushed with 2 mL of 0.1 M sodium phosphate buffer (prepared from Na_2HPO_4 and NaH_2PO_4) at pH 6.5 and 4 °C. The tubes were subjected to centrifugation at 6000 rpm for 20 min at 4 °C. PO activity was determined by mixing 100 µmol of the filtrate with 1.5 mL of 0.05 M pyrogallol and 100 µmol of hydrogen peroxide (1 % V/V). The absorbance was recorded using a spectrophotometer at a wavelength of 420 nm, taking the average of four replicates (22). The data were recorded one day after inoculation with the pathogenic fungus and on days 5, 10, 15 and 20.

Statistical analysis

CRD was used for the experiments and GenStat Discovery Edition 10 software (VSN International Ltd, Rothamsted Experimental Station, UK) was used to analyze the data after Analysis of Variance (ANOVA). Least Significant Difference (LSD) was used to compare means at the 0.05 probability level.

Results and Discussion

Isolation and identification of the causative agent of leaf blight disease

The findings of isolation and identification indicated the existence of several fungi (Table 1). *A. cucumerina* was predominant, with appearance rates in samples of 70.7 %, 65.8 % and 74.2 % in the governorates of Erbil, Diyala and Salah Al-Din respectively, with frequency rates of 51 %, 41.5 % and 55.6 % respectively (Table 1). Fungal spores may be transmitted via air, insects, birds or irrigation water from infected to healthy fields, especially under favourable conditions and without protective management (Fig. 1). *A. cucumerina* exhibited colonies ranging from dark brown to dark green, characterized by a thick mycelium on PDA. Microscopic analysis of isolates cultivated on V4 medium after 7 days of incubation revealed club-shaped conidia that were longitudinally and transversely split, with an average size of 75-90 × 20-23 µm and a spur length ranging from 43 to 140 µm (Fig. 2). The spores measured 45-66 × 17-20 µm on PDA medium. These measurements were consistent with previous studies (14, 23).

Pathogenicity assessment of *A. cucumerina* isolates

The findings from this experiment indicated distinct variation in disease severity among the 44 *A. cucumerina* isolates on zucchini plants cultivated under greenhouse conditions. Disease severity rates ranged from 16 % to 80.5 % for the treatments and 0 % for the control treatment, which did not contain the pathogenic fungus (Table 2). With a disease severity rate of 80.5 % after treatment, isolate Eac-12 outperformed the other isolates. This superiority implies that the pathogenicity and disease-causing potential of fungal isolates vary significantly. Some studies have reported that *Alternaria* species exhibit significant variability in pathogenicity depending on the isolate and environmental conditions. The pathogenicity of this isolate may be attributed to its distinct physiological and molecular features, including the

Table 1. Fungal isolates associated with the diseased plants

Fungi/ governorates	Appearance (%)			Frequency (%)		
	Erbil	Diyala	Salah Al-Din	Erbil	Diyala	Salah Al-Din
Aspergillus flavus Link ex Gray	20.0	0.0	12.5	16.6	0.0	4.2
Aspergillus niger Van Tieghem	21.1	8.6	11.0	13.8	4.0	3.7
Aspergillus spp.	15.5	13.0	7.0	10.4	8.5	3.0
Alternaria alternata (Fr.) Keissl. 1912	14.0	20.0	10.0	8.5	19.7	5.4
A. cucumerina (Ellis & Everh.) J.A.	70.7	65.8	74.2	51.0	41.5	55.6
Alternaria spp.	0.0	0.0	8.0	0.0	0.0	3.8
Dreshslera halodes	0.0	14.4	10.0	0.0	07.0	4.0
Rhizopus spp.	12.0	8.0	16.0	7.7	2.5	8.6

Table 2. Pathogenicity testing of *A. cucumerina* isolates

Isolate code	Disease severity %	Isolate code	Disease severity %	Isolate code	Disease severity %
Control	0.0	Dac-02	30.8	Dac-17	27.5
Eac-01	21.8	Dac-03	35.5	Sac-01	40.0
Eac-02	26.0	Dac-04	19.0	Sac-02	35.0
Eac-03	39.0	Dac-05	29.0	Sac-03	33.0
Eac-04	28.8	Dac-06	34.3	Sac-04	64.5
Eac-05	55.3	Dac-07	54.5	Sac-05	56.5
Eac-06	52.3	Dac-08	63.5	Sac-06	46.3
Eac-07	59.0	Dac-09	66.0	Sac-07	21.8
Eac-08	16.0	Dac-10	59.0	Sac-08	29.3
Eac-09	29.5	Dac-11	41.0	Sac-09	42.5
Eac-10	69.5	Dac-12	51.3	Sac-10	46.8
Eac-11	31.5	Dac-13	43.8	Sac-11	33.3
Eac-12	80.5	Dac-14	54.0	Sac-12	24.5
Eac-13	39.0	Dac-15	47.3	Sac-13	34.0
Dac-01	25.8	Dac-16	32.5	Sac-14	40.5
LSD (0.05) = 2.5					

Fig. 1. Symptoms of leaf blight disease on affected zucchini leaves.

Fig. 2. Conidia spores of *A. cucumerina* under a light microscope.

generation of host-selective toxins and the release of cell wall-degrading enzymes such as pectinase and cellulase. These attributes significantly improve its ability to penetrate host tissues and spread disease (24). One important factor affecting the pathogenicity of *Alternaria* is the production of specific toxins. Moreover, the enhanced isolate could demonstrate an increased capacity to generate conidia with higher viability and faster germination rates, thereby improving its effectiveness in initiating primary infections and facilitating disease dissemination within the greenhouse (25). This result is consistent with previous studies that emphasized the critical role of toxins and fungal components in determining the severity of different *Alternaria* species (26). Regarding the relationship between isolate specificity and host compatibility, the high severity of infection may be explained in part by the genetic compatibility between this isolate and the zucchini variety used (27).

Antagonism assay *in vitro*

The findings indicated that NO, at a concentration of 0.66 mg/L, effectively inhibited the growth of the pathogenic isolate (Eac-12) by 100 % compared with the control treatment, in which the fungus filled the dish after 7 days of incubation (Table 3). The findings also showed that higher concentrations of Cu were associated with an increase in the inhibition rate. The inhibition rate varied from 10 % to 100 % among various treatments, with the maximum inhibition rate attained at concentrations of 50 mg/L or higher. Fig. 3 shows that the Ab treatment had an inhibition rate of 51.5 %, whereas the Av treatment had an inhibition rate of 60.5 %.

Table 3. Impact of biocontrol agents on the proliferation of the pathogenic fungus *A. cucumerina*

Treatment	Inhibition (%)
Control (Eac-12 alone)	0.00
Ab × Eac-12	57.50
Av × Eac-12	64.66
NO (0.66 mg/L) × Eac-12	100.00
Cu (10 mg/L) × Eac-12	19.17
Cu (20 mg/L) × Eac-12	32.78
Cu (30 mg/L) × Eac-12	55.56
Cu (40 mg/L) × Eac-12	75.83
Cu (50 mg/L) × Eac-12	100.00
Cu (60 mg/L) × Eac-12	100.00
Cu (70 mg/L) × Eac-12	100.00
LSD (0.05)	3.75

Management of leaf blight disease in the greenhouse

All treatments achieved a significant difference in increasing the seed germination rate in the presence of the pathogenic fungus *A. cucumerina*, with values ranging from 70–100 % compared with the positive control treatment (fungus alone), which reached 60 %. However, the four-way interaction treatment consisting of Ab and Av, Cu at a concentration of 50 mg/L and NO at a concentration of 0.66 mg/L achieved the highest germination rate of 100 %, followed by the three-way interaction treatment, in which the germination rate ranged from 85–90 % (Table 4). The results showed that the four-way interaction treatment (Ab + Av + Cu + NO) significantly reduced disease incidence rate and severity to 0 %, compared with the positive control treatment, which reached 90 % and 84 % respectively. This was followed by

Table 4. Control of the pathogenic fungus *A. cucumerina* under greenhouse conditions

Treatment	Seed germination (%)	Disease incidence (%)	Disease severity (%)
Negative control (Plant alone)	100.0	0.0	0.0
Positive control (Eac-12 alone)	60.0	90.0	84.0
Ab (Alone)	100.0	0.0	0.0
Av (Alone)	100.0	0.0	0.0
Cu (50 mg/L alone)	100.0	0.0	0.0
NO (0.66 mg/L alone)	100.0	0.0	0.0
Eac-12 × Ab	70.0	67.5	51.5
Eac-12 × Av	72.5	65.0	50.8
Eac-12 × Cu	75.0	55.0	48.3
Eac-12 × NO	72.5	65.0	54.8
Eac-12 × (Ab + Av)	80.0	50.0	43.5
Eac-12 × (Ab + Cu)	82.5	37.5	28.5
Eac-12 × (Ab + NO)	80.0	45.0	33.0
Eac-12 × (Av + Cu)	82.5	35.0	28.5
Eac-12 × (Av + NO)	77.5	40.0	31.8
Eac-12 × (Cu + NO)	82.5	35.0	28.5
Eac-12 × (Ab + Av + Cu)	90.0	30.0	25.5
Eac-12 × (Ab + Av + NO)	85.0	37.5	28.0
Eac-12 × (Ab + Cu + NO)	90.0	27.5	22.8
Eac-12 × (Av + Cu + NO)	90.0	25.0	21.0
Eac-12 × (Ab + Av + Cu + NO)	100.0	0.0	0.0
LSD (0.05)	4.5	8.0	12.0

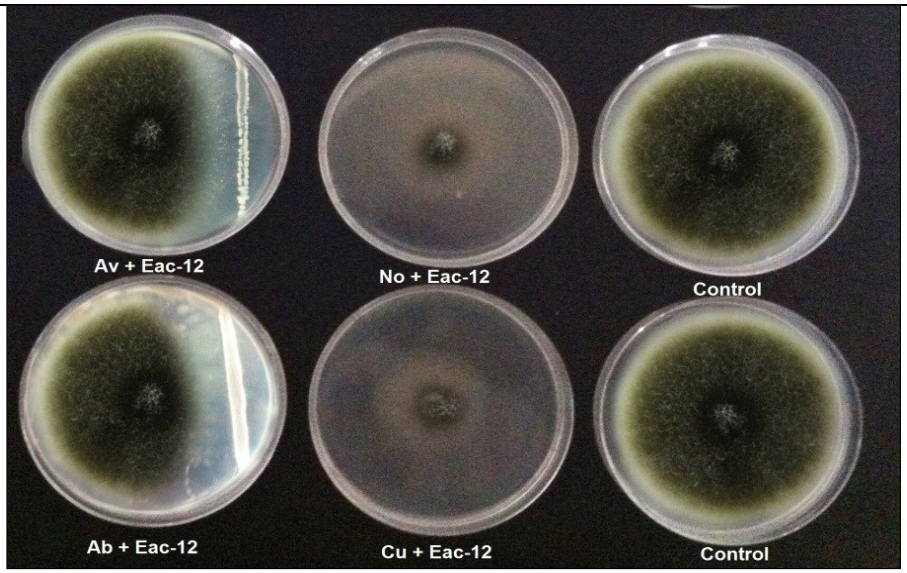

Fig. 3. Evaluation of the inhibitory efficacy of biological and chemical treatments against *A. cucumerina*.

the three-way interaction treatment, in which disease incidence and severity ranged between 25–30 % and 21–28 % respectively. This was followed by the second interaction treatment, which reached 35–50 % and 28.5–43.5 % respectively. However, none of the individual treatments achieved significant differences in reducing the disease incidence and severity. This result reflects the high efficiency of integrating biological agents, plant extracts and chemical compounds in the management of plant diseases. *A. vinelandii* is a highly efficient nitrogen-fixing bacterium that improves soil fertility and increases nitrogen availability to plants, in addition to secreting growth-stimulating compounds such as vitamins and plant hormones (28). These changes prime the plant's defense system via induced systemic resistance (ISR) and improve plant health. *A. brasilense* enhances root growth and ISR by producing auxins and cytokinins, helping the plant limit fungal development (29). Copper compounds directly inhibit fungal spore and mycelium germination (30). Meanwhile, active substances such as azadirachtin, found in NO, have been demonstrated to suppress fungal growth and reduce the severity of plant diseases (31). NO contains various bioactive compounds, including azadirachtin and limonoids, which likely contribute to its antifungal effectiveness (32). *Alternaria alternata*, *Candida* spp., *Epidermophyton* spp., *Geotricum* spp., *Thanatephorus cucumeris* and *Trichophyton* spp. are among the plant and human pathogenic fungi against which NO has been demonstrated to be effective (33). Additionally, it has demonstrated efficacy against *Klebsiella* species (34). Copper sulphate has also proven effective in directly inhibiting pathogenic fungi (30). In comparison with alternative treatments, the synergistic effect of these chemical and biological agents was demonstrated by a notable decrease in the incidence and severity of the disease. Combining chemical or natural agents with plant growth-promoting microbes can result in an efficient and long-lasting approach to integrated plant disease management (35).

Enzyme activity assessment

Table 5 indicates a notable increase in PO enzyme levels in zucchini plants treated individually with bacterial isolates, achieving 16.5 and 18.3 min/g fresh weight for the Ab and Av treatments respectively. Treatments combining bacteria with Cu or NO exhibited a notable increase, ranging from 17.1 to 22.7 min/g fresh weight, compared with the negative control treatment, which recorded 7.2 min/g fresh weight. However, treatments with Cu and NO, whether administered individually or in combination,

Table 5. Assessment of defence enzyme activity in zucchini subjected to the pathogenic fungus *A. cucumerina*

Treatment/ day	Absorbance change (min/g fresh weight)					Average
	1	5	10	15	20	
Negative control (Plant alone)	3.1	5.7	8.2	9.7	9.1	7.2
Positive control (Eac-12 alone)	5.0	7.5	9.2	11.3	10.5	8.7
Ab (Alone)	10.2	15.4	17.1	20.4	19.5	16.5
Av (Alone)	11.5	16.0	19.4	22.5	22.0	18.3
Cu (50 mg/L alone)	3.2	5.9	8.0	10.2	9.5	7.4
NO (0.66 mg/L alone)	3.6	6.0	8.3	10.0	9.3	7.4
Eac-12 × Ab	13.7	16.5	19.4	22.6	20.3	18.5
Eac-12 × Av	14.8	18.8	22.5	24.8	21.7	20.5
Eac-12 × Cu	5.5	7.8	10.6	11.1	10.8	9.2
Eac-12 × NO	6.2	8.1	10.9	11.8	11.0	9.6
Eac-12 × (Ab + Av)	15.0	21.6	24.6	27.4	25.0	22.7
Eac-12 × (Ab + Cu)	10.9	16.2	17.9	21.0	19.9	17.2
Eac-12 × (Ab + NO)	11.0	15.8	17.9	21.1	19.8	17.1
Eac-12 × (Av + Cu)	11.9	17.0	20.2	22.8	20.5	18.5
Eac-12 × (Av + NO)	12.2	16.7	20.3	23.4	21.3	18.8
Eac-12 × (Cu + NO)	3.8	6.5	9.2	10.5	9.0	7.8
Eac-12 × (Ab + Av + Cu)	15.3	21.5	25.2	28.0	25.7	23.1
Eac-12 × (Ab + Av + NO)	15.9	22.0	24.9	27.3	26.4	23.3
Eac-12 × (Ab + Cu + NO)	14.2	16.9	20.3	22.9	19.9	18.8
Eac-12 × (Av + Cu + NO)	15.5	19.7	22.4	25.5	22.3	19.1
Eac-12 × (Ab + Av + Cu + NO)	15.9	21.5	25.8	28.7	26.4	23.7
LSD (0.05)	Between days = 1.3, between transactions = 2.1, overlap between days and transactions = 2.8					

did not demonstrate any significant difference compared with the negative control treatment. The triple combination treatment, comprising two bacterial isolates with Cu or NO, resulted in the highest PO enzyme levels, attaining 32.1 and 32.3 min/g fresh weight and did not differ significantly from the quadruple combination treatment, which reached a maximum of 23.7 min/g fresh weight. These outcomes show how well rhizobacterial treatments work to increase the production of vital defence enzymes in zucchini plants. The significant increase in PO activity observed in treatments contaminated by pathogens implies that these bacteria may cause plants to become more prepared, enabling a more successful defence against pathogen attacks. The antioxidant enzymes PO, phenylalanine ammonia lyase (PAL) and polyphenol oxidase (PPO) in plants have been reported to be strongly correlated with disease suppression, suggesting that these enzymes may act as elicitors of ISR (36). Strains of fluorescent pseudomonads have been shown to improve the resistance of cotton plants to blight disease and markedly increase the activities of PO and PAL (37).

Conclusion

The study findings indicated that the combination of *A. vinelandii* and *A. brasilense* with a copper compound and NO was the most efficacious in diminishing the incidence and severity of leaf blight caused by *A. cucumerina* on zucchini plants under greenhouse conditions. This advantage is attributed to the synergistic interaction between the biological mechanisms of PGBs, which enhance systemic resistance and promote plant development and the direct antifungal effects of the copper compound and NO. The results suggest that the integration of biological and chemical agents may offer an effective approach for the sustainable management of plant diseases, thereby reducing dependence on conventional chemical pesticides. Expanded field trials are recommended to validate the efficacy of this combination across various environmental and agricultural settings.

Acknowledgements

The author expresses gratitude to Mustansiriyah University, Iraq, for its support and contributions to scientific and technological advancement.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

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