Research Article

Biological and Boron-mediated Control and the Induction of Systemic Resistance in Cucumber against Root Rot Disease caused by *Pythium aphanidermatum*

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Abstract | Extensive use of synthetic fungicides against diseases of horticultural crops poses serious threat to environment and human health, necessitating searching for alternate biocontrol strategies. This research work aimed to determine the bioefficacy of microbial biocontrol agents, *i.e.*, *Bacillus paramycoides*, *Trichoderma virens*, and *T. harzianum*, and the boron element against cucumber root rot caused by the pathogenic fungus *Pythium aphanidermatum*. Pathogenicity bioassay results demonstrated the ability of all *Pythium* sp. isolates to cause significant plant infections, manifested by wilting and leaf yellowing. Isolate Py2 had the highest infection severity (69.00%), while isolate Fu3 (*Fusarium* sp.) exhibited the lowest pathogenicity (15.50%). Molecular analysis confirmed the presence of *P. aphanidermatum* isolated from the root system of the diseased cucumber plants. Phenotypic and molecular identification results of the biocontrol fungi isolated from the soil revealed the biocontrol fungus *T. virens*. Shade experiment exhibited that the microbial biocontrol treatments and boron (Boromin Gel) significantly inhibited the proliferation of *P. aphanidermatum* and increased the growth parameters of cucumber plants, particularly combined application of *T. virens*, *B. paramycoides*, and boron resulted in maximum reduction in *P. aphanidermatum* infection severity (6.25%) as compared to control treatment with 75.00% infestation. Moreover, all treatments, whether applied individually or in combination, resulted in a significant increase in cucumber plant growth characteristics, represented by the fresh and dry weight and length of shoots and roots, as compared to the control treatment. In addition, binary application of microbial agents and boron also exhibited inducing systemic resistance in cucumber plants with maximum peroxidase (POD) and polyphenol oxidase (PPO) levels up to 14th day post-treatment. Overall study results demonstrate significant potential of all fungal and bacterial biocontrol agents and boron supplementation against cucumber root rot by *P. aphanidermatum*.

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Introduction

Cucumber (*Cucumis sativus* L.) belongs to the family Cucurbitaceae, and is one of the major summer vegetables, that is being widely cultivated in tropical and subtropical parts of the world including Iraq (Rahim *et al.*, 2023). Each 100 g of fresh cucumber fruit contains approximately 96 g of

water, 1 g of protein, 3 g of carbohydrates, 1 mg of phosphorus, 0.03 mg of iron, 1 g of vitamins B, 0.20 mg of niacin, 0.04 mg of vitamin B2, 8 mg of ascorbic acid, and 12 calories (Chakraborty and Rayalu, 2021).

Cucumber plants are susceptible to various phytopathogens, particularly to the fungi belonging to the genera *Rhizoctonia*, *Pythium,* and *Fusarium*. Cucumber root rot is one of the most drastic diseases of cucumber caused by *Pythium aphanidermatum* (Al-Mahmooli *et al*., 2024). The disease symptoms appear depending upon the prevailing environmental situations and the plant age at the time of pathogenic infection. Symptoms begin with rotting of the stem base and root scales on mature plants, followed by yellowing of the vegetative system after the root system is destroyed. Wilting signs appear during times of high daytime temperatures. They also infect plants at various stages of growth and eventually cause plant death (Postma *et al*., 2000; El-Sheekh *et al*., 2021).

The extensive use of hazardous and persistent synthetic pesticides on fruits and vegetables poses many ecological and health issues (Majeed *et al*., 2025; Zhou *et al*., 2025). This necessitates looking for alternative disease control strategies that would be more biorational and environment-friendly, such as microbial biocontrol agents, including *Bacillus paramycoides*, *Trichoderma virens*, and *T. harzianum* (Hassan *et al*., 2021; Zhou *et al*., 2021). Microbial biocontrol agents are the microorganisms isolated from the soil and are often used as biopesticides and fertilizers to sustain, enhance, and protect crop production. Bacterial species from the genus *Bacillus* are usually effective against different pathogenic microorganisms (Albayrak, 2020). It has received significant attention from researchers in many countries, where it has been introduced as a key component of biological control programs to control many pathogens affecting various crops (Shoaei *et al*., 2012; Serrão *et al*., 2024).

Moreover, the role of beneficial microorganisms in increasing soil fertility and enhancing plant characteristics, which contributes to increased organic agricultural production, is well known. This is particularly true for the fungus *Trichoderma* spp., which is considered a resistance factor against many pathogens (Narayanasamy, 2013; Poveda, 2021). This biocontrol fungus can boost resistance in plants against different phytopathogens (Kipngeno, 2015; Aslam *et al*., 2023). In addition, it exhibits a promising ability to ameliorate the plant growth characteristics, such as weight of the root and vegetative systems, dry and fresh, branching, and yield (Syed *et al*., 2020; Abdullah *et al*., 2021).

Nevertheless, research interest has increased in studying the relationship between nutrients and pathogens in order to induce resistance mechanisms against phytopathogens such as different fungal, bacterial, and viral diseases (Tripathi *et al*., 2022; Jeevanraj *et al*., 2025). This is due to the nutrients' key role in enhancing phyto-resistance against these pathogenic infections, which positively impacts yield increases, improves quality, and protects the environment (Anwar *et al*., 2023).

Keeping in view the afore-mentioned information, this research work aimed to assess the biological potential of *T. virens, T. harzianum*, and *B. paramycoides* applied alone and in interaction with the boron in inhibiting the *P. aphanidermatum*, and to determine the role of these treatments in enhancing the antioxidant enzymes, *i.e.,* peroxidase and polyphenol oxidase, at different post-inoculation time intervals.

Materials and Methods

Isolation and identification of pathogenic fungi from cucumber roots

Isolation was performed from the wilted cucumber seedlings taken randomly from different greenhouses of Babylon Province. The plants that showed clear wilt symptoms were uprooted along with their intact root systems. The samples were put in sterilized zip-lock polyethylene envelopes and were brought to the laboratory for isolation and identification. Disease symptoms included watery, infected root tissues with soft consistency, which led to the easy detachment of the outer root cortex with minimal mechanical pressure.

The seedlings were rinsed first with tap water, then with sterile distilled water, and then were drained on sterile filter paper to remove the excess water. Pieces of infected seedlings were taken from the crown area, and a portion of the seedling petiole (1-2 cm long) using a sterile knife and forceps, and these pieces were lined in sterilized Petri-plates (@ five pieces per plate). The plates were arranged in an incubator at 25 ± 2°C. Three days later, these plates were inspected for the fungal

colonies growing from the infected seedling parts and these colonies were purified on PDA medium. They were then examined under a light microscope at 40X. These fungal species were identified based on their morphological characteristics, colony morphology, mycelial type, sporophyte shape and structure, spore shape, and other structures as mentioned in Domsch and Gams (1980). The identified and purified isolates were stored in a refrigerator at 4°C on a slanted PDA medium in test tubes for further studies.

Isolation of microbial biocontrol agents from soil

Soil samples were collected randomly from the rhizospheric soil around the healthy cucumber plants from different fields located in Babylon Province, Iraq. These soil samples were air-dried for 24 h at room temperature (26°C), after which they were sieved through a sieve (with 1 mm mesh size). Then, the serial dilutions (10^{-1} and 10^{-5}) were prepared from these soil samples. One milliliter of the 5th serial dilution was transferred to a sterile Petri-plate (with 9 cm diameter) containing sterile PDA medium having tetracycline (antibiotic) @ 250 mg/L. Such plates were prepared in quadruplicate. The plates were set on slow rotation for the even distribution of the diluted soil suspension. These Petri-plates were put for 4 days in an incubator at 25 ± 1°C.

Purification of the isolate fungal strains was done by transferring some portions of the fungal colony from the edges to sterile PDA medium in sterilized Petri-plates with a sterile inoculation loop. These plates were placed for 5 days in an incubator at 25 ± 1°C (Santoyo *et al*., 2024). *Trichoderma* isolates were recognized with the help of authentic taxonomic keys (Rifai, 1969). Furthermore, their antagonistic ability was assessed following the scale of Bell *et al*. (1982).

Score = Characteristics

1 = Antagonistic fungus spread over the entire surface of the Petri-plate, preventing the pathogenic fungus from growing.

2 = Antagonistic fungus covers two-thirds of the surface of the Petri-plate, and the pathogenic fungus covers the remaining third.

3 = Antagonistic fungus covers half of the surface of the Petri-plate, and the pathogenic fungus covers the other half.

4 = Antagonistic fungus spreads over 1/3rd of the Petri-plate, while the pathogenic fungal strain spreads over the remaining 2/3rd of the Petri-plate.

5 = Pathogenic fungus spreads over the entire Petri-plate.

If a biological agent exhibits an antagonism score of 2 or less against the pathogenic fungal isolate under study, it is considered antagonistically effective (Picardal, 2019).

Phenotypic identification of pathogenic fungal isolates

The isolated fungi were identified from the root group to the genus level based on the morphometric parameters of the fungal colonies, such as colony diameter, color, shape, and height, in addition to the pigments produced on the PDA culture medium. Microscopic characteristics were also relied upon, including the shape and structure of the spores, the nature of the fungal hyphae, the size of the conidia, and the color and other structures. The fungus was identified after identifying the physical structures of the asexual and sexual reproductive organs and the characteristics of the mycelium, based on the taxonomic key of Domsch and Gams (1980).

Identification of the trichoderma fungal isolates

The biocontrol fungal isolates were re-cultured for their morphological identification in sterilized Petri-plates lined with PDA. The first isolate, *T. harzianum*, was acquired from the Department of Bio-Control Techniques, Al-Mussaib Technical College, Al-Furat Al-Awsat Technical University, Babylon Province, Iraq, and had been previously identified. The second isolate *T. virens* was obtained by dilution from the soil medium of cucumber plants, which exhibited high and distinct plant growth. The Petri-plates were put in an incubator at 25 ± 2°C for five days. The identification of the fungus was performed according to the authentic taxonomic keys based on morphological characteristics, nature, and spore formation of the fungal culture. Afterwards, the fungal isolates were identified on a molecular basis.

Molecular identification of fungal isolates

DNA extraction

For molecular diagnostic purposes, DNA of the fungal isolates, *T. virens* and *P. aphanidermatum,* was extracted at the molecular biology facility of Wahj Al-Dana Molecular Research Company, Babylon, Iraq. ZR Fungal/Yeast/Bacterial DNA MiniPrep™ kit (Zymo Research, USA) was employed for this DNA extraction from the fungi grown in Petri-plates on PDA culture medium, following the company's

recommendations for standard DNA extraction from fungal isolates. The purity and concentration of the extracted DNA were determined using the Nanodrop® 2000 spectrophotometer based on optical absorbance.

PCR amplification

For this amplification by the PCR technique, ITS1 and ITS4 primers were used, which were synthesized by the Integrated DNA Technology® (Canada). Each PCR assay (iNtRON Biotechnology, Seoul, Korea) contained 25 µl reaction mixture having template DNA, primers, and other ingredients. The thermal protocol used for the DNA amplification in the thermocycler is described in Table 1. PCR amplicons were stained with ethidium bromide and were visualized under UV light on agarose gel using the electrophoresis technique.

Table 1: *Thermal protocol for PCR amplification of DNA fragments of Trichoderma virens and pythium aphanidermatum.*

Step No.	Phase	Temp. (°C)	Time	No. of cycle
1	Initial denaturation	94°C	5 min.	1 cycle
2	Denaturation -2	94°C	45sec	35 cycles
3	Annealing	52°C	45sec	
4	Extension-1	72°C	45sec	
5	Extension -2	72°C	7 min.	1 cycle

DNA sequencing

For molecular identification of the fungal species, the nucleotide sequences of PCR products of the fungal ITS4 and ITS1 genes were further sequenced using the Illumina HiSeq4000 system by Macrogen (Daejeon, Korea). Analysis of the sequences obtained from the Macrogen company was done by the National Center for Biotechnology Information (NCBI) website under the Blast subwindow, followed by the Nucleotide blast subwindow.

Preparation of T. virens and T. harzianum inoculum

For this preparation, water-soaked (6 h) clean millet seeds were placed on a cloth to remove the excessive water, and distributed into sterilized glass bottles, @ 50 g per 150 mL bottle. The bottles were sealed with cotton plugs. The seeds were treated in an autoclave at 15 psi and 121°C for 1 h, then cooled to room temperature (27 °C). The seeds were sterilized the next day for another 1 h (Dewan and Sivasithamparam, 1989). Five discs (with 0.5 cm diameter) were inoculated with *T. virens* and *T. harzianum*, respectively, from seven-day-old colonies. The seeds were incubated at 25°C, with the bottles shaken every 2-3 days to ensure even distribution of the fungus among the seeds and to avoid seed clumping with the mycelium.

Preparation of the P. aphanidermatum inoculum

The fungal inoculum was prepared using cucumber fruits as a natural medium for inoculum preparation. Ripe, soft, and wound-free fruits were taken and washed thoroughly with water. They were then sterilized externally with alcohol using sterile cotton. A 2-3 cm deep and 6 cm long incision was then made in the center of the fruit using a sharp, flame-sterilized knife. A portion of the 3-day-old of *P. aphanidermatum* colony was placed on the PDA culture medium using a flame-sterilized inoculum needle, inserted into the fruit through the wound. The fruits were packed in polyethylene sacks and were put in an incubator at 25 ± 2°C for 3 to 5 days.

After abundant fungal growth occurred on fruit surfaces, these fruits were chopped into small pieces and placed in an electric blender at a slow speed for five seconds to avoid affecting the viability of the pathogenic fungus. About 750 mL of sterile water was added to every 250 g of cucumber fruit, and the mixture was collected in 1-L glass beakers for later use as inoculum (Satour and Butler, 1987).

Preparation of plants for the pathogenicity test

This pathogenicity test was conducted at the Al-Mussaib Technical College of the Al-Furat Technical University. The experiment was conducted using plastic pots (with a 20 cm diameter), each filled with 2.5 kg of soil. Peat moss and soil, sterilized with 40% commercial formalin (@ 3 L m^{-3} soil), were mixed in pots in a 2:1 ratio. Unsterilized cucumber seeds, surface-sterilized with a sodium hypochlorite solution, were sown @ 25 seeds per pot. A fungal inoculum was added to each of the *P. aphanidermatum* isolates (Py1, Py2, Py3, and Py4) carried on cucumber fruits and the fungal isolates. *Fusarium* sp. isolates (Fu5, Fu6, and Fu7), *Verticillium* sp. (Ve8), *Alternaria* sp. (Al9), and *Macrophomina* sp. (Mo10) were inoculated onto local millet seeds in plastic pots with 1% (w/w) concentration.

Each treatment was repeated four times, with four replicates as control with no fungal inoculation.

These pots were carefully watered and severity of the infection was determined. The percentage of root infection severity was calculated according to the 5-point pathology index (Souza *et al*., 2010) and according to the following equation of McKinney (1923).

$$Infection\ (\%) = \frac{number\ of\ infected\ plants}{total\ number\ of\ plants\ examined} \times 100$$

Preparation of B. paramycoides suspension

The previously identified *B. paramycoides* isolate was acquired from the Pathological Laboratory of the Department of Biocontrol Techniques, Al-Mussaib Technical College of the Al-Furat Technical University. It was propagated for 15 min in an autoclave in 500 mL sterilized glass flasks on Nutrient Broth medium at 1.5 kg/cm^2 and 121°C. It was then inoculated with the desired bacteria with a sterile pad from the previously prepared 48 h-old bacterial growth on Nutrient Agar. The flask components were mixed well and incubated for 3 to 4 days at 32 ± 3°C in an incubator.

Biocontrol assay

This experiment was conducted at the shade site of the Al-Mussaib Technical College in Babylon Governorate on September 1, 2024. The experiment used plastic pots with capacity of 5 kg each. Mixed soil and peat moss were sterilized after mixing with 40% formalin (sterilized). The soil was then covered with a 100-micron thick polyethylene bag for seven days. Then, it was left for three days before use to allow formalin residues to evaporate. The seeds were distributed in plastic pots @ 4 kg/pot, and were planted with local cucumber seedlings, including a non-inoculated control treatment. Fertilization and irrigation were provided to seedlings as per need. The bioassay was carried out using a complete random design (CRD) with three replications per treatment. Plants were treated with the following treatments at the age of 5 weeks.

T. virens alone
T. harzianum alone
B. paramycoides alone
Boron alone
T. virens + *P. aphanidermatum*
T. harzianum + *P. aphanidermatum*
B. paramycoides + *P. aphanidermatum*
Boron + *P. aphanidermatum*
T. virens + *T. harzianum*
T. virens + *B. paramycoides*
T. virens + Boron
T. harzianum + *B. paramycoides*
T. harzianum + Boron
Boron + *B. paramycoides*
T. virens + *T. harzianum* + *P. aphanidermatum*
T. virens + *B. paramycoides* + *P. aphanidermatum*
T. virens + Boron + *P. aphanidermatum*
T. harzianum + *B. paramycoides* + *P. aphanidermatum*
T. harzianum + Boron + *P. aphanidermatum*
Boron + *B. paramycoides*
P. aphanidermatum alone
Plant + pesticide (metalaxyl)
Plant alone

The inoculum of the pathogenic fungus *P. aphanidermatum* was prepared using cucumber fruits as a natural propagation medium, as described in the previous section. Addition of the fungal inoculum was done to the pots @ 400 mL per pot, and the soil was watered and maintained at an appropriate humidity level to ensure the viability of the pathogenic fungus (Shukla *et al*., 2022). The inoculum was prepared one week after inoculation with the biological control agents. Meanwhile, the inoculum of the biocontrol fungi, *T. virens* and *T. harzianum*, was applied to the local millet seeds for all treatments, @ 2 g per kg (Nawrocka and Malolepsza, 2013).

B. paramycoides suspension was applied to soil @ 25 mL per pot, with a concentration of 6.8 × 10^7 colony-forming units/mL (CFU/mL). One day after the addition of the pathogenic fungi, the boron was applied to the soil @ 2.5 mL L^{-1}, while the commercial pesticide metalaxyl (25% EC) was applied @ 1 mL L^{-1} (De Meyer *et al*., 1998). The results were calculated 40 days after the addition of the pathogenic fungi by estimating the severity and incidence of cucumber root rot inflicted by the pathogenic fungus *P. aphanidermatum*, according to the previously mentioned scale. The remaining plant parameters were calculated, including the fresh and dry weight and length of the shoots and roots of the cucumber plant.

Determination of the induction of antioxidant enzymes in cucumber plants

Samples were taken from the stem and leaf parts of healthy plants treated with biocontrol agents to estimate the levels of some systemic induction

compounds in these plants, namely the polyphenol oxidase (PPO) and enzymes peroxidase (POD). This was done to determine the degree of influence of these biocontrol agents on plant parameters and to compare them with the control treatment containing only the pathogenic fungus.

Table 2: *Isolation and identification of fungi associated with the cucumber (Cucumis sativus) roots.*

No.	Fungi	Frequency %
1	*Pythium* sp..	67.13
2	*Fusarium* sp..	12.10
3	*Verticillium* sp..	4.16
4	*Alternaria* sp..	4.12
5	*Macrophomina* sp..	1.22

Estimation of peroxidase and polyphenol oxidase activities
Enzymatic activity was determined at the Al-Mussaib Technical College, Pathology Laboratory for Postgraduate Studies, according to the method described by Sekmen and Turkan (2010). The activity of PPO was determined by the spectrophotometric method based on the initial rate of increase in absorbance at 420 nm. Estimation was performed using the previously described method of Simões (2015). PPO was estimated as international units /g fresh weight (μg. g^{-1}fwt).

Results and Discussion

Isolation and identification of fungi associated with cucumber plants
Cucumber plants that showed wilt symptoms were found associated with the presence of five fungal genera: *Pythium* sp., *Verticillium* sp., *Fusarium Macrophomina*, and *Alternaria* sp. (Table 2). *Pythium* sp. showed the highest replication rate among the isolated fungal isolates, with a rate of 67.136%, followed by *Fusarium* sp., which reached a replication rate of 12.10%. *Verticillium* sp. and *Alternaria* sp. with rates of 4.16 and 4.12%, respectively. The *Macrophomina* sp. had the lowest replication rate, reaching 1.22%. This fungus possesses the capability of producing huge numbers of reproductive units, enhancing its ability to subsist in adverse environmental situations. The high replication rate of *Pythium* sp. may be attributed to the fact that when environmental conditions are unfavorable, the fungus produces thick-walled oospores for longer survival. These remain dormant within soil till their germination under favorable conditions is present (Karunasinghe *et al.*, 2025). Or the spores remain for a short period, and through the asexual phase, the sporangium is formed, which either can germinate directly or indirectly by forming thin-walled, water-borne, floating spores (Malloch and Blackwell, 1992). This is considered the main method of dispersal of these spores, as a result of the secretion of substances by these hairs that act as chemotactic stimulants, which attract the fungus towards them. These spores then encyst, germinate, and cause infection by attacking sensitive young seedlings and newly formed root tissues through direct penetration (Nishat *et al.*, 2022).

Pathogenicity of fungal isolates on cucumber seedlings
Plastic pot experiment regarding the pathogenicity assessment of fungal isolates against cucumber seedlings showed that all of these isolates were capable of infecting cucumber seedlings, with infection rates ranging from 22-91% and 15.50-69.00%, respectively (Table 3). These treatments differed significantly from the control treatment, which had an infection rate and severity of 0.0%. The isolate (Py2) of the *Pythium sp*. parasitoid showed significant pathogenicity, significantly outperforming the other isolates, recording a high infection rate and severity of 91-69.00%, respectively. Meanwhile, the isolate Fu3 had the lowest pathogenicity, with an infection rate and severity of 22-15.50% (Table 3).

Table 3: *Pathogenicity of fungal isolates on cucumber (Cucumis sativus)seedlings grown in plastic pots.*

No.	Fungi	Code	Infection ratio (%)	Infestation rate (%)
1	*Pythium* sp.	Py1	77*	67.25
2	*Pythium* sp.	Py2	91	69.00
3	*Pythium* sp.	Py3	83	67.50
4	*Pythium* sp.	Py4	67	54.00
5	Fusarium oxysporum	Fu1	57	52.00
6	*Verticillium* sp.	Ve	47	27.50
7	*Alternaria* sp.	Al	53	34.50
8	*Macrophomina* sp.	Ma	40	24.00
9	*Fusarium* sp.	Fu2	52	32.25
10	*Fusarium* sp.	Fu3	22	15.50
11	Control		0	0
LSD 0.05			17.00	7.52

**each value in the table represents the average of four replicates.*

Other isolates varied in their pathogenicity on

Table 4: *The accession numbers of the fungal isolates deposited to the GenBank and their matching isolates.*

Fungi name	Accession number	Matching percentage	Country	International code
Pythium aphanidermatum	PV579842	91.57	Oman	MT51040 0.1
Trichoderma virens	PV579841	99.72	China	MK8705 72.1

Table 5: *Efficacy of Trichoderma virens, T. harzianum, Bacillus paramycoides and boron on the infestation of pathogenic fungus Pythium aphanidermatum and on some growth parameters of cucumber plants under greenhouse conditions.*

Treatment	infestation rate %	Length of pant (cm)	Weight of plant (g)		Length of root (cm) fresh	Weight of plant (g)	
			Fresh	Dry		fresh	dry
T1	0.00	120.37	145.63	40.98	16.37	14.50	4.50
T2	0.00	119.23	144.13	35.46	14.17	13.97	3.97
Ba	0.00	116.04	143.50	34.04	13.45	13.38	3.38
Bo	0.00	113.50	142.23	33.15	12.23	11.99	2.37
T1+Py	12.50	113.03	134.83	32.05	10.53	12.37	2.14
T2+Py	12.50	111.37	127.97	28.32	9.23	11.67	1.99
Ba+Py	12.50	107.47	123.63	24.88	9.17	10.72	1.38
Bo+Py	27.78	105.73	120.74	18.48	8.33	8.78	1.22
T1+T2	0.00	156.70	227.33	98.56	24.78	24.17	13.05
T1+Ba	0.00	139.23	214.44	79.32	22.53	21.17	10.17
T1+Bo	0.00	131.83	175.73	65.59	20.08	19.47	9.13
T2+Ba	0.00	133.57	185.67	70.92	21.53	20.17	9.83
T2+Bo	0.00	131.77	171.03	61.00	18.96	18.38	8.05
Ba+Bo	0.00	130.10	168.77	58.44	18.12	17.83	7.38
T1+T2+Py	6.25	127.63	166.93	56.91	16.45	16.44	6.11
T1+Ba+Py	6.25	125.27	164.77	54.79	15.37	15.33	4.34
T1+Bo+Py	8.33	122.80	158.03	47.64	13.45	13.72	3.38
T2+Ba+Py	6.25	123.20	163.63	53.58	14.17	14.18	3.85
T2+Bo+Py	8.33	121.20	153.23	43.51	12.23	12.63	2.63
Ba+Bo+Py	8.33	119.30	153.50	43.07	10.88	11.72	2.00
Pathogen	75.00	57.43	63.47	10.52	1.05	3.86	0.92
Plant + pesticide	12.5	95.60	109.77	13.48	6.87	9.52	1.72
Plant only	0.00	103.53	117.40	17.86	8.04	10.92	1.55
LSD 0.05	11.90	21.79	17.082	14.709	1.63	2.2023	2.7061

*T1 = T . viren, T2 = T. harzianum, Ba = B. paramycoides, Bo = Boron, Py = P. aphanidermatum; *each number presents average of three independent replicates.*

cucumber seedlings. The effect of the fungus *Pythium* sp. on the incidence and severity of cucumber seedling infection may be attributed to its high pathogenicity and parasitic nature. This is in agreement with Sopher (2012), who found that the pathogenic fungus *P. aphanidermatum* is a major cause of damping-off of seedlings, their wilting, and stem and root rot in many economic plants, particularly vegetables, as well as fruit rot, leading to fruit spoilage (Utkhede and Koch, 1999). Jenkins and Averre (1983) reported that the pathogenicity of the fungus is rapid, with severe root and stem rot occurring within 3–9 days after inoculation, followed by wilting and plant death. Similarly, Harvey (2002) explained that the fungus is fast-growing and highly pathogenic, killing plants at the seedling stage. Surviving seedlings have damaged root systems, resulting in reduced yield and poor growth. It was also found to cause root necrosis in general, inhibit root elongation and plant growth, and ultimately cause seedling death (Wulff *et al.*, 1998; Elazzazy *et al.*, 2012). Therefore, the isolate *Pythium* sp. (Py2) was chosen for the subsequent experiments.

Phenotypic identification of P. aphanidermatum

P. aphanidermatum is characterized by its coenocytic, translucent, branched, and rapidly growing hyphae on

potato dextrose agar. It grows rapidly, has a spherical or oval appearance, is unspecialized, and contains thick-walled, spherical sexual organs (oospores). This is consistent with the findings of Agrios (2005) and Karunasinghe *et al*. (2025).

Phenotypic identification of T. virens

It is characterized by its translucent hyphae that proliferate swiftly on PDA medium. Colonies are initially white and then turn green to dark green due to spore production. Colonies have a cottony or powdery texture. Conidia may appear unicellular, spherical to oval, and green upon maturity. They form on short branches, and conidiophores typically branch in a tree-like pattern. This is consistent with the findings of Pacheco-Trejo *et al*. (2022).

Molecular identification of the fungal isolates

The results of Table 4 show that the isolate of the pathogenic *P. aphanidermatum* was registered under accession number PV579842. This isolate matched the isolate from Oman registered under accession number MT510400.1, with a 91.57% match rate. The *T. virens* isolate was registered under accession code PV579841, and it matched the Chinese isolate registered under accession code MK870572.1 with a 99.72% match rate.

Efficacy of biocontrol agents against P. aphanidermatum and on growth parameters of cucumber plants under greenhouse conditions

The results showed that the biocontrol agents, including the fungi *T. virens* and *T. harzianum*, the bacteria *B. paramycoides*, and boron application, suppressed the infection severity of cucumber root rot caused by *P. aphanidermatum* as compared to the control treatment (plant alone) (Table 5). The binary treatments of *T. virens* + *T. harzianum*, *B. paramycoides* + *T. virens*, and *B. paramycoides* + *T. harzianum,* along with *P. aphanidermatum,* outperformed all other treatments. The infection severity reached 6.25% for all treatments above, followed by the treatment *B. paramycoides* + *T. harzianum* and *B. paramycoides* + boron, which achieved a significant reduction in the percentage of pathogenic fungal infection severity, reaching 8.33%, respectively, as compared to the fungus alone, which showed 75.00% infection.

Significant reduction of pathogenic fungal infection by binary treatments is due to the reason that each biocontrol agent may employ different mechanisms to combat the pathogen. The combined mode of action of both biocontrol agents results in greater suppression of the pathogenic fungus than if the biocontrol agent were used alone (Domenech *et al*., 2006; Anwar *et al*., 2023). This result is in agreement with previous studies, which have shown that binary treatments more effectively reduced the severity of phytopathogenic infection than if a biocontrol agent were used alone (Latha *et al*., 2009; Nehra *et al*., 2022). The single treatments of *T. virens*, *T. harzianum*, and *B. paramycoides* against the *P. aphanidermatum.*

Moreover, the results exhibited a significant reduction in the severity of the infection for all treatments, reaching up to 12.50% as compared to the control treatment. This would be because of the fact that the biological control agent *T. virens* is a highly antagonistic fungus capable of environmentally competing against pathogenic fungi when colonizing sites (Sreenivasaprasad and Manibhushanrao, 1990; Howell, 2002). The reduction in infection rate with the biocontrol *T. harzianum* is also due to its ability to secrete the antibiotics gliotoxin, alamethicins, and viridol, which inhibit the proliferation of *P. aphanidermatum*. Furthermore, its ability to parasitize and directly compete with the phytopathogenic fungus may contribute to the suppression of infection rate (Yassin *et al*., 2022; Lyousfi *et al*., 2023).

The reason for reducing the severity of infection when treated with *B. paramycoides* is that the species of the genus *Bacillus* spp. are efficient agent for biological resistance against many plant pathogens, including fungi that are highly sensitive to these bacteria, due to their production of many anti-compounds that affect the growth of microorganisms that cause harm to plants. Thus, they are highly efficient in inhibiting the metabolic activities of competitive organisms, including fungi that inhabit the soil. They are also characterized by their stimulation of the growth and enhancement of the productivity of plants both quantitatively and qualitatively (Kinsella *et al*., 2009; Sansinenea, 2019; Patani *et al*., 2024).

Combing boron with *P. aphanidermatum* showed a significant reduction of infection severity (27.78%) as compared to the control treatment (75.00%). This is because boron may participate in the formation of bonds between pectin molecules in the plant cell wall, increasing its rigidity and reducing the penetration of plant pathogens (O'Neill, 2004). Boron plays a role

in improving calcium distribution within plant cells, which helps regulate hormonal signals associated with defense responses such as jasmonic and salicylic acids (Cakmak and Römheld, 1997; Shireen *et al*., 2018).

Moreover, results demonstrated that the biological control agents, *i.e., B. paramycoides*, *T. virens*, *T. harzianum,* and boron significantly increased the growth parameters of cucumber plants as compared to the pathogenic fungus alone. The binary treatment *T. virens* + *T. harzianum*, along with *P. aphanidermatum,* produced better results regarding the growth characteristics of cucumber plants, with the fresh and dry weight and length of the shoots and roots reaching up to 127.63, 16.45 cm, 166.93, 56.91, 16.44, and 6.11 g, respectively. The second significant treatment was the binary treatment *T. virens* + *B. paramycoides* along with *P. aphanidermatum*. This treatment exhibited better results regarding the growth characteristics of cucumber plants, with the fresh and dry weight and length of the shoots and roots reaching up to 125.27, 15.37 cm, 164.77, 54.79, 15.33, and 4.34 g, respectively, as compared to the control treatment, which were 57.43, 1.05 cm, 63.47, 10.52, 3.86, and 0.92.

Many previous studies have shown that the use of different microbial biocontrol agents together increased the plant yield as compared to their separate application (El-Saadony *et al*., 2022). This increase in cucumber growth characteristics by binary treatments would be due to the synergistic impact of the mode of action of the fungi and bacteria used in the experiment. The biotic factors were administered alone including the presence of *P. aphanidermatum*, the *T. virens* treatment yielded the maximum values of all growth traits for cucumber plants as compared to the control treatment, with the fresh and dry weight and length of the shoots and roots of plants reaching up to 113.03, 10.53 cm, 134.83, 32.05, 12.37, and 2.14 g, respectively.

This is because the fact that this biocontrol fungus would have a crucial role in stimulating plant growth, improving vegetative and root growth indicators, and increasing production in terms of quality and quantity. It grows within the rhizosphere (plant roots) and interacts with them in a non-parasitic manner, creating a healthy microbial environment around the roots that helps the plant grow better (Zhang *et al*., 2023). *T. virens* solubilizes phosphate and increases iron availability to the plant, which enhances growth and productivity, especially in nutrient-poor soil conditions (Mukherjee *et al*., 2022; Kabir *et al*., 2023).

Indeed, microbial biocontrol agents have excellent capability to safeguard cucumber plants from pathogenic fungal infections. This is due to their numerous chemical compounds known as antibiotics (Harman, 2000), and their remarkable ability to synthesize different enzymes that degrade the cellular walls of phytopathogenic fungi. Among their enzymes, protease is an important one which helps colonize within the soil nutrients (Tziros *et al*., 2007; Ayaz *et al*., 2023).

Furthermore, *B. paramycoides* treatment along with *P. aphanidermatum* resulted in a high efficiency in the growth characteristics of cucumber plants compared to the control treatment, showing the fresh and dry weights and length of the roots and shoots as 107.47, 9.17 cm, 123.63, 24.88, 10.72, and 1.38 g, respectively. This is attributed to the bacteria's competitive ability, as *B. paramycoides* strains compete with fungal pathogens for nutrients. They also limit their growth and colonization in the same environment and stimulate systemic resistance in plants. This enhances the defense mechanisms of plants against the fungal pathogens, helping plants resist fungal attacks more effectively (Chakraborty *et al*., 2022).

In addition, the fungicide metalaxyl also reduced the fungal infection in cucumber plants treated as compared to the treatment with *P. aphanidermatum* alone, which significantly impacted the growth characteristics of cucumber plants. These findings are in agreement with many previous research works that have confirmed the inhibitory action of metalaxyl against pathogenic fungi. Metalaxyl is particularly effective against oomycetes, such as *Phytophthora* spp. and *Pythium* spp., and is used for the treatment and prevention of root rots and wilt diseases (Vargas *et al*., 2022). This is consistent with what Wollgiehn *et al*. (1984) and Sierotzki *et al*. (2019) showed that metalaxyl inhibits the activity of oomycetes by disrupting RNA synthesis, thus hindering the vital processes essential for fungal growth and spread within the plant.

Effectiveness of biological factors in inducing antioxidant enzymes in cucumber plants

Results presented in Table 6 indicate a significant

effect of the biological treatments studied on the activity of POD enzyme in the shade experiment, which included the bacterium *B. paramycoides* and the two biogenic fungi *T. virens* and *T. harzinum*, applied either alone or in combination with each other, along with *P. aphanidermatum*. The enzyme was measured in the shade experiment at different time intervals, namely 7, 14, and 21 days after adding the biological control elements to the shade experiment. The *T. virens* + *T. harzianum* treatment outperformed in changing the level of the peroxidase (POD) enzyme, along with *P. aphanidermatum,* as compared to the other treatments under shade conditions. The highest POD reading observed at the 14th day reaching 4.83 µg g^{-1} fw, and then decreased on the 21st day to 3.79 µg g^{-1} fw, compared to the control treatment, which contained the pathogenic fungus alone.

Adding more than one biocontrol agent had an effect in increasing the concentration of the POD enzyme. This is attributed to the binary effect between the biocontrol agents used in the experiment against *P. aphanidermatum*. They worked synergistically to stimulate the systemic plant resistance against the pathogen, which results in an increase in the levels of the inducible enzymes. These results are consistent with Ibrahim (2024) found an increase in the POD enzyme after the 7th day after adding the pathogenic fungus, followed by a decrease in the reading on the 11th day after adding the pathogenic fungus. The *B. paramycoides* + *T. harzinum* treatment, in the presence of the pseudo-pathogenic fungus *P. aphanidermatum* and under shade conditions, yielded highly significant differences compared to the control treatment. Moreover, the boron treatment along with *P. aphanidermatum* also yielded highly significant differences. The change in absorption was 1.71 µg g^{-1} fw on the seventh day after adding the biocontrol. The highest reading was at 14th day, reaching 2.50 µg g^{-1} fw, and then decreased on the 21st day to 2.13 µg g^{-1} fw, as compared to the control treatment (Table 6).

The effectiveness of biological factors in changing the levels of antioxidant enzymes for the PPO enzyme in cucumber plants indicates the presence of highly significant differences for all biological resistance elements as compared to the control treatment of *P. aphanidermatum*. The *T. virens* + *T. harzinum* treatment, along with *P. aphanidermatum,* as compared to other treatments. The highest reading (39.95 µg g^{-1} fw) was recorded at the 14th day, which then decreased on the 21st day to 29.66 µg g^{-1} fw. This was followed by the *B. paramycoides* + *T. harzinum* treatment along with *P. aphanidermatum*. This is attributed to the fact that the systemic resistance stimulated by the presence of bacteria complements the action of antibiotics (Bakker *et al*., 2003). It was found that resistance induced by bacteria is accompanied by a systemic increase in the activity of enzymes or phytoalexins. This is consistent with the findings of Zhang *et al*. (2023), which indicated that bacteria stimulated cotton plants treated with them to produce phytoalexins, followed by treatment with the *T. virens* along with *P. aphanidermatum.*

Table 6: *Effect of Trichoderma virens, T. harzianum, Bacillus paramycoides and boron on antioxidant enzymes Peroxidase (POD) and polyphenol oxidase (PPO) in the cucumber plants under greenhouse conditions.*

Treatments	POD level			PPO level		
	7 days	14 days	21 days	7 days	14 days	21 days
T1	1.71	2.43	2.12	15.60	25.16	16.62
T2	1.55	2.37	2.09	14.78	23.73	15.52
Ba	1.40	2.33	1.96	12.74	22.59	13.57
Bo	1.32	2.20	1.80	11.83	18.38	12.41
T1+Py	1.99	2.57	2.26	20.57	32.94	21.32
T2+Py	1.88	2.53	2.24	19.61	31.63	20.22
Ba+Py	1.78	2.50	2.18	17.86	30.57	18.67
Bo+Py	1.71	2.50	2.13	16.71	29.67	17.49
T1+T2	2.26	3.93	2.81	25.80	34.60	26.91
T1+Ba	2.01	3.67	2.47	24.46	33.63	25.45
T1+Bo	1.81	3.07	2.34	21.26	31.67	22.15
T2+Ba	1.93	3.27	2.40	23.85	32.57	24.20
T2+Bo	1.74	3.40	2.29	20.87	30.73	21.76
Ba+Bo	1.67	3.43	2.30	20.50	30.25	21.10
T1+T2+Py	3.06	4.83	3.79	32.58	41.97	34.44
T1+Ba+Py	2.92	3.80	3.58	28.74	39.95	29.66
T1+Bo+Py	2.62	3.53	3.26	28.00	38.93	28.79
T2+Ba+Py	2.78	3.60	3.21	27.55	38.23	28.50
T2+Bo+Py	2.53	3.40	3.11	27.02	36.63	27.75
Ba+Bo+Py	2.42	2.87	2.27	26.65	34.71	27.05
Pathogen	1.62	2.20	2.05	13.58	16.75	14.37
Plant only	1.45	1.90	1.87	10.34	13.76	11.64

*T1 = T . viren, T2 = T. harzianum, Ba = B. paramycoides, Bo = Boron, Py = P. aphanidermatum; *each number presents average of three independent replicates.*

T. virens has a high ability of this fungus to stimulate the systemic induced resistance in plants, enhancing their ability to resist a wide range of pathogens, including fungi, bacteria, and viruses. This is achieved through the activation of hormonal signaling pathways, such as salicylic acid, jasmonic acid, and ethylene (Martínez-Medina *et al*., 2013; Gan *et al*., 2022). Similarly, *T. harzianum* helps to regulate the oxidative balance within plants by reducing the accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), and increasing the activity of antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD), thus enhancing plant resistance to environmental stresses and pathogens (Chen *et al*., 2019; Boamah *et al*., 2021; Nawrocka *et al*., 2022).

Conclusions and Recommendations

The study concludes that all treatments comprising microbial biocontrol agents and boron applied individually or in combination, exhibited a significant increase in cucumber plant growth parameters, represented by the average length, fresh and dry weight of shoot and root, as compared to the control treatment having plants inoculated with a semi-pathogenic fungus alone. Moreover, the results regarding the peroxidase (POD) and polyphenol oxidase (PPO) enzymes showed the superiority of the binary treatments with the biological resistance elements represented against the pathogenic fungus in inducing systemic resistance, through increasing the effectiveness of the antioxidant enzymes in the canopy as compared to the control treatment of the pathogenic fungus.

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Novelty Statement

Novelty and results of this research demonstrates significant potential of all fungal and bacterial biocontrol agents and boron supplementation against cucumber root rot by *P. aphanidermatum*.

Author's Contribution

M. Ghaith Ghayyib: Wrote the first draft and analysed the data
Kadhim Z.K. Al-Karaawi: Editted the draft, supervised the project.

Generative AI or AI assisted technology statement
The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest
The authors have no conflict of interest.

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