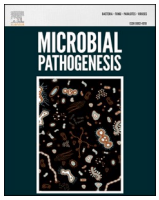
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Investigation of the effects of *Bacillus subtilis* and *Bacillus thuringiensis* as Bio-agents against powdery mildew (*Podosphaera xanthii*) disease in zucchini (*Cucurbita pepo* L.)

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ABSTRACT

Fungi, bacteria, and viruses cause highly devastating diseases in species of the Cucurbitaceae family. Powdery mildew, a fungal disease, is one of the most important diseases of cucurbits. The pathogen, *Podosphaera xanthii*, is the most common causal agent of powdery mildew disease within cucurbits. The aim of this study was to investigate the effectiveness of the combined formulations of two biological agents, *B. subtilis* and *B. thuringiensis*, in combating powdery mildew disease, which represents a significant threat to *C. pepo* cultivation in Kayseri, Türkiye. The efficacy of six different treatments in controlling the disease agent *P. xanthii* was evaluated in susceptible zucchini genotypes. It was found that full-dose bacteria dilution application, before and after powdery mildew infection, as well as three- and five-fold bacteria dilutions application significantly prevented (1–2 scale value) powdery mildew disease on infected zucchini plants than the control application. There was a decrease in vegetative growth in the control-treated crops while plant growth increased significantly in bacterial-treated crops. Also, our findings showed that combined formulations made from *Bacillus subtilis* (61.29e and 3.3a strains) and *Bacillus thuringiensis* (2B3-1 and 2B2-2 strains) significantly increased the synthesis of plant defense enzymes such as DPPH, antioxidant, proline production, total phenolic substance, and total flavonoid content. The application of B + PM resulted in the highest enzyme contents, quantified as follows: 22.91 mg AAE/g antioxidant, 2.01 mg/g KU proline, 10.03 mg GAE/g TPC, and 7.756 mg CE/g TFC. These enzymes may have played vital roles in triggering zucchini defense mechanisms, thereby significantly preventing powdery disease in the bacteria-treated crops.

1. Introduction

The family Cucurbitaceae consists of important vegetable species naturally distributed in the temperate, tropical, and subtropical regions of the Americas and widely grown throughout the world [1,2]. The family consists of 119 genera and 825 species cultivated worldwide [3], with pumpkins being the most cultivated species [1]. Among pumpkins, summer squash (*Cucurbita pepo* L.) and winter squash (*C.maxima* Duch. and *C.moschata* Poir.) are commercially produced in large quantities. According to 2021 data, 23.8 million tons of pumpkin were produced on 1.5 million hectares (ha) of land worldwide [4]. In Turkey, 683,000 tons of pumpkin were produced on 91,839 ha of land. In addition, 61,000 tons of pumpkin seeds were produced through the cultivation of *C. pepo* in an area of 78,000 ha in Turkey [5]. Turkey ranks sixth among the top pumpkin-producing countries in the world [4]. In 2022, pumpkins (summer squash, winter squash, and seed pumpkin) were grown on 36,035.3 ha of land in the Central Anatolia region of Kayseri, with seed pumpkin for snacks accounting for approximately 98 % of this area [5].

Cucurbit species have high water consumption and require irrigation for commercial production in arid and semi-arid regions. This leads to an increase in relative humidity in the cultivation areas, thereby creating favorable conditions for the development of fungal and bacterial diseases [6]. Pathogenic fungi, bacteria, and viruses cause diseases and biotic stresses that lead to significant yield and economic losses. Among these is a fungal disease called powdery mildew, which is one of the most harmful diseases of cucurbits [2]. In the *Cucurbitaceae* family,

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powdery mildew is caused by two main species; *Podosphaera xanthii* (Castagne) U. Braun and N. Shishkoff (syn. *Sphaerotheca fuliginea) and Golovinomyces cichoracearum* var. *cichoracearum* (D.C.) V.P. Heluta, syn. *Erysiphe cichoracearum* D.C [7,8]. *P. xanthii,* one of the two species responsible for powdery mildew disease, is commonly found in pumpkin growing areas and the reason for this is reported to be the high temperature and relative humidity in the cultivation areas [9]. The disease-causing fungus is known as a biotroph because it only feeds on living cells and cannot be cultured in artificial nutrient media [10]. The disease can cause a 100 % yield loss in young plants, especially in seedlings, and most often the plants die before they can reach the reproductive stage [6]. Powdery mildew disease generally affects the above-ground parts of the plant such as leaves, stems, and sometimes fruits, while no disease formation has been observed on the root and root collar [7]. Initial symptoms of the disease can be observed as circular white spots on both the lower and upper surfaces of infected leaves [2]. For a living organism to cause disease in plants; a suitable host, virulent pathogen, and appropriate environmental conditions must be present simultaneously. In the absence of even one of these three, powdery mildew does not occur in the plant [6]. Fungal disease agents are common on non-wet leaf surfaces, in warm and dry climates with low relative air humidity [6]. While *P.xanthii* is a common disease agent in many countries, such as France and Turkey, *Golovinomyces cichoracearum* is frequently found in temperate regions [11]. Dry climates aid the dispersal of spores, while humid climates encourage spore germination [6].

Generally, powdery mildew is not only spread by dry atmosphere and soil conditions. Moderate temperatures, low light intensity, fertile soils, and succulent plant tissues also promote its development [7]. Powdery mildew thrives better in shade than in full sunlight because the relative humidity is higher in shade than in illuminated areas. Powdery mildew spores are superficial and develop only on the surfaces of plant tissues' cells [6,2]. The mycelium and spores of the fungus are sensitive to environmental factors such as wind and heavy rain [12–14]. Sanitary and cultural practices for controlling powdery mildew diseases in cucurbits reduce the incidence of the disease [15], but these practices alone are not sufficient to completely eradicate the disease. For this reason, large amounts of commercially available synthetic chemicals are employed to combat powdery mildew pathogens. Despite the development of new fungicides to combat powdery mildew diseases, it remains a temporary solution as the pathogenic fungus can develop resistance to the chemical fungicides. In addition, chemical control of the disease is time-consuming and expensive, damages the ecological balance, and may lead to yield losses as it may cause some damage to the outer surface of fruits. Chemical control may also be insufficient due to the negative environmental impacts of the chemicals and the rapid development of resistance to these chemicals [16]. For these reasons, the use of resistant varieties in addition to chemical control is recommended as the best and relatively permanent solution in the control of the disease [17].

Plants, by their nature, have mechanisms to protect themselves from these stress factors that occur in their environment and negatively affect their normal growth and development. One of these mechanisms is the stress response mechanism created by the synthesis of molecules called secondary metabolites, which include groups such as terpenes, phenolic compounds, flavonoids, lignins, anthocyanins, and steroids. In addition to responding to pathogenic microorganisms with these compounds, they also defend the plant against different herbivores and various abiotic stress factors [18,19].

Secondary metabolites protect the plant against environmental stresses that occur around the plant by providing the plant with the ability to continue its vital activities, protection against insects and weeds with the chemicals they secrete, and competitive properties with allelopathy [20]. Phenolic compounds have a functional structure in the aromatic ring in their structure. They are phytomolecules containing OH- (hydroxyl) groups, which are responsible for the formation of color, taste and odor in plants in the defense mechanism of plants [21,22].

It is essential to use environmentally friendly alternative control methods in the control of powdery mildew diseases and to develop different methods for sustainable agriculture and the environment. In this study, the usability of *B. subtilis* and *B. thuringiensis* strains for the control of powdery mildew disease agent *P. xanthii,* which is a major problem in the pumpkin production areas of Kayseri province, was investigated, and it was determined that these bacteria, which do not cause any disease in zucchini, significantly prevent powdery mildew.

2. Material and method

2.1. Cultivation of plant materials and plants

A commercial squash (*Cucurbita pepo* L.) variety called Intfa was used in this study. Seeds were washed three times with sterile distilled water to remove coating materials. Three seeds were sown in each pot filled with peat and perlite in a 1:1 ratio. The experiment was carried out on September 6, 2022, in a greenhouse at the research field of the Faculty of Agriculture, Erciyes University, Kayseri, Turkey, in a randomized block design with three replications. Once plants had fully emerged (when the cotyledons on the leaves were parallel to the soil surface), they were thinned to one healthy plant per pot. For the duration of the experiment, an average temperature of 26 ± 2 °C was recorded in the greenhouse. Routine watering of the plants was done manually.

2.2. Activation of bacteria

A four-isolate mixture of *Bacillus subtilis* isolates (61.29e and 3.3a) and *Bacillus thuringiensis* isolates (2B3-1 and 2B2-2) was formulated. Bacteria strains were activated on Nutrient Agar (NA) by streak plate procedure, from −80 °C stock culture at the School of Natural and Applied Science, Erciyes University, Turkey. Bacteria were then grown in Nutrient Broth (NB) media at 20–30 °C and 150 rpm shaking speed for 24 h. Media used in the activation and growth of the bacteria were prepared as; 1) NA medium (pH 6.8 ± 0.2): prepared by suspending 20 g of solid medium in 1 L of distilled water and sterilized by autoclaving at 121 °C for 20 min, and 2) NB medium (pH 6.8 ± 2): prepared by dissolving 8 g of solid medium in 1 L of distilled water and autoclaving at 121 °C for 20 min [23].

Bacterial solutions were serially diluted at concentrations of 3×10^8, 3×10^5, and 3×10^3. Six different application strategies were used by spraying the solution on the leaves;

1. Application of five-fold diluted bacterial solution four days after powdery mildew inoculation (3×10^{-5}: $1/10^5(10^5)$
2. Application of three-fold diluted bacterial solution four days after powdery mildew inoculation (3×10^{-3}:$1/10^3(10^3)$
3. Application of full dose bacteria solution four days after powdery mildew inoculation (PM + B/3×10^8).
4. Nutrient medium application four days after powdery mildew inoculation (PM + MEDIA).
5. Application of a full dose of bacterial solution two days before powdery mildew inoculation (PM + B/3×10^8)
6. Control/powdery mildew infected plants only (C) (Powdery mildew spores reproduced on the susceptible zucchini plants were transmitted to the moistened leaves using a brush.).

The leaf surfaces of plants in the greenhouse were inoculated with *P. xanthii* spores on September 26, 2022. Powdery mildew spores were obtained from a pumpkin production area in Yeşilhisar district of Kayseri province. Due to the favorable environmental conditions in this region from spring to summer, powdery mildew diseases commonly occur and cause significant crop losses.

2.3. Morphological identification of powdery mildew agent

Whether the powdery mildew leaf spores collected from pumpkin fields were *Golovinomyces cichoracearum* or *Podosphaera xanthii* was determined through microscopic examination and measurements. The conidiospores of powdery mildew on zucchini leaves were evaluated morphologically in 3 % KOH under a microscope for the presence of fibrosis structures [24,25]. Observation of conidia in 3 % KOH indicated the presence of fibrosin bodies, which are commonly found in the cucurbit powdery mildew genus *Podosphaera*.

2.4. Molecular characterization of powdery mildew agent

Powdery mildew-infected leaf samples were collected from the pumpkin production field. Molecular characterization of powdery mildew agents was carried out on sample leaves taken from the pumpkin production area in Kayseri, in the Molecular Mycology Laboratory of the Department of Plant Protection, Faculty of Agriculture, Akdeniz University. Different staining techniques and Internal Transcribed Spacer (ITS) molecular techniques were applied to the samples as detailed in a previous study [9]. Both microscopic and molecular techniques were used to characterize powdery mildew agents on pumpkin samples. In brief, primer sets ITS1 (5′ TCCGTAGGSTGAACCTGCGG 3′)/ITS4 (5′TCCTCCGCTTATTGATATGC3′) and IT2 (5′ CCTCCGCTTATTGATATGCTTAGG 3′)/ITS5 (5′ CTTGGTCATTTAGAGGA 3′) specific to the conserved ribosomal subunit of powdery mildew fungi were used to amplify the target region by Polymerase Chain reaction (PCR). The DNA sequence, which was a 540 bp DNA fragment produced as a result of PCR, was analyzed with the Nucleotide BLAST program in the National Center for Biotechnology Information (NCBI) system.

2.5. Determination of powdery mildew prevention efficacy of bacterial solution

The prevention rates of powdery mildew disease of the above-mentioned bacterial applications were determined in zucchini plants infected with powdery mildew. The development of powdery mildew disease on leaves was evaluated 29 days after inoculation, on a scale of 0–4 as indicated in Table 1 [26]. Disease inoculation and evaluation were performed in triplicate and results are presented as means of replicates. Disease development on each plant was separately scored and their means were computed.

After evaluation according to the severity of the powdery mildew disease, plants were harvested on October 10, 2022, and they were separated into organs (shoot and root). Then the plant height (cm), root depth (cm), and fresh weights of roots and shoots (g) were determined. Also, plant materials were dried at 65 °C for 48 h and their dry weights (g/plant) were recorded.

Table 1
Disease reaction scale to score disease phenotypes of Cucurbit powdery mildew disease [26].

Disease reaction category	Diseased plant phenotype
0	No or very limited sporulation, fungus, and conidiospore are not visible to the naked eye and are usually seen with necrotic lesions (HR).
1	A small amount of powdery mildew on the leaf margin with low sporulation. Sometimes HR observed.
2	Moderate or delayed sporulation, 10–30 % of the leaf surface is covered with powdery mildew. HR is not observed
3	More than 30 % of the leaf surface is covered with powdery mildew with intense sporulation. There is no HR.
4	Almost the entire leaf surface is covered with powdery mildew spores.

*HR: Hypersensitive response.

2.6. Biochemical analysis of defense enzymes

2.6.1. Preparation of plant extracts

For bioactivity analyses, the method proposed by Martins et al. [27] was used with partial modifications. Thus, dried plant samples were crushed into powder form using liquid nitrogen. For the extraction of the samples, 0.2 g of green zucchini leaves were weighed and 10 ml of 70 % ethanol/water mixture was added. The mixture was then homogenized in a shaking incubator at 25 °C and 150 rpm overnight. Samples were then centrifuged at 5000 g for 5 min and the supernatant was transferred to clean Eppendorf tubes for further analysis.

2.6.2. Determination of proline content

The method proposed by Karabal et al. [28] was modified and applied to determine the amount of proline in dried samples obtained from the plants. For this purpose, 0.05 g of zucchini plant leaves were crushed with liquid nitrogen in a laboratory mortar and pestle until powdered and homogenized with 5 ml of 3 % sulfosalicylic acid solution. The solution was then centrifuged at 15000 g for 10 min at room temperature to form supernatant. 2 ml of the supernatant was transferred to each tube and two parallels were performed for each tube. Then 2 ml of glacial acetic acid and 2 ml of acetic acid, phosphoric acid, and ninhydrin solution were added to the tubes and vortexed. The tubes were then boiled (for 1 h) followed by rapid cooling in ice water. After cooling, 4 ml toluene was added and vortexed for 30 s. Then they were kept for 5 min, and the absorbance of the pink phase formed at the upper part was read and recorded in a spectrometer (520 nm). The proline contents in plant samples were determined by a standard curve as detailed by Karabal et al. [28].

2.6.3. Determination of antioxidant content by the phosphomolybdenum method

To determine the content of antioxidants, the phosphomolybdenum method proposed by Prieto et al. [29] was used with a few modifications. First, the reagent solution was prepared by adding 100 mM sodium phosphate (28 ml), 10 mM ammonium molybdate (40 ml), and 2 M sulfuric acid (30 ml) and then topping up to 100 ml with distilled water. It was also ensured that the solution was fresh. Afterward, 0.4 ml of plant sample was added to 4 ml of reagent solution and thoroughly mixed. The test tubes were vortexed. They were then incubated in a water bath at 95 °C for 90 min. Plant samples were measured by UV–vis spectrophotometer at 695 nm absorbance. The antioxidant activity of samples was calculated as mg AAE/g sample using the equation of the line obtained from the ascorbic acid standard graph as described by Prieto et al. [29]. The experiment was conducted in four replicates.

2.6.4. Analysis of total phenolic content (TPC)

To determine the total phenolic content, the method developed by Singleton and Rossi [30] was modified and used. In brief, 200 μl of plant sample extract was topped up to 2 ml with 1800 μl of distilled water. Then 1 ml of diluted (1/10) Folin-Ciocalteau reagent (Merck, Germany) and 2 ml of sodium carbonate (2 % w/v) were added. The samples were then allowed to react for 2 h at room temperature in the dark. After the waiting period, the absorbance values of the samples were recorded using a spectrophotometer (UV1800 Shimadzu, Japan) at a wavelength of 765 nm. The total phenolic content of the samples was calculated as mg GAE/g sample using the equation of the line obtained from the gallic acid standard graph [30]. The experiment was conducted in four replicates.

2.6.5. Analysis of total flavonoid content (TFC)

TFC of the samples was measured as described by Zhishen et al. [31]. 0.5 ml of sample was mixed with 2 ml of distilled water and 150 μl of sodium nitrite (5 % w/v). After 5 min, 150 μl of $AlCl_3$ (5 % w/v) was added into the tubes and after 6 min, 1 mL of NaOH (1 M) and 1.2 mL of distilled water were added. The final mixture was vortexed and the

absorbances were recorded at 510 nm by a UV–vis spectrophotometer (Shimadzu, 1800; Japan). The total flavonoid content of the samples was calculated as mg catechin equivalent (CE)/g dry sample.

2.6.6. Determination of DPPH radical scavenging activity

The DPPH radical scavenging activity of the samples was determined according to the method described by He et al. [32]. Plant sample extract (100 μl) and 3900 μl of 0.1 mM DPPH (Sigma, USA) radical solution were mixed with methanol and thoroughly homogenized by vortexing. The samples were incubated at room temperature in the dark for 30 min and then absorbance values were measured using a UV–Vis spectrophotometer (UV1800 Shimadzu, Japan) at a wavelength of 517 nm. It was calculated as % inhibition using the following equation for the DPPH radical scavenging capacity. This study was repeated four times in two parallel experiments.

$$\%\ \text{Inhibition} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} X100$$

2.7. Statistical analysis

Data were analyzed with the SAS statistics program Version 9.00 (SAS Institute, USA). The data were subjected to analysis of variance and the means were compared with the least significant difference test (LSD) at a 0.05 significance level.

3. Results and discussion

3.1. Morphological and molecular characterization of powdery mildew agent

In this study, the observed conidia showed the same patterns observed in previous research with the same species [33], i.e., they were observed to produce chains ranging from ellipsoid to oval form. Analysis with 3 % KOH showed the presence of fibrosin bodies commonly found in Cucurbit powdery mildew genus *Podosphaera*. In addition to its morphological characteristics, it was concluded that the powdery mildew isolate used in the study belongs to *P. xanthii* species.

Molecular characterization was performed by PCR analysis through the amplification of a 540 bp portion of the fungal DNA using *P. xanthii*-specific PCR primers PMITS1 and PMITS2 [34]. The DNA sequence of the 540 base pairs obtained as a result of polymerase chain reactions using ITS1-2 primers specific for powdery mildew was analyzed using the Nucleotide BLAST program in NCBI. The Nucleotide BLAST result (left) and the similarity map of the DNA sequence to other sequences in the NCBI database (right) clearly show the identity of the powdery mildew isolate used in this study is *P. xanthii*. Similar results were found by Kousiket al. [35], with 100 % identity to the ITS sequences of *P. xanthii* in the GenBank database.

3.2. Effectiveness of bacterial solution against powdery mildew disease

Results showed that the symptoms observed on the samples of pumpkin leaves were typically associated with powdery mildew. All samples had similar morphological characteristics caused by powdery mildew disease. While severe disease formation was observed in the control plants, the best results (completely healthy leaves) were achieved with the full-dose bacteria application before and after powdery mildew infestation. Although low infection rates were observed on the leaves (1–2 scale value), 3- and 5-fold diluted bacterial solutions significantly prevented the development of powdery mildew disease compared to the control.

It was found that disease severity increased as the concentration of bacteria in the diluted solution decreased. Relatively higher infection rates were observed in plants treated with the 5-fold bacteria dilution diluted solution (Fig. 1 and Table 2). After testing for disease, the control plant showed a significant decline in vegetative growth due to powdery mildew infection, while all plants treated with the bacterial formulation exhibited better vegetative growth. In particular, the highest plant height, root length, and plant weight (fresh and dry weight) were determined in plants where full-dose application of bacteria dilution before and after infection. While the lowest fresh root weight (4.07 g/plant) was recorded in the nutrient medium application, full-dose

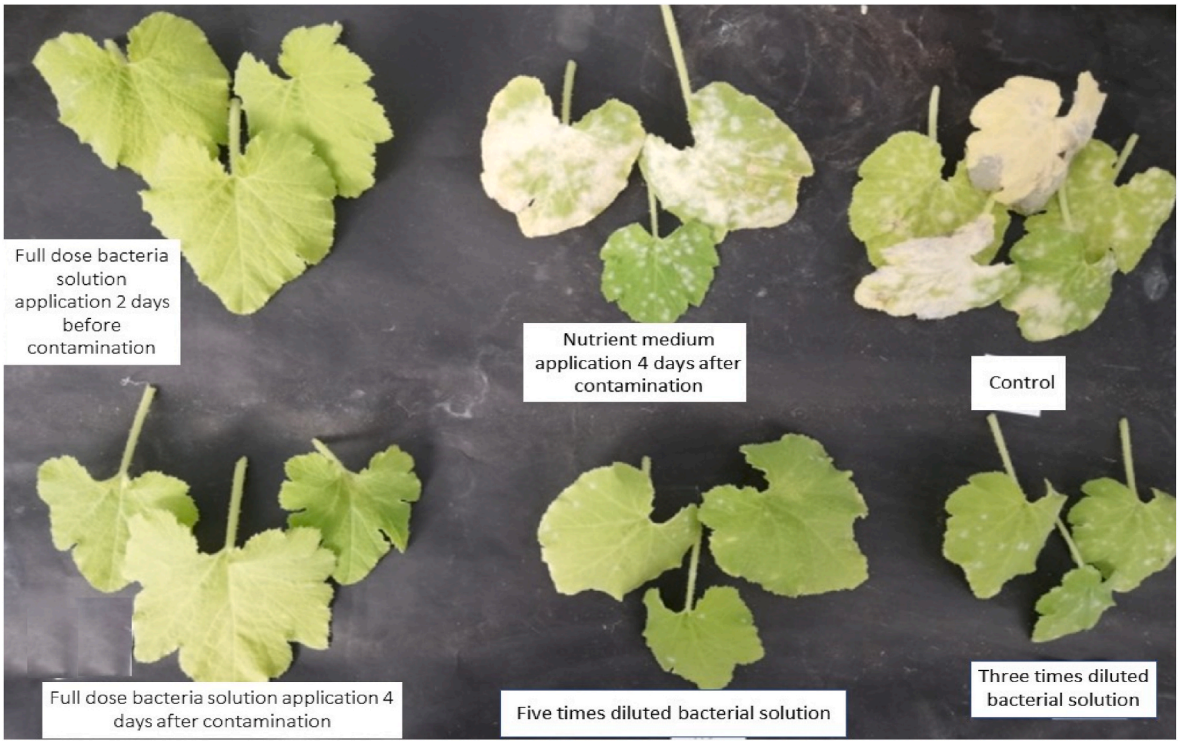


Fig. 1. Formation of powdery mildew after application of bacterial formulations.

Table 2
Quantified data of vegetative growth and disease symptoms in zucchini.

Treatments	Plant Height (cm)	Root depth (cm)	Root fresh weight(g/plant)	Shoot fresh weight (g/plant)	Root dry weight (g/plant)	Shoot dry weight (g/plant)	Scale
10^5	20b	21.33bc	7.82b	17.41d	5.75c	5.36cd	2
10^3	24.33a	24.33a	15.79a	22.26c	7.57b	8.3c	1
PM + B	24.66a	22.33 ab	15.62a	26.34 ab	10.53a	12.54b	0
B + PM	24.66a	24.66a	16.49a	33.87a	11.19a	13.81a	0
Media	20b	20.66c	4.07d	13.51de	6.84bc	6.48cd	4
Control	17.33c	19.66c	7.28b	9.31e	1.28d	1.01d	4
CV	1.02	1.66	13.14	12.5	0.98	1.08	
LSD	5.05	5.45	8.25	8.75	9.84	10.5	

PM + B: Powdery mildew infection before bacteria solution application, B + PM: Bacteria dilution application before Powdery mildew infection, 10^3: Three-fold bacterial dilution, 10^5: Five-fold bacterial dilution.
**Means in the same column followed by the same letter are not significantly different ($P \leq 0.05$).*

bacteria application, before and after powdery mildew (16.49 and 15.62 g/plant, respectively), and 3-fold bacteria dilution application (15.79 g/plant) gave the highest fresh root weight (Table 2) (see Fig. 2).

Shoot fresh weight was significantly affected by the treatments. While the highest fresh shoot weight was achieved by full-dose application of the bacteria before and after powdery mildew contamination, control plants produced the lowest shoot fresh weight. When compared to full-dose bacterial treatments, the powdery mildew disease-related reductions in shoot fresh weight in the control plants ranged from 288 % to 400 %.

The highest values of root and stem dry weight, similar to fresh weight, were obtained when bacteria were applied at full-dose. As previously mentioned, the cucurbit family occupies a very important place in vegetable cultivation both in Turkey and in the world. Cucurbits are cultivated on very large scales, with powdery mildew disease being the culprit behind heavy product losses across the world. Developing environmentally friendly methods to combat the disease is an important contribution to reducing crop losses and protecting the environment.

3.3. Induction of pumpkin defense enzyme production

This study found that the application of bacterial strains as PGPR increased the proline content, TFC, TPC, DPPH, and antioxidant enzymes, which play vital roles in plant defense mechanisms (Table 3). The application of B + PM resulted in the highest enzyme contents, which were: 22.91 mg AAE/g antioxidant, 2.01 mg/g KU proline, 10.03 mg GAE/g TPC, and 7.756 mg CE/g TFC. Also, the application of PM + B, B + PM, and 10^3-fold bacterial dilutions increased DPPH inhibition percentages by 41.86 %, 41.44 %, and 40.59 %, respectively. Overall, plants treated with bacterial dilution had significantly higher enzyme contents than those treated with control or media applications. According to Zhu et al [36], PGPR can boost induced systemic resistance (ISR) in plants against pathogen infections. The results presented in Table 3 indicate that the levels of enzymes such as antioxidant (11.548 mg AAE/g), proline (0.3013 mg/g KU), TPC (5.32 mg GAE/g), TFC (3.589 mc CE/g) and DPPH inhibition (7.61 %) were significantly lower in plants treated with control application, as compared to those treated with bacteria dilution applications. Thus, the application of bacteria dilutions significantly enhanced pumpkin defense enzyme production and activity.

PM + B: Powdery mildew infection before bacteria solution

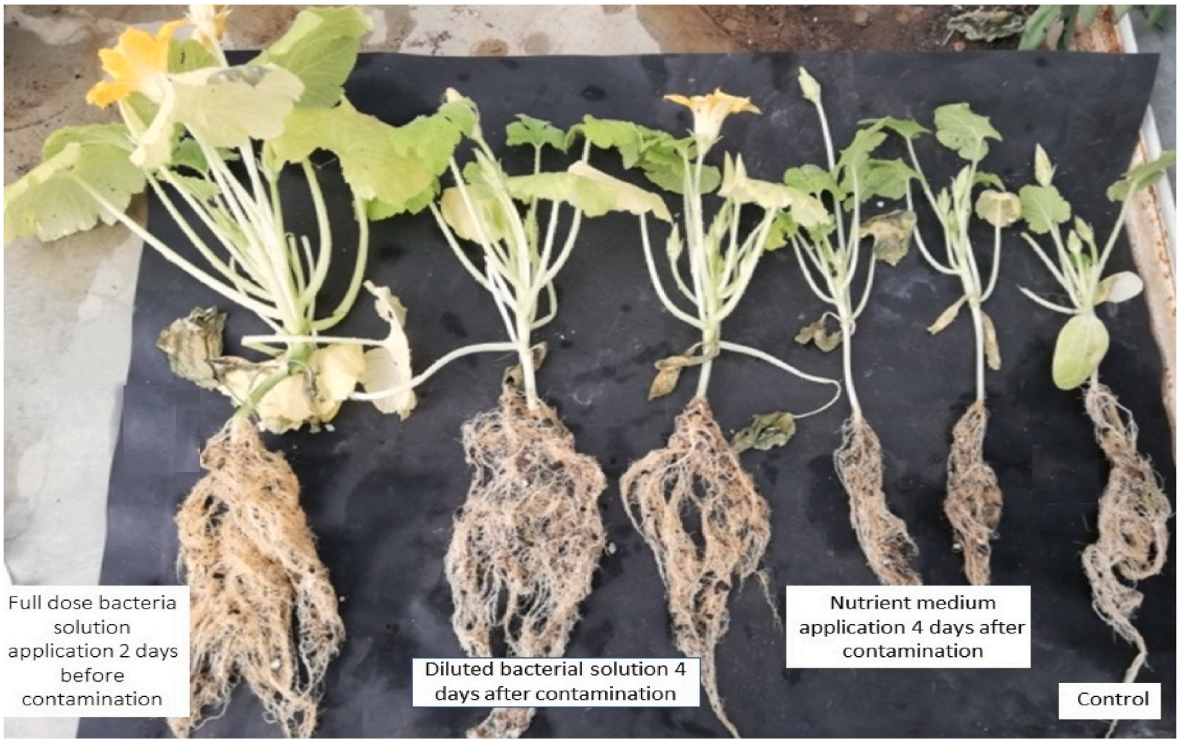


Fig. 2. Vegetative gr f plants inoculated with powdery mildew after bacterial solution applications.

Table 3
Zucchini enzyme content examined after powdery mildew infection and bacteria dilution application.

Application	Antioxidant (mg AAE/g)	Proline (mg/g KU)	TPC (mg GAE/g)	DPPH (% inhibition)	TFC (mg CE/g)
10^5	14.633b	1.109b	7.323bc	35.906b	4.783b
10^3	15.671b	1.313b	7.87bc	40.59a	5.626 ab
PM + B	18.982 ab	1.526b	8.773b	41.44a	6.553 ab
B + PM	22.91a	2.013a	1003a	41.86a	7.756a
Media	12.408c	0.502c	5.81c	10.09c	3.028c
Control	11.548cd	0.3013c	5.32c	7.61d	3.589c
CV	14.06	53.25	34.45	42.15	18.84
lsd	12	1.98	30.65	31.45	5.5

**Means in the same column followed by the same letter are not significantly different ($P \leq 0.05$).*

application, B + PM: Bacteria dilution application before Powdery mildew infection, 10^3: Three-fold bacterial dilution, 10^5: Five-fold bacterial dilution.

The powdery mildew-causing fungal species was identified using both morphological examination under a microscope and molecular characterization through PCR amplification of the ITS region at the Molecular Mycology Laboratory in the Department of Plant Protection at Akdeniz University. The 540 bp target region was sequenced and compared against sequences in the NCBI database using Nucleotide BLAST. Based on the BLAST results shown in Fig. 1, it is evident that the powdery mildew isolate can be identified as *Podosphaera xanthii*. *P. xanthii* is a pest that causes significant economic losses worldwide to crops in the Cucurbitaceae family [3].

In this study, *B. subtilis* and *B. thuringiensis* formulations were utilized as eco-friendly applications and biocontrol agents to combat powdery mildew disease, which poses a significant challenge to pumpkin cultivation in Kayseri and Turkey, where pumpkin is extensively grown.

Similar to the present study, previous studies have explored the potential of *B. subtilis* and *B. thuringiensis* as bioagents in the control of some plant diseases [37–39]. *Bacillus* species including *B. megaterium, B. thuringiensis, B. pumilus, B. subtilis*, and *B. cereus* exhibit high levels of antagonistic activities against seed-, soil-, and leaf-borne fungal and bacterial pathogens in various host plants. The antagonistic activities of these organisms is generally due to their ability to produce antimicrobial compounds such as siderophores, proteases, ammonia, biosurfactants, antimicrobial peptides, and fungal enzymes including chitinases, β-1, 3-glucanases, and β-1,4-glucanases [40–49].

Other studies have evaluated the efficacy of *B. subtilis*, nano TiO2, ZnO, and Topas100 fungicide against powdery mildew caused by P. xanthii in cucumber plants under *in vitro* and *in vivo* conditions.

The effectiveness of Bio-agent *B. subtilis*, nano titanium dioxide (TiO_2), zinc oxide (ZnO), and commercial fungicide (Topas100) against powdery mildew caused by *P. xanthii* in cucumber plant (*Cucumis sativus* L.) *in vitro* and *in vivo* (greenhouse) conditions. Most of the treatments prevented spore germination of *P. xanthii*. The combination of *B. subtilis* and nano-ZnO resulted in the best outcomes, followed by the application of a commercial fungicide. Yaser et al. [39] observed changes in the histo-biochemical and growth parameters of cucumbers due to bacterial applications. Roberts and Kucharek [50] found significant improvements in plant growth parameters, including plant height, number of leaves, and fresh and dry weight, as a result of the PGPR applications.

The plant defense response can be triggered by an increase in antioxidant and phenolic enzymes, leading to the accumulation of phenolic compounds [51]. Other studies have reported that some plant infections are prevented and/or reduced due to the increase in defense enzyme production as a result of bacterial application [52–54].

Plant tissues accumulate amino acids, proline, and metabolic components in response to pathogenic stress [55]. In previous studies, it has been reported that biotic factors, along with their direct antagonistic effects in suppressing fungal diseases, also promote plant defense mechanisms [56,57]. Some biotic and abiotic stimuli activate resistance genes in the host that induce resistance by facilitating interaction between the pathogen's receptors and the host plant's receptors [58].

It is crucial to create eco-friendly applications and agents that can replace chemical methods for controlling powdery mildew. Such alternatives can help achieve a sustainable environment and increase crop yield. The bacterial strains used in the current study were found to have the potential to control powdery mildew disease and enhance plant growth.

4. Conclusion

The emergence of powdery mildew as a devastating disease of cucurbits is mainly attributed to global climate changes and the fact that these pathogens have the ability to reproduce sexually and asexually. Because powdery mildew disease agents are biotrophic and change their genetic structures rapidly, they are difficult to control and study. For these reasons, studies with PGPR against these diseases in cucurbits in Turkey and around the world are limited. The present study found *Bacillus subtilis* and *Bacillus thuringiensis* formulations to be significantly efficient in combating *P. xanthii*. Within the scope of this study, the bacteria formulations were utilized as both disease control agents to combat powdery mildew disease and PGPR to enhance pumpkin growth. Our findings showed that formulations made from *Bacillus subtilis* (61.29e and 3.3a strains) and *Bacillus thuringiensis* (2B3-1 and 2B2-2 strains) significantly increased the synthesis of DPPH, antioxidant, proline production, total phenolic substance, and total flavonoid content. These enzymes play vital roles in the activation of the plant defense mechanism against powdery mildew disease in zucchini. Thus, these formulations have the potential to significantly prevent yield losses and serve as an environmentally friendly alternative to chemical pesticides for disease control in economically important cucurbit crops.

Future studies may focus on large-scale field trials by comparing the bacterial formulations with chemical pesticides. It is also recommended to conduct studies on host-pathogen interactions to better understand the genes that activate pumpkin defense mechanisms.

CRediT authorship contribution statement

Emel Ünlü: Writing – original draft, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing. **Özer Çalış:** Writing – review & editing, Methodology, Data curation. **Ahmet Say:** Data curation, Formal analysis, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Abdul Aziz Karim:** Conceptualization, Data curation, Formal analysis, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Halit Yetişir:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Data curation, Conceptualization. **Semih Yılmaz:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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