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Mitigation of Powdery Mildew Disease by Integrating Biocontrol Agents and Shikimic Acid with Modulation of Antioxidant Defense System, Anatomical Characterization, and Improvement of Squash Plant Productivity

Nour El-Houda A. Reyad [1], Samah N. Azoz [2], Ayat M. Ali [3] and Eman G. Sayed [4,*]

[1] Plant Pathology Department, Faculty of Agriculture, Cairo University, Giza 12613, Egypt
[2] Agricultural Botany Department, Faculty of Agriculture, Cairo University, Giza 12613, Egypt
[3] Central Lab of Organic Agriculture, Agricultural Research Center, Giza 12619, Egypt
[4] Vegetable Crops Department, Faculty of Agriculture, Cairo University, Giza 12613, Egypt
* Correspondence: eman.ali@agr.cu.edu.eg; Tel.: +20-100-636-2604

Abstract: Squash (*Cucurbita pepo* L.) is a globally important vegetable, the production of which is severely constrained by powdery mildew caused by *Podosphaera xanthii*. In this study, we examined the effects of *Trichoderma asperellum* (MW965676), *Streptomyces rochei* (MN700192), and a mixture of the two foliar sprays with or without shikimic acid seed priming treatment on powdery mildew severity, plant growth, and total yield during the 2020–2021 and 2021–2022 growing seasons. We also studied their immune eliciting properties by examining their enzymatic, phenolic, and hormonal functions. The combination of *Trichoderma asperellum*, *Streptomyces rochei*, and shikimic acid triggered plant defense responses, which elicited enzyme activities such as peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT), phenolic compound accumulation, and increased salicylic acid (SA) and jasmonic acid (JA) content. This approach yielded high-quality results in the control of powdery mildew during the two growing seasons under greenhouse conditions. Additionally, relatively large statistical differences in plant growth, total yield, mineral components, and physiological traits were observed. A GC–MS analysis of *Trichoderma asperellum (*MW965676) showed hemin cation as a major component, while *Streptomyces rochei (*MN700192) contained 2,4-di-tert-butyl phenol and the hexadecenoic acid methyl ester. With respect to the morphological changes induced by powdery mildew and the treatments, plants treated with a mixture of *Trichoderma asperellum*, *Streptomyces rochei* and shikimic acid showed an improvement in the thickness of the midvein, increased dimensions of the main midvein bundle, a larger number of xylem rows in the main midvein bundle, greater mean diameters of vessels and of parenchyma cells in the ground tissues, as well as increased thickness of the upper and lower epidermis, lamina, palisade tissue and spongy tissue. This extensive, new study is the first step toward a more profound understanding of the use of *Trichoderma asperellum* and *Streptomyces rochei* with shikimic acid-primed seeds as a potential alternative technique for attenuating powdery mildew infection in squash.

Keywords: *Cucurbita pepo* L.; *Podosphaera xanthii*; *Trichoderma* spp.; *Streptomyces* spp.; seed priming; phenols; antioxidant enzyme

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1. Introduction

Squash (*Cucurbita pepo* L.) is an important vegetable crop. It is widely cultivated and consumed as food in many countries worldwide. It is a good source of vitamins A, C, and B and is rich in antioxidants and minerals like potassium, magnesium, and manganese [1]. Powdery mildew is the most serious disease affecting squash plants, accounting for 30–50% of yield losses [2]. Resistant varieties, chemical fungicides, natural products, and biological control are used to combat plant diseases [3]. Disease control is primarily based on the use

of recommended chemical fungicides that are hazardous to humans, animals, plants, and useful organisms [4].

To address these issues, appropriate control measurements and alternative safe methods, such as biological control, must be implemented. *Trichoderma* spp. and *Streptomyces* spp. are widely used as bioagents. Some *Trichoderma* species have been used to control mildew; for example, *T. viride* culture filtrates were effective in controlling *Leveillula taurica* [5], and *T. asperellum* and *Metarhizium anisopliae* were effective in controlling *L. taurica* on peppers [6]. In addition, *T. asperellum* IZR D-11 efficiently protects common oak leaves from powdery mildew caused by *Erysiphe alphitoides* [7]. The growing interest in *Trichoderma* spp results from its various complex mechanisms, *i.e.,* competition for nutrients and space, mycoparasitism, the degradation of pathogen cell walls, and the induction of plant resistance [8].

On the other hand, the genus *Streptomyces* is a promising candidate for controlling phytopathogenic microorganisms, including powdery mildew pathogens, through a range of mechanisms, including competition for nutrients, secretion of antibiotics and lytic enzymes, and stimulation of plant defenses [9]. For example [10], reported that *Streptomyces* sp. (AcH 505) reduced oak powdery mildew disease by activating plant defense responses. Additionally, wuyiencin produced by *S. albulus* CK-15 significantly reduced the disease incidence and severity of powdery mildew in cucumber plants [11].

Shikimic acid is the well-known precursor of the lignin building blocks, L-phenylalanine and L-tyrosine. Plants use phenylalanine, leucine, and tyrosine to suppress pathogen growth, promote the production of antimicrobial chemicals [12], and improve crop protection under stress conditions by altering the enzymes involved in their defense responses [13].

Several studies have shown that by activating their complex defense responses, plants can evolve appropriate defense mechanisms to recognize and resist fungal infections [14,15]. One of these reactions is the rapid formation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH), and superoxide anions (O2) [15]. ROS scavenging enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) play an essential role in regulating ROS levels and the extent of oxidative damage [15]. In higher plants, another defense response is the phenylpropanoid pathway [16]. Phenylalanine ammonia-lyase is the first enzyme involved in a number of structurally diverse, defensive phenolic and lignin compounds [17]. Polyphenol oxidase (PPO) is another key enzyme involved in the formation of phenolic compounds to defend against pathogens in plants [18]. In addition, plant hormones such as jasmonic acid (JA) and salicylic acid (SA) are involved in all plant defense reactions. However, increasing JA levels in plants can improve resistance to necrotrophic infections and make plants more sensitive to biotrophic pathogens [19].

The purpose of this study was to estimate the efficacy of several potential biocontrol agents (BCAs) alone and combined with shikimic acid to control squash powdery mildew. We also examined their impact on squash plant growth, yield, and quality to determine the potential of using these two control options as an integrated disease management strategy, as well as to measure oxidative enzyme activity, plant hormones, photosynthesis, and mineral uptake to determine their role in powdery mildew control.

2. Materials and Methods

2.1. Plant Material and Tested Compounds

A squash (*Cucurbita pepo* L. cv Eskandarani) cultivar was employed as an experimental model in all experiments throughout this study. Seeds were obtained from Mecca Trade Co. (Cairo, Egypt), and shikimic acid was purchased from Sigma-Aldrich, (Humberg, Germany).

2.2. Biocontrol Agents (BCAs) Preparation

We investigated the potential benefits of *T. asperellum (MW965676) and S. rochei (MN700192)* for squash powdery mildew control. These bioagents were previously iso-

lated from pepper [20] and sugar cane [21] plants, respectively. For improved growth of *T. asperellum*, three fresh fungal discs (5 mm) of three-day-old culture were grown in a 250 mL conical flask containing 200 mL liquid gliotoxin fermentation medium consisting of 25 g dextrose, 2 g KH_2PO_4, 2 g ammonium tartrate, and 0.01 g $FeSO_4$. The inoculated flasks were incubated in a rotary shaker at 25 °C for 11 days. On the other hand, *S. rochei* was grown in 100 mL of starch-casein medium consisting of 10 g of soluble starch, 0.3 g casein, 2 g KNO_3, 2 g NaCl, 2 g K_2HPO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCO_3$, 0.01 g $FeSO_4 \cdot 7H_2O$, and 1000 mL distilled water in an Erlenmeyer flask (250 mL). The pH was adjusted to 7.3 and the flasks were incubated on a rotary shaker at 30 °C for 7 d.

2.3. Identification and Inoculation of *P. xanthii*

Based on its morphological features, we identified the pathogen according to the method described in [22]. *P. xanthii* conidia were collected from infected squash plants by rubbing infected leaves with a soft brush and rinsing with distilled water; this water was then collected and used as an inoculum. The conidial suspension was adjusted to 10^5 conidia mL^{-1} by a hemocytometer and sprayed onto the leaves of squash seedlings using a hand sprinkler at a rate of 5 mL per leaf.

2.4. Seed Priming Preparation

Before sowing, summer squash seeds (*Cucurbita pepo* L. cv. Eskandarani) were sterilized in 7% sodium hypochlorite for 10 min and then fully washed with distilled water. The sterilized seeds were divided into four groups. The control treatment (group 1) was primed in water, while groups 2, 3, and 4 were soaked in shikimic acid at 20, 40, and 60 ppm, respectively. For priming, summer squash seeds were immersed in a solution of shikimic acid at 20 °C for 5 h in the dark. Following the treatments, the seeds were washed four times in distilled water for 5 min each time and then dried using blotting paper. They then received a flow of dry air at 30 °C until the original moisture content was approximated [13]. Following priming, seeds were used for the pot experiments.

2.5. Pot Experiments

Two pot experiments were conducted in the greenhouse of the Plant Pathology Department, Faculty of Agriculture, Cairo University, Giza, Egypt, to evaluate the effect of shikimic acid at 20, 40, and 60 ppm and/or foliar spraying of certain bioagents (*T. asperellum*, *S. rochei*, and *T. asperellum* + *S. rochei*). The experiment comprised four main groups. The first was the control treatment, with seeds primed in water. The other three groups (2, 3, and 4) were primed with shikimic acid at 20, 40, and 60 ppm. The three groups of primed squash seeds and control were sown in pots 50 cm^2 in diameter, which were filled with a 1:1:1 mixture of peat moss, vermiculite, and perlite. Each group was subdivided into two subgroups. The first group was sprayed with water alone and served as the control. The second group was separately sprayed with *S. rochei*, *T. asperellum*, and a mixture of the two bioagents (*S. rochei* + *T. asperellum*) at a rate of 1/50 L of water (1 mL of each bioagent was adjusted to 20×10^6 CFU). The initial foliar spraying with the bioagent was performed 30 d after planting, and spraying was performed every 15 d in the early morning. All groups of plants were artificially inoculated with an aqueous conidial suspension of *P. xanthii* 24 h after the first treatment (freshly collected *P. xanthii* conidia were suspended in deionized water to a concentration of 10^5 conidia mL^{-1} water) as described in [23]. All other horticultural practices were performed as recommended for summer squash. The experiment was performed twice. A split plot design was used to analyze all of the data obtained from six replicates (total of six plants per replicate). Plant height (cm), number of leaves, and chlorophyll (SPAD) readings were assessed for all treatments in both experiments 45 d after sowing. Photosynthesis, transpiration rate, and leaf stomatal conductance analyses were performed using an infrared gas analyzer, the LICOR 6400 Portable Photosynthesis System (IRGA, Licor Inc., Lincoln, NE, USA), on the fourth leaves of 10 squash plants chosen from each treatment, with six pot replications. Measurements were taken from

10 a.m. to 2 p.m., with a light intensity of around 1300 mol m^{-2} s^{-1} and 85% RH. The leaf chamber temperature ranged from 26.2 °C to 27 °C, and the volume gas flow rate was 400 mL min^{-1}. The CO_2 content in the air was 399 µmol mol^{-1}.

2.6. Greenhouse Experiments

Two greenhouse experiments were performed in the vegetable crop greenhouse of the Department of Cairo University, Faculty of Agriculture in Giza (located at 31°1′13″ N, 30°13′5″ E), during two successive growing seasons (October to February 2020–2021 and 2021–2022) to evaluate the impact of 40 ppm shikimic acid seed priming and/or foliar spraying of certain bioagents (*T. asperellum*, *S. rochei*, and *T. asperellum* + *S. rochei*) on the powdery mildew, vegetative growth, and total yield of squash plants. Squash seeds primed with 40 ppm of shikimic acid were sown directly in the greenhouse on October 6 and 10 during the 2020–2021 and 2021–2022 seasons, respectively. Squash seeds were sown on ridges of 1.5 m width, 7 m length on one side of the ridge, 50 cm apart. Control squash seeds (not primed with shikimic acid at 40 ppm) were grown under the same conditions as the primed seeds. Initial foliar spraying with the bioagent was performed 30 d after planting, and spraying was performed every 15 d in the early morning. All agricultural and farming practices for squash crops in the greenhouse were performed as recommended by the Egyptian Ministry of Agriculture. A drip irrigation system was used. The farm soil type was clay loam in texture with 7.6, EC 1.2 dS m^{-1} and contained 118 ppm N, 23 ppm P, and 30 ppm K. The pH value was 7.02, and the soluble cation values were 4.5, 3.2, 2.2, and 3.3 meq L^{-1} for Ca^{++}, Mg^{++}, K^{+}, and Na^{+}, respectively, and 0.8, 4.8, and 7.1 meq L^{-1} for HCO^{3-}, Cl^{-}, and SO_4, respectively. The treatments were arranged in a randomized complete block design with three replicates.

2.7. Data Recorded

2.7.1. Disease Assessment

Disease severity, whether in pots or in the greenhouse experiments, was determined-weekly as described in [24], based on a scale ranging from 1 to 5: 1 = no detectable infection; 2 = 1–5% of leaf area infected; 3 = 6–25% of leaf area infected; 4 = 26–50% leaf area infected; and 5 = more than 50% of leaf area infected. Disease severity percentage was estimated using the following equation [25]:

$$\text{Disease severity (\%)} = [\sum (n \times v)/5N] \times 100$$

where n refers to the number of infected leaves in each category, v denotes the numerical values of each category, and N is the total number of infected leaves.

The mean area under the disease progression curve (AUDPC) for each replicate was calculated using Equation [25] (Pandey et al., 1989).

$$\text{AUDPC} = D\,[1/2(Y1 + Yk) + (Y2 + Y3 + \ldots \ldots + Yk - 1)]$$

where D = time interval, Y1 = first disease severity, Yk = last disease severity, and Y2, Y3, and Yk − 1 = intermediate disease severity.

2.7.2. Vegetative Growth

Four plants were randomly picked with roots from each plot 50 days after seeding to measure the vegetative growth parameters. Plant height, the number of leaves per plant, and the fresh and dry weight of the leaves per plant were all measured. A SPAD meter was used to determine the chlorophyll content in the sample.

2.7.3. Yield and Its Components

The total number of fruits per plant, total yield per plant, average fruit weight, and total yield per hectare were calculated.

2.7.4. Mineral Content in Squash Leaves

The total nitrogen concentration of the dried samples was measured using the modified micro-Kjeldahl method [26]. Phosphorus was determined calorimetrically by using the chloro stannous molybdophosphoric blue color method in sulfuric acid, as described in [27]. The total potassium and calcium contents were determined using the flame photometer apparatus (CORNING M 410, Germany). Concentrations of Mg, Zn, B, Fe, and Mn in leaf samples were measured by atomic absorption spectrophotometry with air-acetylene and fuel (PyeUnicam, model SP-1900, USA).

2.7.5. Activity of Antioxidant Enzymes, Salicylic Acid and Jasmonic Acid

Salicylic acid and jasmonic acid were measured according to the method described in [28]. For enzyme analysis, at 45 days after sowing (24 h after the last spray), 0.5 g leaf tissues were homogenized in 3 mL of 0.05 M Tris buffer (pH 7.8), containing 0.001 M EDTA–Na2 and 7.5% Polyvinylpyrrolidone at 0–4 °C. The homogenates were centrifuged (12,000× *g* rpm, 20 min, 4 °C), and the total soluble enzyme activity in the supernatant was measured colorimetrically using a UV-160A spectrophotometer (Shimadzu, Kyoto, Japan). Catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) activities were determined according to [29]

2.7.6. GG/MS Analysis of *T. asperellum* Culture Filtrate

The chemical composition of the *T. asperellum* culture filtrate was determined using a Trace GCTSQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a TG5MS direct capillary column (30 m, 0.25 mm, 0.25 m film thickness). The column oven temperature was initially maintained at 50 °C and then increased at a rate of 5 °C/min to 250 °C and held for 2 min. It was subsequently increased at 30 °C/min to the final temperature of 30 °C and held for 2 min. The injector and MS transfer line temperatures were maintained at 270 and 260 °C, respectively. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The solvent delay was 4 min, and diluted 1 L samples were injected automatically by the AS1300 autosampler coupled with GC in split mode. EI mass spectra were recorded at 70 eV ionization voltages over the range m/z 50–650 in full-scan mode. The ion source temperature was set at 200 °C. The components were identified by comparing their mass spectra to those of the WILEY 09 and NIST 14 mass spectral databases, as described in [30].

2.7.7. Anatomical Studies

The tested materials included the blade of the fifth leaf developed on the main stem of summer squash (cv. Eskandarani) affected by powdery mildew as a control and treated with shikimic acid, bioagents *(S. rochei+ T. asperellum*), and mix of the two. Samples were taken throughout the second growing seasons of 2021 at 50 days after sowing. About 1.0 cm of specimens were killed and fixed in FAA solution (5 mL glacial acetic, 10 mL formalin, 35 mL water, and 50 mL ethyl alcohol 70%) for at least 48 h. The selected materials were washed in 30% ethyl alcohol, dehydrated in a normal ethanol and butyl alcohol series, embedded in paraffin wax with a melting point 56 °C, sectioned to a thickness of 15 micrometers (µm), stained with crystal violet-erythrosin, cleared in xylene, and counted in Canada balsam [31]. Transverse sections were done with a Leica Microtome RM 2125 and photographed and measured using the Leica Light Image Analysis System DM 750 at the Faculty of Agriculture, Cairo University-Research Park (CURP). The following parameters were recorded:

- Thickness of midvein (µm)
- Thickness of upper periderm (µm)
- Thickness of lower periderm (µm)
- Thickness of lamina (µm)
- Thickness of palisade tissue (µm)
- Thickness of spongy tissue (µm)
- Dimension of main midvein bundle (µm)

- No of xylem rows in main midvein bundle
- Mean diameter of vessels (µm)
- Mean diameter of parenchyma cells in the ground tissue (µm)

2.7.8. Statistical Analysis

Using the MSTAT-C computer software package prepared by [32], the data were subjected to analysis of variance. The LSD test was used to examine differences between treatment means at a 5% level of probability [33]. Furthermore, principal component analysis (PCA) was carried out using all data points of individual response variables using Origin pro-2021 version software. A heatmap was generated to visualize variations and similarities in all response variables combined with a two-way hierarchical cluster analysis using the standardized means of all results matrices for the tested treatments.

3. Results

3.1. Pot Experiments

3.1.1. Morphology of *P. xanthii*

Our microscopic examinations of the anamorphic stage of the causal pathogen showed that mycelium is ectophytic. Conidiophores were un-branched, bearing conidia in long chains varying from ellipsoid to ovoid forms of 25.30–33.20 × 14.95–22.70 µ (17.54 × 30.75 µ on the average) in size. Observations of conidia in 3% KOH indicated the presence of fibrosin bodies, which are commonly found in the cucurbit powdery mildew genus *Podosphaera*, as shown in Figure 1.

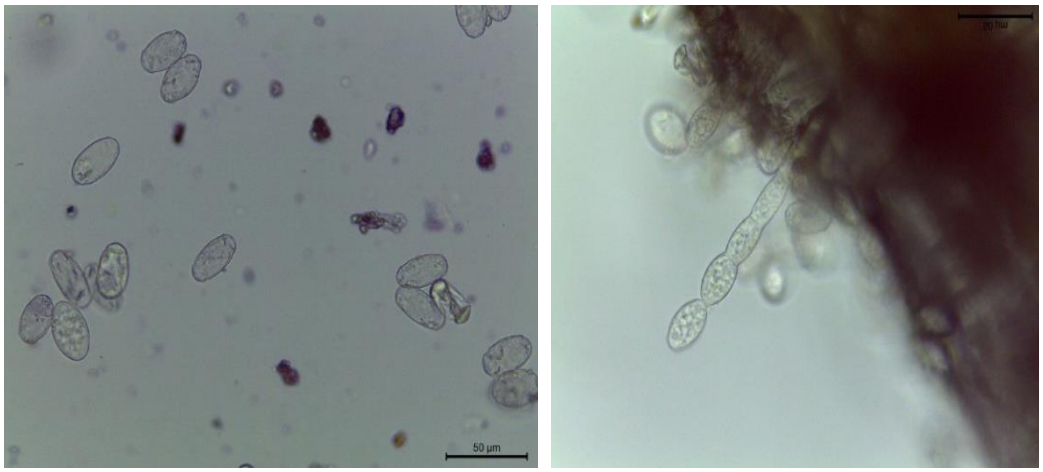

Figure 1. Morphological features of anamorphic stage of *P. xanthii*.

3.1.2. Disease Assessment

Under artificial inoculation with powdery mildew, the response of squash to shikimic acid as seed priming at 20, 40, and 60 ppm with or without foliar application of BCAs was observed. As shown in Table 1, all tested treatments had a highly significant effect on powdery mildew disease severity (DS%) in comparison to the control during the two trials. In this regard, significant disease severity reduction was obtained with 40 and 20 ppm shikimic acid, through seed priming combined with a mixture of BCAs (*S. rochei* + *T.asperellum*) as a foliar application, while the application of 60 ppm shikimic acid through seed priming, followed by the application of *S. rochei* through foliar spray as individual treatments, recorded the lowest disease severity reduction

Table 1. Effect of the interaction between seed priming of shikimic acid (0, 20, 40, 60 ppm) and foliar applications with *S. rochei* (SR), *T. asperellum* (TS), *S. rochei* + *T. asperellum* (SR + TS), and water (control) on powdery mildew severity % on squash plants during the first and second experiments.

Shikimic Acid Concentrations	Bioagents Treatments	1st Experiment		2nd Experiment	
		Disease Severity %	Reduction %	Disease Severity %	Reduction %
0 ppm	Control	37.04 ^{a}	-	36.30 ^{a}	-
	T. asperellum	30.37 ^{cd}	18.01	30.37 ^{bc}	16.34
	S. rochei	31.85 ^{bc}	14.01	32.59 ^{ab}	10.22
	T. asperellum +*S. rochei*	28.89 $^{c–e}$	22.00	25.9 $^{c–e}$	28.57
20 ppm	Control	28.15 $^{c–e}$	24.00	24.44 $^{d–f}$	32.67
	T. asperellum	23.70 ^{fg}	36.02	22.22 $^{e–g}$	38.75
	S. rochei	26.67 $^{d–f}$	28.00	22.96 $^{e–g}$	36.75
	T. asperellum +*S. rochei*	20.74 ^{g}	44.01	19.26 ^{gh}	46.94
40 ppm	Control	28.15 $^{c–e}$	24.00	24.44 $^{d–f}$	32.67
	T. asperellum	22.96 ^{fg}	38.01	19.26 ^{gh}	46.94
	S. rochei	23.70 ^{fg}	36.02	21.48 $^{e–g}$	40.83
	T. asperellum +*S. rochei*	20.00 ^{g}	46.00	16.30 ^{h}	55.10
60 ppm	Control	34.82 ^{ab}	05.99	30.37 ^{bc}	16.34
	T. asperellum	25.93 ^{ef}	29.99	22.22 $^{e–g}$	38.79
	S. rochei	29.63 $^{c–e}$	20.01	28.15 $^{b–d}$	22.45
	T. asperellum +*S. rochei*	22.96 ^{fg}	38.01	20.74 $^{f–h}$	42.87
LSD value at 0.05:		4.17	-	4.47	-

Mean values in each column followed by a different letter are significantly different according to the LSD test ($p \leq 0.05\%$).

3.1.3. Plant Growth Parameters of Squash Plants

Under artificial inoculation with powdery mildew, the response of squash to shikimic acid as seed priming at 20, 40, and 60 ppm, with or without foliar application of BCAs, was observed. As shown in Tables 2 and 3, all tested treatments had a highly significant effect on enhancing plant growth parameters in comparison to the control in the two trials. In this regard, the maximum plant height, number of leaves, and chlorophyll content were noted following the application of 40 ppm shikimic acid through seed priming combined with exogenous foliar spray with a mixture of BCAs in both seasons.

3.1.4. Physiological Traits of Squash Plants

Transpiration rate, stomatal conductance and photosynthesis were significantly influenced by seed priming with shikimic acid and the foliar application of BCAs (Figure 2). However, no statistically significant differences were observed between 60 ppm shikimic acid and untreated squash plants in terms of transpiration rate. The values obtained for the physiological traits were higher at 40 ppm shikimic acid Overall, the mixture of BCAs as a foliar application treatment showed statistically significant ($p \leq 0.05$) transpiration rate, stomatal conductance, and photosynthesis. In this case, the untreated plants had the lowest transpiration rate, stomatal conductance and photosynthesis. The combination of seed priming with shikimic acid 40 ppm and the mixture of BCAs exhibited better transpiration rate, stomatal conductance, and photosynthesis (Figure 2A–D).

Table 2. Effect of the interaction between seed priming of shikimic acid (0, 20, 40, 60 ppm) and foliar applications with *S. rochei* (SR), *T. asperellum* (TS), *S. rochei* + *T. asperellum*(SR + TS), and water (control) on the vegetative growth characteristics of squash plants during the first experiment.

Shikimic Acid Concentrations	Bioagents Treatments	Plant Height (cm)	Leaves Numbe per Plant	Chlorophyll (SPAD) Reading
0 ppm	Control	22.73 ^{j}	9.433 ^{i}	21.73 ^{g}
	T. asperellum	47.73 $^{d–g}$	12.77 $^{f–h}$	25.10 ^{ef}
	S. rochei	38.40 ^{hi}	11.43 ^{h}	25.23 ^{ef}
	T. asperellum +S. rochei	51.07 ^{cde}	13.50 ^{fg}	35.60 ^{c}
20 ppm	Control	44.33 $^{f–h}$	11.20 ^{h}	27.00 ^{e}
	T. asperellum	54.33 $^{b–d}$	13.87 ^{f}	35.03 ^{c}
	S. rochei	47.00 $^{e–g}$	11.87 ^{gh}	35.57 ^{c}
	T. asperellum +S. rochei	61.0 ^{b}	16.87 ^{de}	46.00 ^{b}
40 ppm	Control	50.33 $^{c–f}$	16.87 ^{de}	30.73 ^{d}
	T. asperellum	59.33 ^{b}	20.20 ^{b}	46.23 ^{b}
	S. rochei	50.00 $^{c–f}$	17.87 ^{cd}	45.23 ^{b}
	T. asperellum +S. rochei	69.00 ^{a}	23.20 ^{a}	57.10 ^{a}
60 ppm	Control	35.60 ^{i}	13.87 ^{f}	23.73 ^{fg}
	T. asperellum	49.00 $^{d–f}$	17.87 ^{cd}	27.10 ^{e}
	S. rochei	42.00 $^{g–i}$	16.20 ^{e}	25.90 ^{ef}
	T. asperellum +S. rochei	56.33 ^{bc}	18.53 ^{c}	36.23 ^{c}
LSD value at 0.05:		6.7	1.646	2.6

Mean values in each column followed by a different letter are significantly different according to the LSD test ($p \leq 0.05\%$).

Table 3. Effect of the interaction between seed priming of shikimic acid (0, 20, 40, 60 ppm) and foliar applications with *S. rochei* (SR), *T. asperellum* (TS), *S. rochei* + *T. asperellum* (SR + TS), and water (control) on the vegetative growth parameters of squash plants during the second experiment.

Shikimic Acid Concentrations	Bioagents Treatments	Plant Height (cm)	Leaves Number per Plant	Chlorophyll (SPAD) Reading
0 ppm	Control	27.33 ^{h}	9.33 ^{i}	21.4 ^{i}
	T. asperellum	35.33 ^{fg}	12.67 ^{gh}	24.43 ^{gh}
	S. rochei	31.33 ^{gh}	11.33 ^{h}	26.23 ^{fg}
	T.asperellum+S. rochei	46.00 ^{cde}	14.00 ^{fg}	35.87 ^{d}
20 ppm	Control	43.0 ^{de}	14.67 ^{f}	27.40 ^{ef}
	T. asperellum	51.67 ^{c}	18.67 ^{cd}	34.90 ^{d}
	S. rochei	46.0 ^{cde}	17.00 ^{e}	37.23 ^{d}
	T.asperellum+S. rochei	60.0 ^{b}	19.33 ^{c}	47.43 ^{b}
40 ppm	Control	49.00 ^{cd}	17.67 ^{de}	29.07 ^{e}
	T. asperellum	58.0 ^{b}	21.00 ^{b}	44.23 ^{c}
	S. rochei	48.67 ^{cd}	18.67 ^{cd}	43.23 ^{c}
	T.asperellum+S. rochei	67.67 ^{a}	24.00 ^{a}	55.10 ^{a}
60 ppm	Control	30.67 $_{gh}$	12.00 ^{h}	22.07 ^{hi}
	T. asperellum	45.67 ^{cde}	14.67 ^{f}	25.43 ^{fg}
	S. rochei	40.00 ^{ef}	12.67 ^{gh}	27.23 ^{ef}
	T.asperellum+S. rochei	59.0 ^{b}	17.67 ^{de}	36.10 ^{d}
LSD value at 0.05:		6.0	1.6	2.58

Mean values in each column followed by a different letter are significantly different according to the LSD test ($p \leq 0.05\%$).

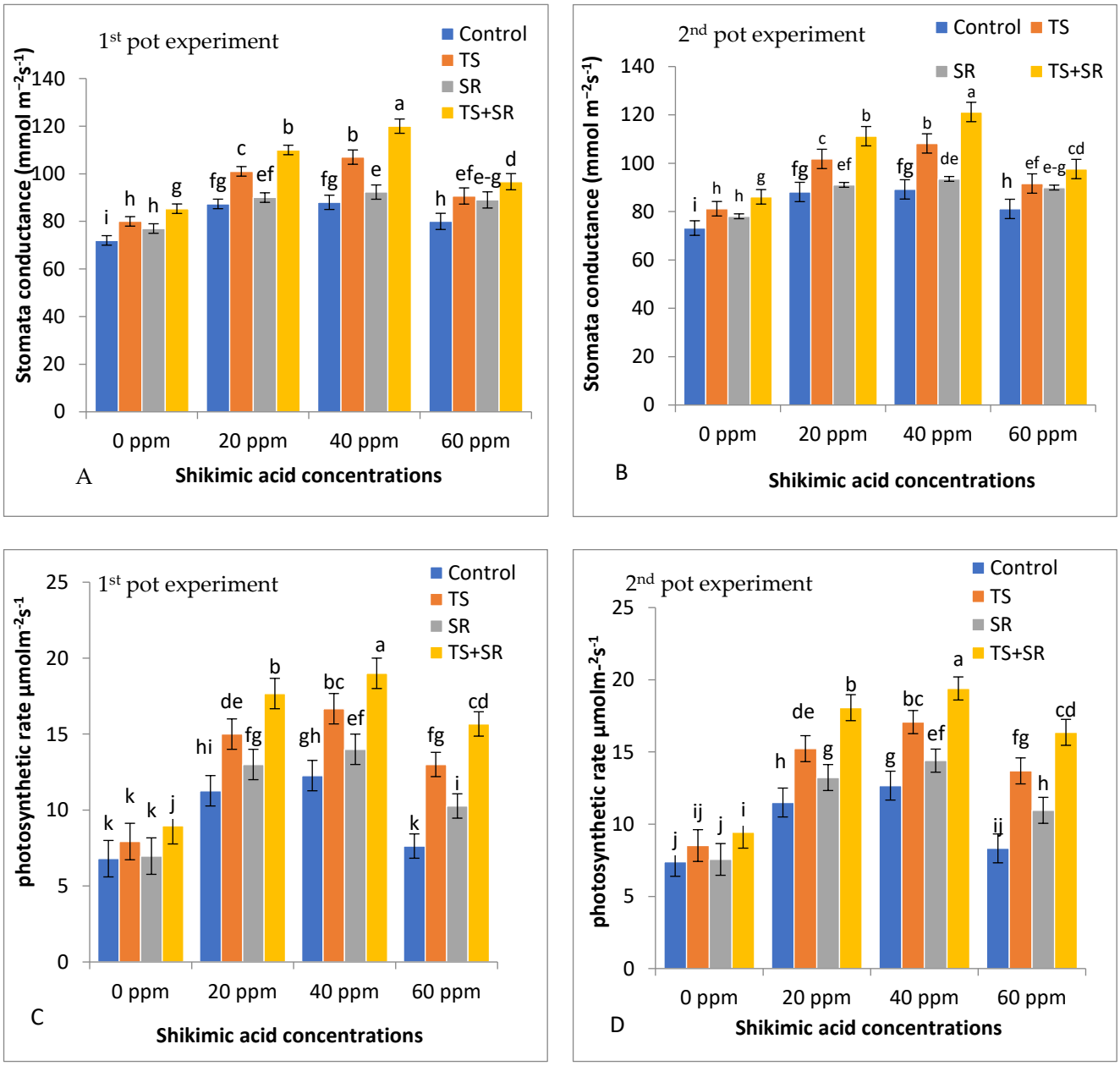

Figure 2. Effect of the interaction between seed priming of shikimic acid concentration (0, 20, 40, 60 ppm) and foliar applications with water (control), *T. asperellum* (TS), *S. rochei* (SR), *T. asperellum* + *S. rochei* + (TS + SR), and on (**A**) Stomata conductance in 1st pot experiment (**B**) Stomata conductance in 2nd pot experiment, and (**C**) photosynthetic rate in 1st pot experiment, (**D**) photosynthetic rate in 2nd pot experiment of squash plants. Standard errors of the mean are shown as vertical bars; the LSD test indicates that differences between values in each bar that are marked by different letters are significant at $p \leq 0.05$.

3.2. Greenhouse Experiments

3.2.1. Disease Assessment

The response of squash to 40 ppm shikimic acid seed priming with or without foliar application of BCAs was evaluated after 50 d of growth under natural infection with powdery mildew. As shown in Table 4, all treatments showed significantly ($p \leq 0.05$) decreased the final powdery mildew severity level (FDL%) and the area under the disease progress curve (AUDPC) compared to the control in both seasons. The lowest disease severity percentages were obtained with 40 ppm shikimic acid, through seed priming combined

with the mixture of BCAs *(S. rochei* + *T. asperellum*) foliar spray (37.22% and 52.78%) and BCA mixture (*S. rochei* + *T. asperellum*) foliar spray alone (41.11% and 57.78%), with 37.38–30.84% and 32.14–25.71% disease reduction in the first and second season, respectively. Furthermore, the AUDPC was decreased by 51.56% and 45.72% following seed priming with 40 ppm shikimic acid treatment in combination with the BCA mixture (*T. asperellum* + *S. rochei*) compared to the control during the first and second season, respectively.

Table 4. Effect of 40 ppm shikimic acid (SA) seed priming and foliar applications of bioagents on the final powdery mildew disease severity level (FDL%) and the area under the disease progress curve (AUDPC) during the 2020–2021 and 2021–2022 growing seasons.

Treatment	FDL %	Reduction %	AUDPC	Reduction %
First season				
control	59.44 ^{a}	-	871.11 ^{a}	-
SA	49.44 ^{b}	16.82	672.78 ^{b}	22.77
SR	47.78 ^{bc}	19.62	614.44 ^{bc}	29.46
SA + SR	46.11 $^{b–d}$	22.43	523.06 ^{cd}	39.95
TS	43.89 $^{c–e}$	26.16	507.50 ^{de}	41.74
SA + TS	43.33 ^{de}	27.10	484.17 ^{de}	44.42
SR + TS	41.11 ^{ef}	30.84	455.00 ^{de}	47.77
SA + SR + TS	37.22 ^{f}	37.38	421.94 ^{e}	51.56
LSD.05	4.23	-	92.85	-
Second season				
control	77.78 ^{a}	-	1165.28 ^{a}	-
SA	71.67 ^{b}	7.86	1071.94 ^{ab}	8.00
SR	67.78 ^{bc}	12.86	974.72 ^{bc}	16.35
SA + SR	63.33 ^{cd}	18.58	869.72 ^{cd}	25.36
TS	61.11 ^{de}	21.43	801.67 ^{de}	31.20
SA + TS	59.44 ^{de}	23.58	718.06 ^{ef}	38.37
SR + TS	57.78 ^{e}	25.71	688.89 ^{f}	40.88
SA + SR + TS	52.78 ^{f}	32.14	632.50 ^{f}	45.72
LSD.05	4.92	-	106.58	-

Mean values in each column followed by a different letter are significantly different according to the LSD test ($p \leq 0.05$%). SA; shikimic acid at rate 40 ppm, TS; *T. asperellum*, SR; *S. rochei*.

3.2.2. Plant Growth

The response of squash to 40 ppm shikimic acid seed priming with or without foliar application of BCAs was evaluated after 50 d of growth under natural infection with powdery mildew. As shown in Table 5, all treatments showed significantly ($p \leq 0.05$) increased plant growth compared to the control in both seasons.

Table 5. Effect of seed priming with 40 ppm shikimic acid (SA) and foliar applications of bioagents on the vegetative growth characteristics of squash plants during the 2020 and 2021 seasons.

Treatment	Plant Hight (cm)	Leaves Number/ Plant	Plant Fresh Weight (gm)	Plant Dry Weight (gm)	Chlorophyll (SPAD) Reading
2020 season					
Control	44.0 ^{g}	12.0 ^{f}	155.7 ^{h}	41.0 ^{f}	30.47 ^{h}
SA	64.0 ^{d}	21.0 ^{c}	310.0 ^{e}	85.0 ^{d}	38.93 ^{e}
TS	60.0 ^{e}	18.3 ^{d}	250.0 ^{f}	72. 7 ^{e}	36.33 ^{f}
SR	50.0 ^{f}	14.7 ^{e}	204.7 ^{g}	67.7 ^{e}	33.73 ^{g}
TS + SR	69.3 ^{c}	22.0 ^{c}	354.3 ^{d}	101.3 ^{c}	42.33 ^{d}
SA + TS	77.3 ^{b}	24.3 ^{b}	445.0 ^{b}	110.3 ^{b}	48.0 ^{b}
SA + SR	75.67 ^{b}	21.7 ^{c}	396.7 ^{c}	106.3 ^{bc}	44.7 ^{c}
SA + TS+ SR	86.33 ^{a}	30.0 ^{a}	543.0 ^{a}	146.3 ^{a}	52.63 ^{a}
LSD.05	3.34	2.2	38.24	5.69	1.20

Table 5. *Cont.*

Treatment	Plant Hight (cm)	Leaves Number/ Plant	Plant Fresh Weight (gm)	Plant Dry Weight (gm)	Chlorophyll (SPAD) Reading
2021 season					
Control	40.3 ^{h}	13.0 ^{e}	157.0 ^{g}	42.0 ^{f}	31.57 ^{h}
SA	60.3 ^{e}	22.7 ^{c}	313.3 ^{d}	85.3 ^{d}	40.0 ^{e}
TS	53.9 ^{f}	19.0 ^{d}	256.7 ^{e}	73.3 ^{e}	37.4 ^{e}
SR	45.9 ^{g}	15.3 ^{e}	207.3 ^{f}	68.3 ^{e}	34.6 ^{e}
TS + SR	71.3 ^{d}	22.7 ^{c}	352.7 ^{d}	102.7 ^{c}	43.1 ^{d}
SA + TS	86.0 ^{b}	25.3 ^{b}	446.7 ^{b}	111.7 ^{b}	48.8 ^{b}
SA + SR	79.0 ^{c}	22.3 ^{c}	399.3 ^{c}	107.3 ^{bc}	45.5 ^{c}
SA + TS + SR	95.0 ^{a}	31.7 ^{a}	546.7 ^{a}	149.0 ^{a}	53.9 ^{a}
LSD.05	1.3	2.4	39.68	6.857	1.2

Mean values in each column followed by a different letter are significantly different according to the LSD test ($p \leq 0.05\%$). SA; shikimic acid at rate 40 ppm, TS; *T. asperellum*, SR; *S. rochei.*

The greatest plant height, number of leaves, fresh weight, and dry weight were obtained with 40 ppm shikimic acid combined with the BCA mixture (*T. asperellum* + *S. rochei*) as a foliar application in both the first and second season. Furthermore, the chlorophyll content was increased by 72.7% and 70.7% following seed priming with 40 ppm shikimic acid treatment in combination with the BCAs mixture (*T. asperellum* + *S. rochei*) as a foliar spray compared to the control during the first and second season, respectively.

3.2.3. Yield and Its Components

The data in Figure 3A–D clearly show that priming of squash seeds with shikimic acid at a rate of 40 ppm and the foliar application of *T. asperellum* with or without *S. rochei* significantly ($p \leq 0.05$) influenced the total yield and its components compared to the control in both seasons. Additionally, 40 ppm of shikimic acid combined with *T. asperellum* + *S. rochei* foliar application increased the average fruit weight (32.43%, 85.8%), number of fruits per plant (62.4%, 69.7%), total fruit yield per plant (83%, 90%), and total yield per hectare, compared with the control in the first and second seasons. However, squash plants treated with 40 ppm shikimic acid seed priming or *T. asperellum* as a foliar application did not significantly differ from the conventional control treatment with respect to total fruit yield per plant in the second season.

3.2.4. Mineral Content in Squash Leaves

The results in Figure 4A–D show that all treatments significantly increased the concentrations of macronutrients, i.e., nitrogen (N), phosphorus (P), potassium (K), and magnesium (Mg), in the leaves of squash plants compared to the control. A marked increase in N (5.9% and 4.6%), P (4.5% and 4.3%), K (4% and 4.2%), and Mg (1.5% and 2.0%) was observed with 40 ppm shikimic acid seed priming combined with foliar application of the BCA mixture (*T. asperellum* + *S. rochei*) compared to the control in the first and second season, respectively. The same figures show that the highest calcium (Ca) concentrations (3.59% and 3.03%) in squash leaves were found for 40 ppm shikimic acid seed priming combined with the BCA mixture (*T. asperellum* + *S. rochei*) as a foliar spray, followed by 40 ppm of shikimic acid seed priming combined with *T. asperellum* as a foliar application in the first season. In addition, application of 40 ppm shikimic acid in combination with the foliar application of the BCA mixture (*T. asperellum* + *S. rochei*), *T. asperellum,* and *S. rochei* showed the most significant increase in Ca concentrations in the leaves (2.9%, 3.03%, and 3.33%), with no significant difference among them compared to the control treatment in the second season.

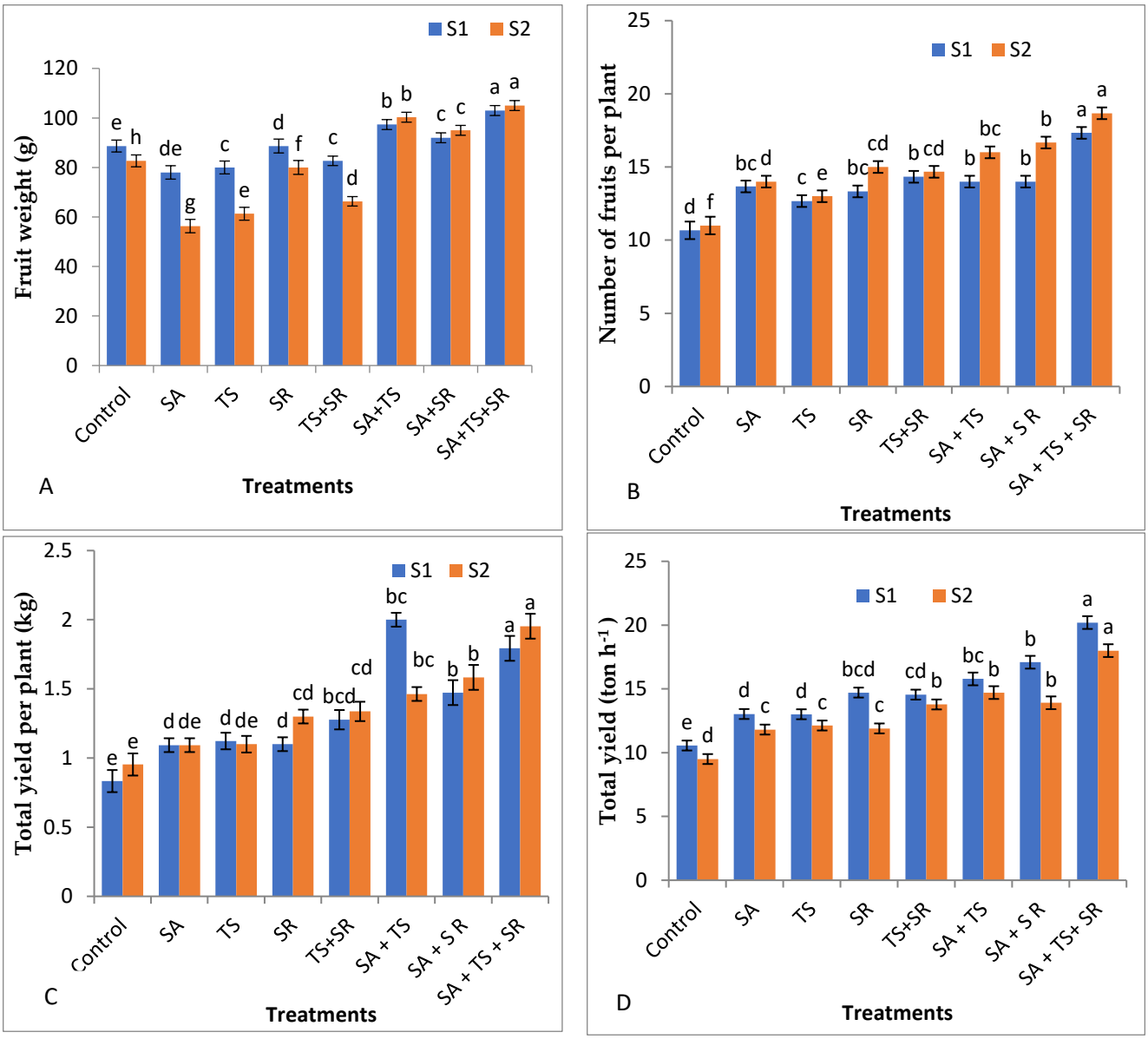

Figure 3. Effect of shikimic acid (SA) seed priming rates 40 ppm and foliar applications of water (control), *T. asperellum* (TS), *S. rochei* (SR), *T. asperellum* + *S. rochei* + (TS + SR), *shikimic acid* + *T. asperellum* (SA + TS), shikimic acid+ *S. rochei* (SA + SR), and shikimic acid + *T. asperellum* + *S. rochei* (SA + TS + SR) on (**A**) average fruit weight (g), (**B**) number of fruits per plant, (**C**) total fruit yield per plant (kg), and (**D**) total fruit yield per hectare during two seasons. Standard errors of the mean are shown as vertical bars; the LSD test indicates that differences between values in each bar that are marked by different letters are significant at $p \leq 0.05$.

In terms of the effect of treatments on the micronutrient content of squash leaves (Figure 5A–D), boron (B), iron (Fe), zinc (Zn), and manganese (Mn) were considerably affected by shikimic acid (40 ppm) seed priming, the BCA mixture (*T. asperellum*+ *S. rochei*) foliar application, and their interactions. Furthermore, the highest concentrations of B (55.47% and 57.17%), Fe (76.12% and 78.02%), Zn (56.15% and 57.18%), and Mn (66.3% and 68.0%) were obtained from plants treated with 40 ppm shikimic acid and combined BCA (*T. asperellum* + *S. rochei)* foliar application compared to the control in the first and the second season, respectively. Nevertheless, the lowest concentrations of B (14.18% and 16.08%), Fe (30.6% and 33%), Zn (13.14% and14.34%), and Mn (27.63% and 28.53%) were obtained from untreated plants during the first and second season, respectively.

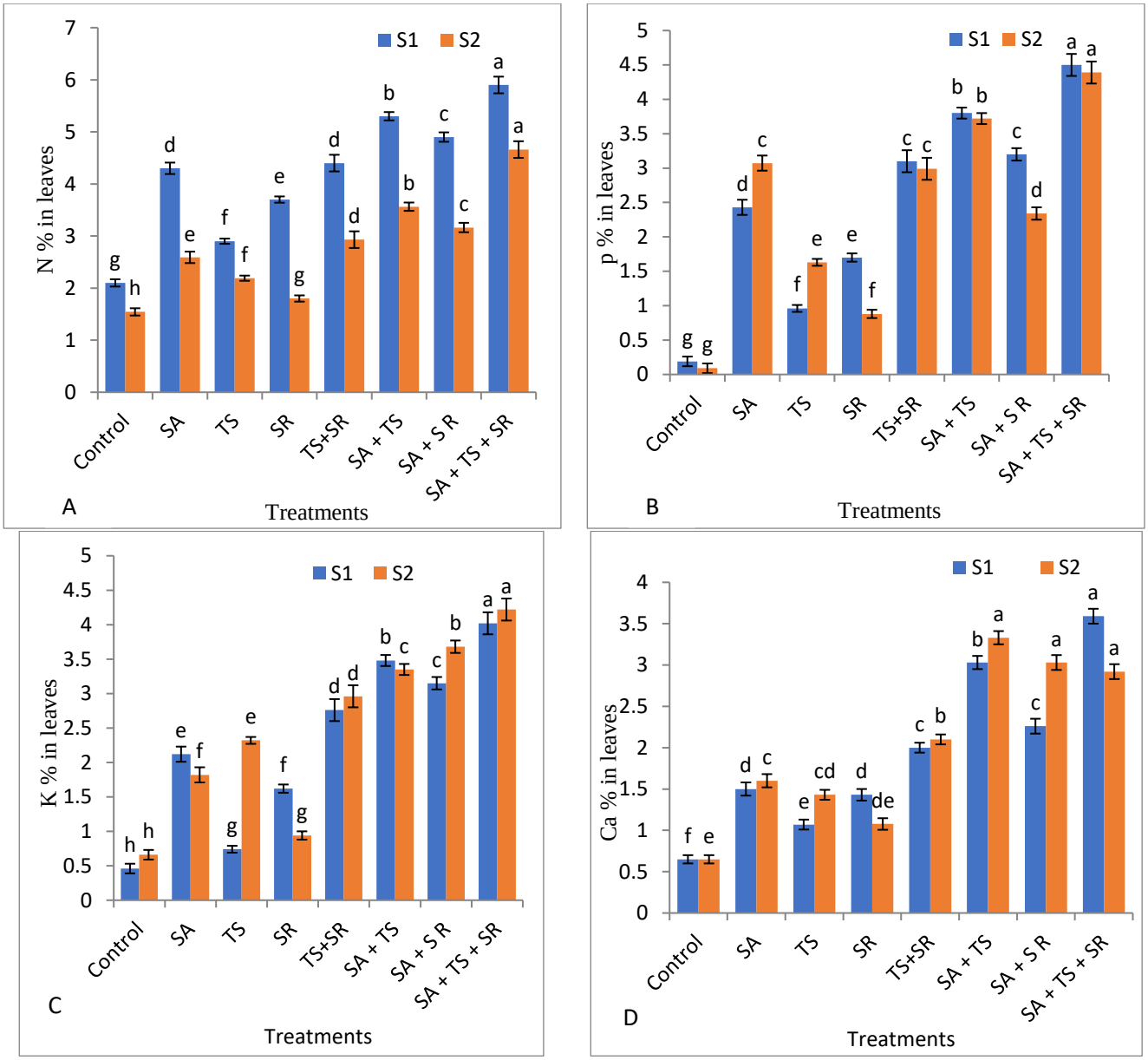

Figure 4. Effect of shikimic acid (SA) seed priming rates 40 ppm and foliar applications of water (control), *T. asperellum* (TS), *S. rochei* (SR), *T. asperellum* + *S. rochei* + (TS + SR), shikimic acid + *T. asperellum* (SA + TS), shikimic acid + *S. rochei* (SA + SR), and shikimic acid + *T. asperellum* + *S. rochei* (SA + TS + SR) on (**A**) N%, (**B**) P%, (**C**) K%, and (**D**) Ca% in leaves during two seasons. Standard errors of the mean are shown as vertical bars; the LSD test indicates that differences between values in each bar that are marked by different letters are significant at $p \leq 0.05$.

3.2.5. Antioxidant Enzymes, Total Phenols, and Plant Hormones in Squash Leaves

POD, SOD, and CAT enzyme activities were assayed in untreated and treated squash plant leaves, as shown in Figure 6A–C. Constitutive SOD, POD, and CAT activity were recorded for all treated plants and compared with the control in both seasons. Maximum SOD (346 and 353 units mg^{-1} protein), POD (686 and 675.5 units mg^{-1} protein), and CAT (189 and 183.2 units mg^{-1} protein) were recorded under a combination of seed priming with shikimic acid at 40 ppm and *T. asperellum* + *S. rochei* as a foliar application during the first and second season, respectively. The lowest activity was observed in the control plants (Figure 6A–F).

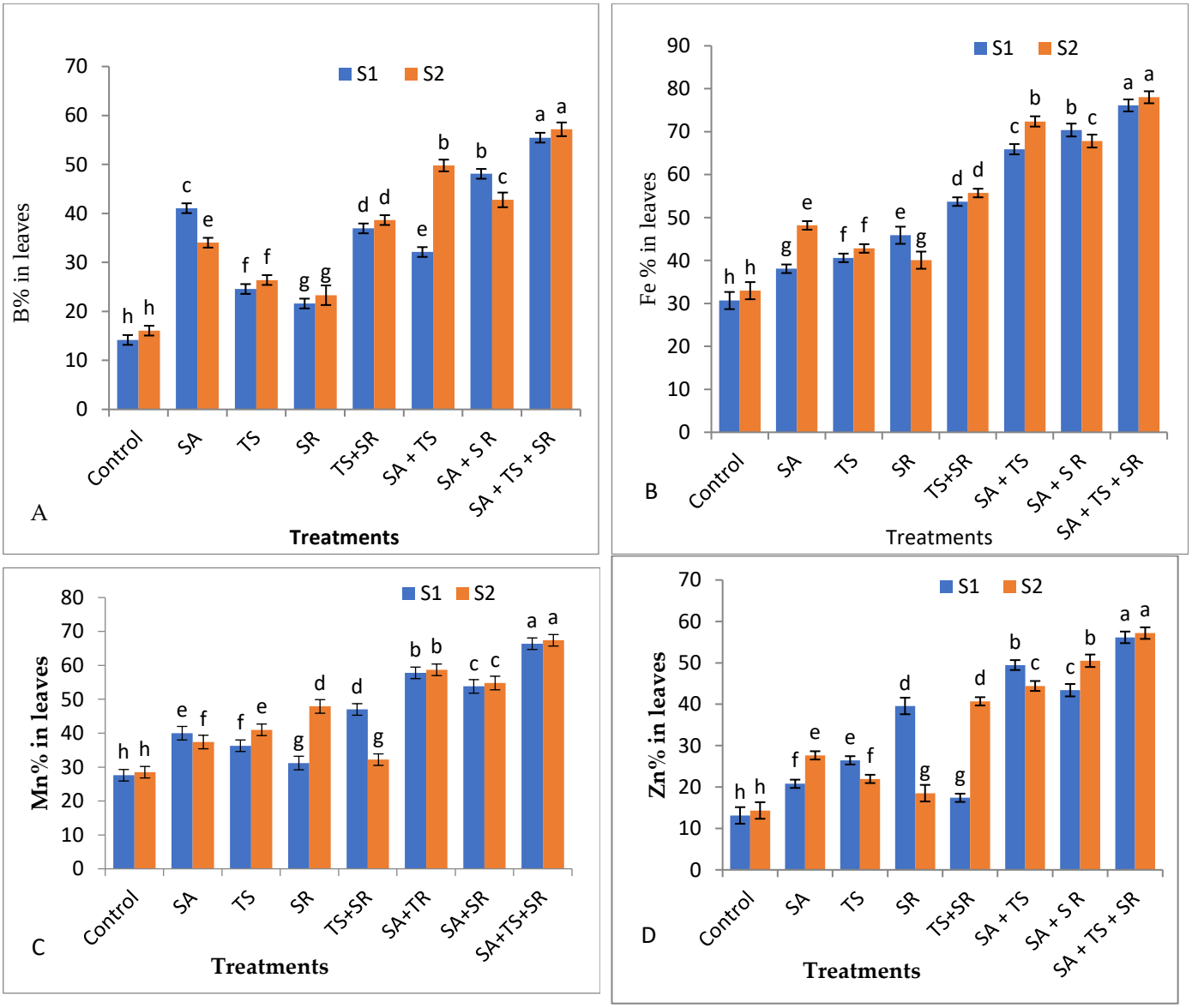

Figure 5. Effect of shikimic acid (SA) seed priming rates 40 ppm and foliar applications of water (control), *T. asperellum* (TS), *S. rochei* (SR), *T. asperellum* + *S. rochei* + (TS + SR), shikimic acid + *T. asperellum* (SA + TS), shikimic acid+ *S. rochei* (SA+SR), and shikimic acid + *T. asperellum* + *S. rochei* (SA + TS + SR) on (**A**) B%, (**B**) Fe%, (**C**) Mn%, and (**D**) Zn% in leaves during two seasons. Standard errors of the mean are shown as vertical bars; the LSD test indicates that differences between values in each bar that are marked by different letters are significant at $p \leq 0.05$.

The highest total phenol content was recorded for 40 ppm shikimic acid combined with BCA mixture foliar application (87.7 and 78.47 mg GAE eq g^{-1}), followed by 40 ppm shikimic acid combined with foliar application of *T. Asperellum* (81.08 and 71.58 mg GAE eq g^{-1}) as compared with untreated plants in the first and second season, respectively. On the other hand, the lowest content of total phenols in leaves (53.41 and 44.21 mg GAE eq g^{-1}) was recorded in untreated plants during the first and second season, respectively (Figure 6D).

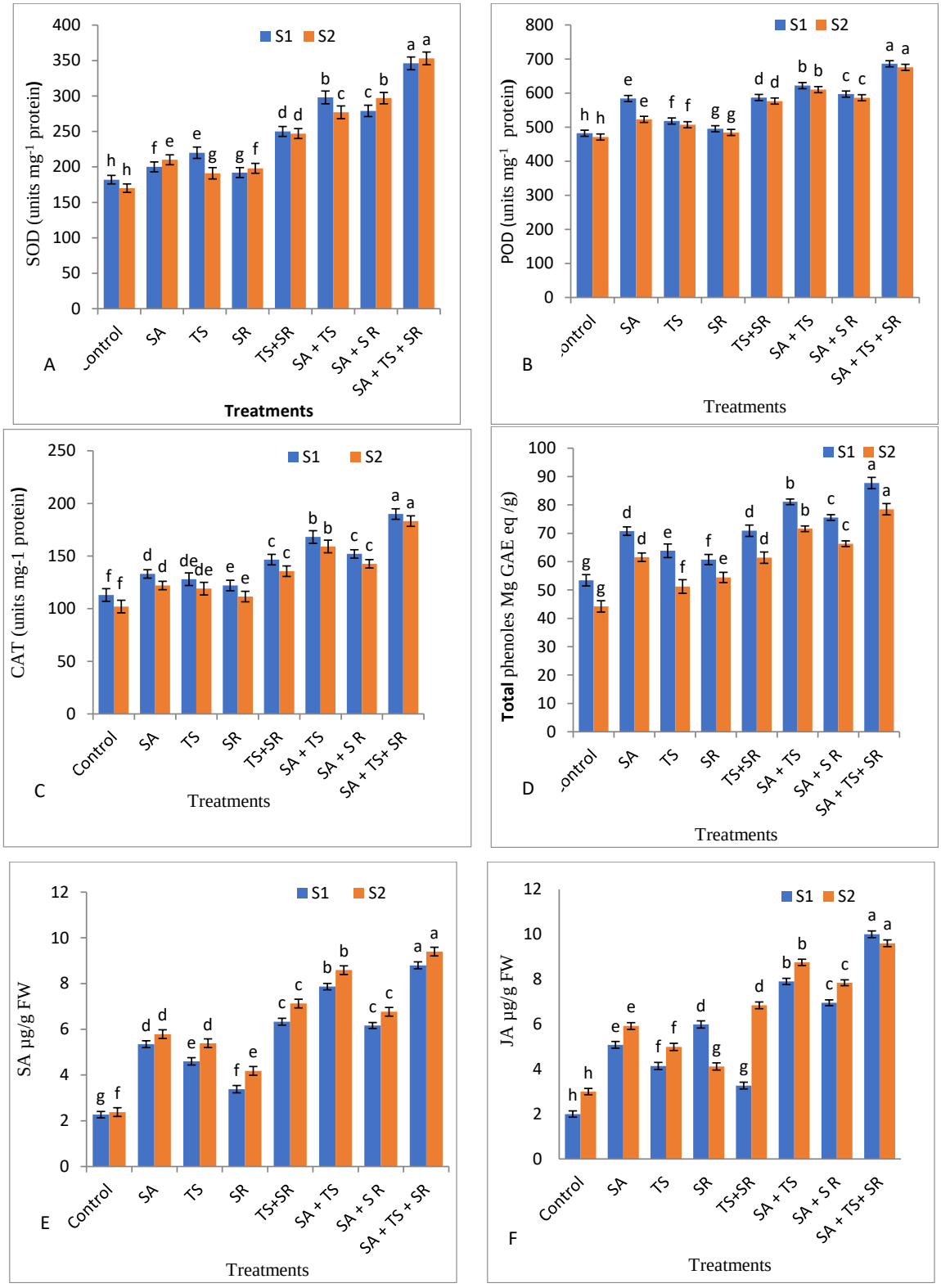

Figure 6. Effect of shikimic acid (SA) seed priming rates 40 ppm and foliar applications of water (control), *T. asperellum* (TS), *S. rochei* (SR), *T. asperellum* + *S. rochei* + (TS + SR), shikimic acid + *T. asperellum* (SA + TS), shikimic acid + *S. rochei* (SA + SR), and shikimic acid + *T. asperellum* + *S. rochei* (SA + TS + SR) on (**A**) SOD, (**B**) POD, (**C**) CAT, (**D**) phenols, (**E**) SA, and (**F**) JA in leaves during two seasons. Standard errors of the mean are shown as vertical bars; the LSD test indicates that differences between values in each bar that are marked by different letters are significant at $p \leq 0.05$.

When comparing treated and untreated plants under natural infection with powdery mildew, lower amounts of JA (2.1 and 3.0 µg g^{-1} FW) and SA (2.26, 2.38 µg g^{-1} FW) were observed in the untreated plants in the first and second season, respectively. Meanwhile, the maximum JA (10 and 9.6 µg g^{-1} FW) and SA (8.8 and 9.4 µg g^{-1} FW) values were recorded under a combination of seed priming with 40 ppm shikimic acid and *T. asperellum* + *S. rochei* foliar application during the first and the second season, respectively (Figure 6E–F). JA is often negatively correlated with SA, and we also observed that the SA level was decreased.

3.3. Clustering Analysis

Our cluster analysis included all growth traits, all yield components, and all mineral compositions of the squash plants along with the activity of antioxidant enzymes, total phenols, SA, and JA. Figure 7 presents a heatmap showing the relationships among the seed priming and foliar application of *T. asperellum*, *S. rochei*, and a mixture of the two, based on the tested parameters. A heatmap analysis clearly identified the overall variations among all treatments. The foliar application of a mixture of *T. asperellum* and *S. rochei* alone showed an increase in all studied traits compared with the control. However, seed priming with shikimic acid at a rate of 40 ppm and treatment with *T. asperellum* + *S. rochei* foliar application decreased oxidative injury and AUDPC values by enhancing plant growth, increasing antioxidant enzyme concentrations, and significantly improving plant growth and yield under natural infection with powdery mildew (Figure 7).

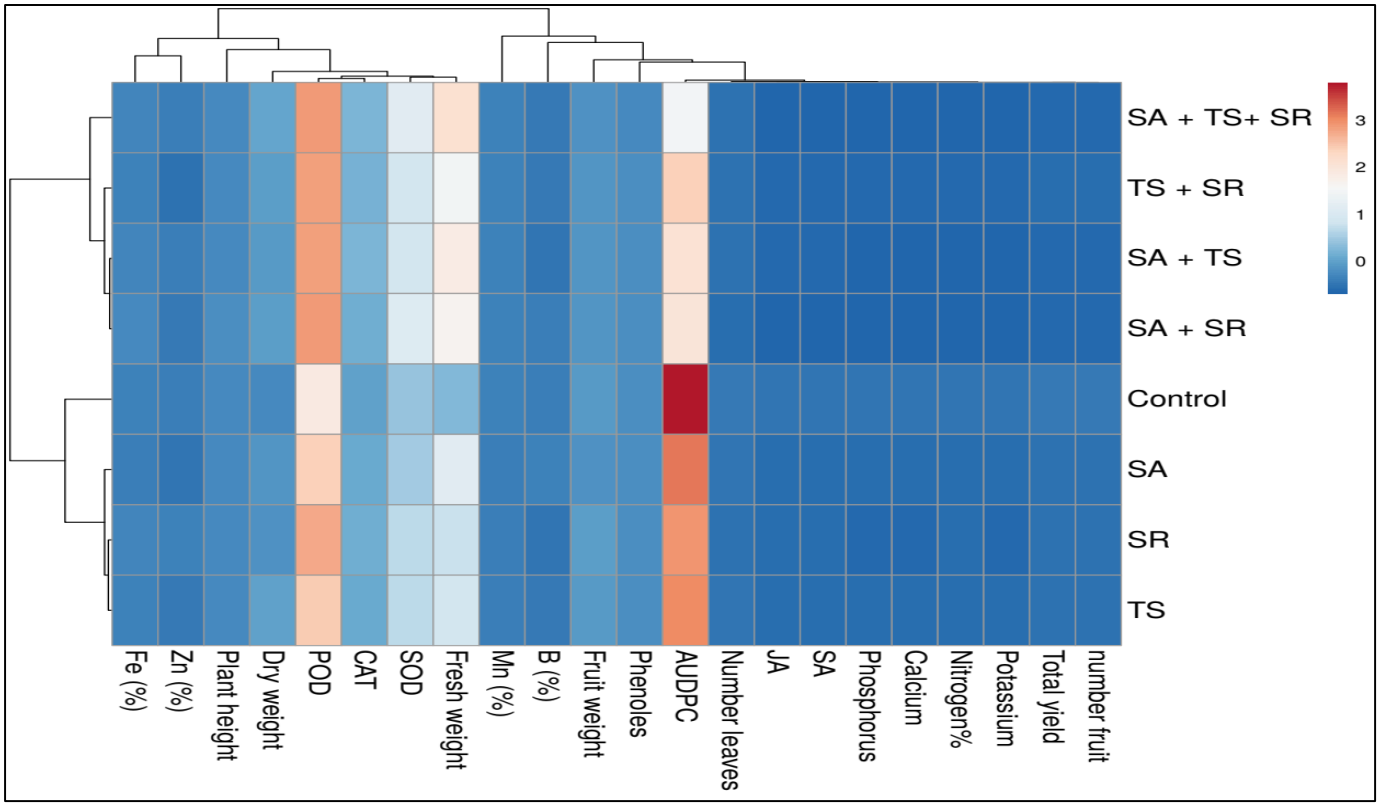

Figure 7. Heatmap of shikimic acid (SA) seed priming rates 40 ppm and foliar applications of *S. rochei* (SR), *T. asperellum* (TS), *S. rochei* + *T. asperellum* (SR + TS), and water (control) and measured parameters of squash plants. The differences in the response variables between all studied treatments are visualized in the heatmap diagram. Columns represent the individual response variables, while rows represent the treatments. Lower numerical values are colored blue, whereas higher numerical values are colored red (see the scale at the top right corner of the heatmap).

3.4. Trait Interrelationships

The association among the evaluated morphological, yield, and physiochemical traits of the squash plants were estimated based on principal component analysis (PCA; Figure 8). Data were analyzed using PCA to establish a relationship between priming with shikimic acid (40 ppm) and the application of *T. asperellum* + *S. rochei* on plant growth and yield parameters. The sum of principal components 1 and 2 (PC1 and PC2) accounted for 93.03% of the variation among plants. PC1, the first component, made up 88.93% of the total variation, and while the second component accounted for 4.1% of the total variation.

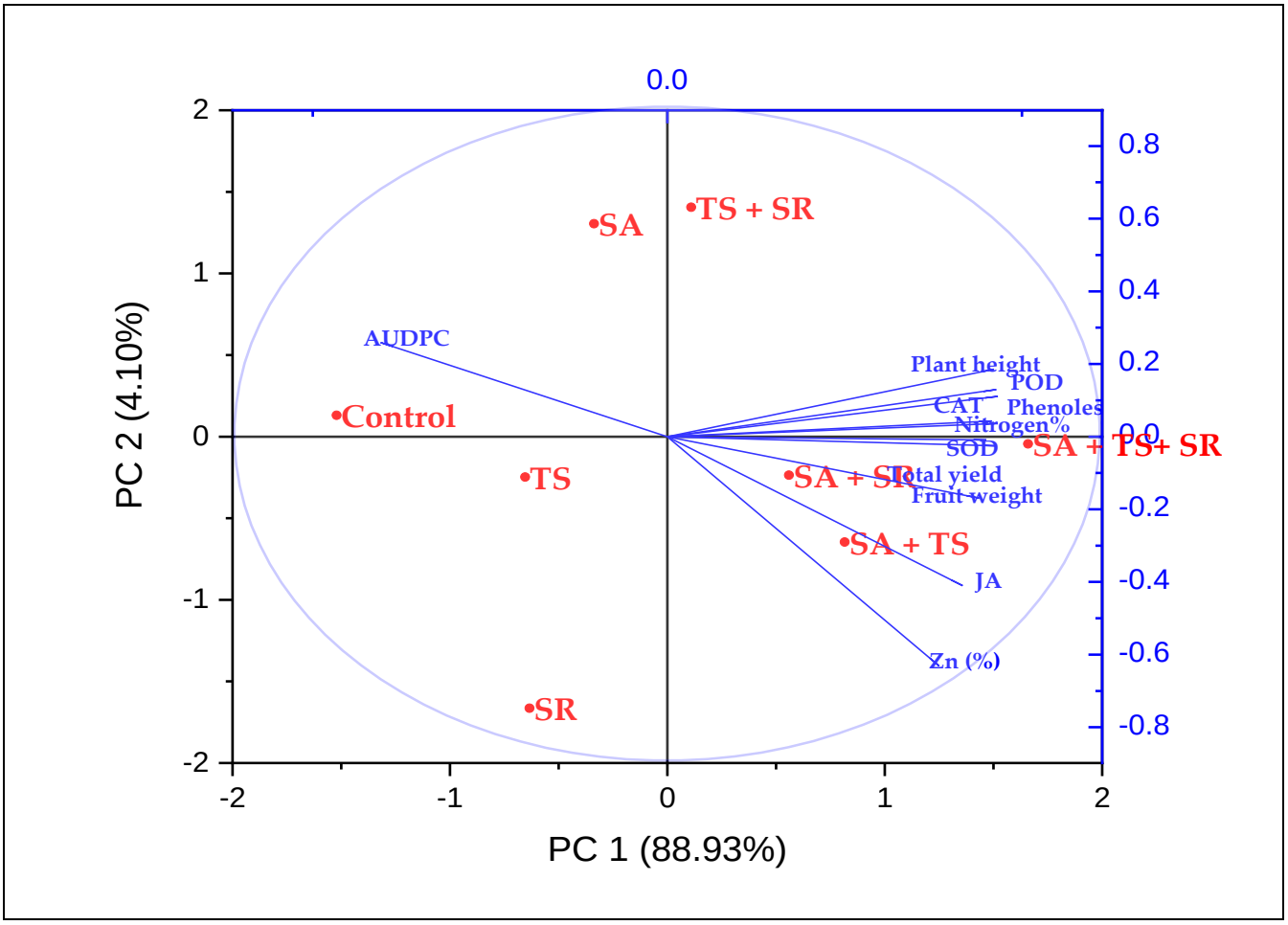

Figure 8. Biplot of the first two principal components for the morphological, yield, AUDPC, and physiochemical traits of sqaush plants. Red circles indicate the eight treatments: shikimic acid (SA) seed priming rates 40 ppm and foliar applications of *S. rochei* (SR), *T. asperellum* (TS), *S. rochei* + *T. asperellum* (SR + TS), and water (control).

3.5. GG/MS Analysis of T. asperellum Culture Filtrate

Our analysis of the *T. asperellum* culture filtrate led to the detection of 28 metabolites, as shown in Table 6. The most abundant component was hemin cation (Heme), at 38.95%.

3.6. Leaf Anatomy

Microscopic counts and measurements of several histological characteristics in transverse sections through the mature fifth leaf developed on the main stem of 50-day-old summer squash plants affected by powdery mildew for the control and treated with shikimic acid, bioagents *S. rochei* and *T. asperellum*, and a mixture of the two are shown in Table 7 and Figures 9–11.

Table 6. Composition of the *T. asperellum* (MW965676) culture filtrate.

No.	RT	Compounds	Area %
1	3.44	1-methoxy-2-acetoxypropa NE	4.97
2	4.27	Cyclohexane, (ethoxymethoxy)-	1.40
3	4.35	Tetracarbonyl) [1-(t-buty l)-2,4-bis{[2′,4′,6′-tris(t-butyl) phenyl] imino}-1-aza-2,4-dip hospha-3-molybdacyclobutane	0.96
4	4.59	Z)-6-deoxy-1,2-o-isopropyliDene-3,4-di-o-methyl-5-à-d-xYlo-hepteno-furannurononItrile	0.96
5	5.49	2H-Pyran, 3,6-dihydro-4-methyl-2-(2-methyl-1-p ropenyl)-	0.86
6	7.13	1-pentene, 3,4-dimethyl-	1.28
7	8.14	1,5-pentanediamine	1.18
8	9.91	6-benzyloxy-7-methoxy-1,2,3,4-tetrahydroisoquinoline	4.91
9	13.08	1,5-Hexadien-3-yne, 2-methyl-	2.11
10	13.13	Benzeneacetic acid	1.02
11	13.20	Cycloheptatrienylium, iodide	2.50
12	13.46	1,5-Hexadien-3-yne, 2-methyl-	0.47
13	14.21	1,6-Heptadien-3-yne	0.78
14	15.29	3-Benzylsulfanyl-3-fluoro-2-trifluoro Methyl-acrylonitrile	1.11
15	15.48	3-pyridinemethanol	0.68
16	15.92	Nà-Z-D-2,3-Diaminopropionic acid	1.23
17	16.18	Progesterone 3-biotin	2.33
18	16.45	Tetrahydro-1,3-oxazine-2-thione	0.60
19	17.52	[5-(4′-Formyl phenyl) -10,15,20-tris(4″-tolyl) porphyrinat o] zinc(ii)	1.46
20	18.67	2)-n-cyclohexyl carbamoyl)-4-methoxy-4-methyl-6,6-diphe nyl-2,9,10-triazatricyclo [5. 2.2.0(1,5)] undeca-4,8,10-trien-3-one	0.64
21	19.45	Gln-pro-arg	1.75
22	22.40	7-Oxabicyclo [4.1.0] heptan-2-one	2.93
23	24.74	1-butanol, 2-nitro	2.77
24	25.80	Oxalic acid, cyclobutyl nonyl ester	1.72
25	28.22	4-hydroxyhexenal	3.48
26	35.84	Norcollatone	2.31
27	36.26	2,4,5-tris)Isopropyl)-1,1,3,3-tetrachlOro-2,4,5-triphospha-1,3-dig Ermolane	4.06
28	36.58	Hemin cation	38.95

Table 7. Counts and measurements in micrometers (μm) of certain histological characters in transverse sections through the blade of the fifth leaf developed on the main stem of summer squash (cv. Eskandarani), aged 40 days, affected by powdery mildew as a control and treated with shikimic acid, bioagent (*S. rochei* + *T. Asperullum*), and a mix between them (Means of three sections from three specimens).

Histological Aspects	Treatments			
	Control	40 ppm (SA)	(SR + TS)	40 ppm SA and (SR + TS)
Thickness of midvein	2020.766	2404.648	2501.365	2812.411
Thickness of upper epidermis	12.132	12.418	13.459	15.422
Thickness of lower epidermis	6.987	7.196	8.633	8.951
Thickness of lamina	120.173	120.688	124.944	145.919
Thickness of palisade tissue	40.700	44.202	42.102	50.911
Thickness of spongy tissue	53.877	57.404	62.465	69.911
No of vascular bundle	4.000	4.000	5.000	6.000
Dimentions of main midvein bundle	454.521	469.419	490.742	559.504
No of xylem rows in main midvein bundle	3.2	3.2	3.3	3.5
Mean diameter of vessels	30.614	30.699	30.888	30.921
Mean diameter of paranchyma cells in ground tissues	104.666	111.945	108.887	120.111

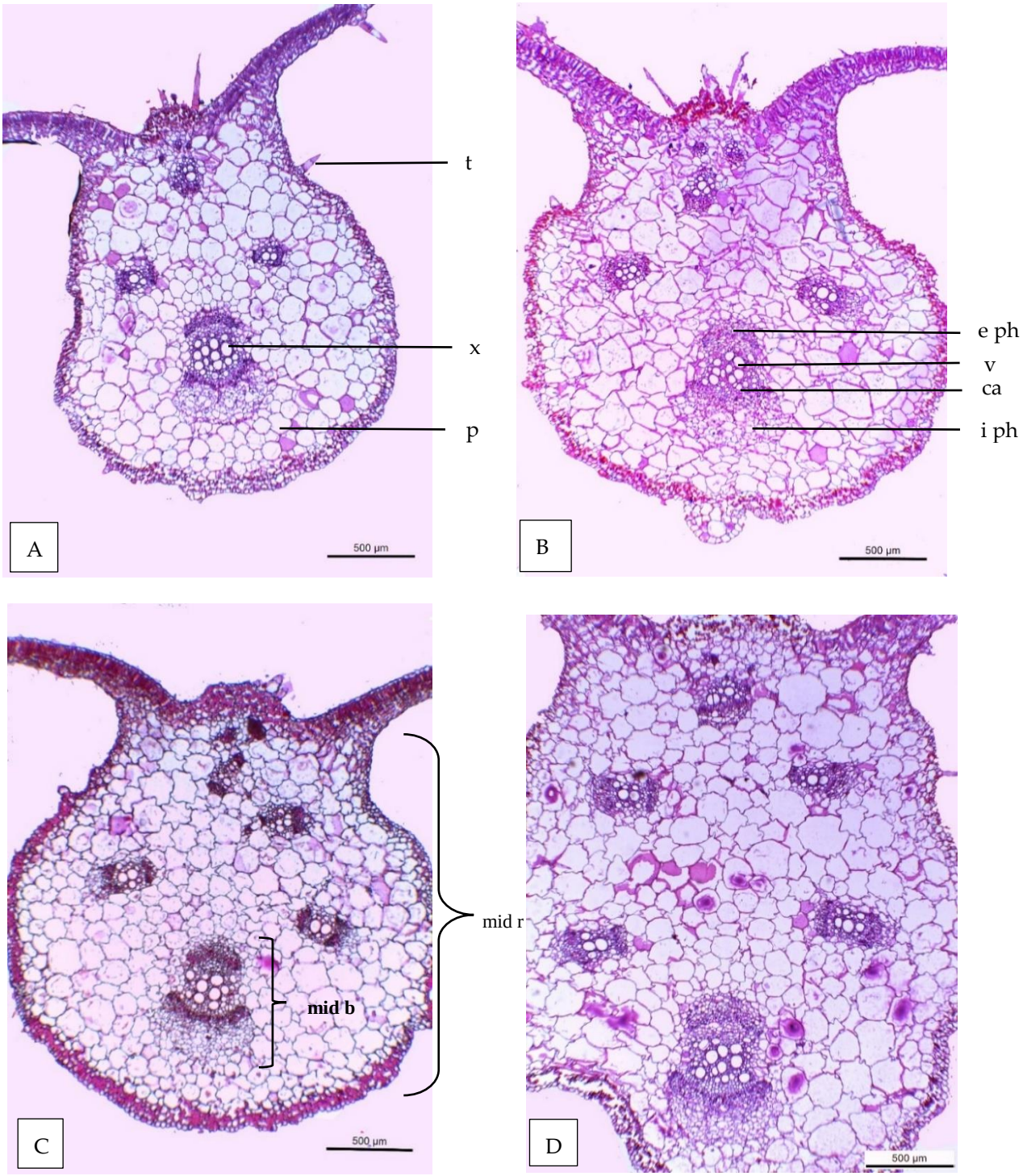

Figure 9. Microphotographs of cross sections through the blade of the fifth leaf developed on the main stem of summer squash (cv. Eskandarani), aged 50 days. Scale bars = 500 µm. (**A**) Plants affected by powdery mildew (control). (**B**) Plants treated by 40 ppm shikimic acid (SA). (**C**) Plants treated by *S. rochei* + *T. Asperullum* (SR + TS). (**D**) Plants treated by mix between 40 ppm SA and (SR + TS). Abbreviations: mid b—midvein bundle; mid r—midvein region; t—trichome; p—paranchyma cells; ca—cambium zone; e ph—external phloem; i ph—internal phloem; v—vessels; and x—xylem.

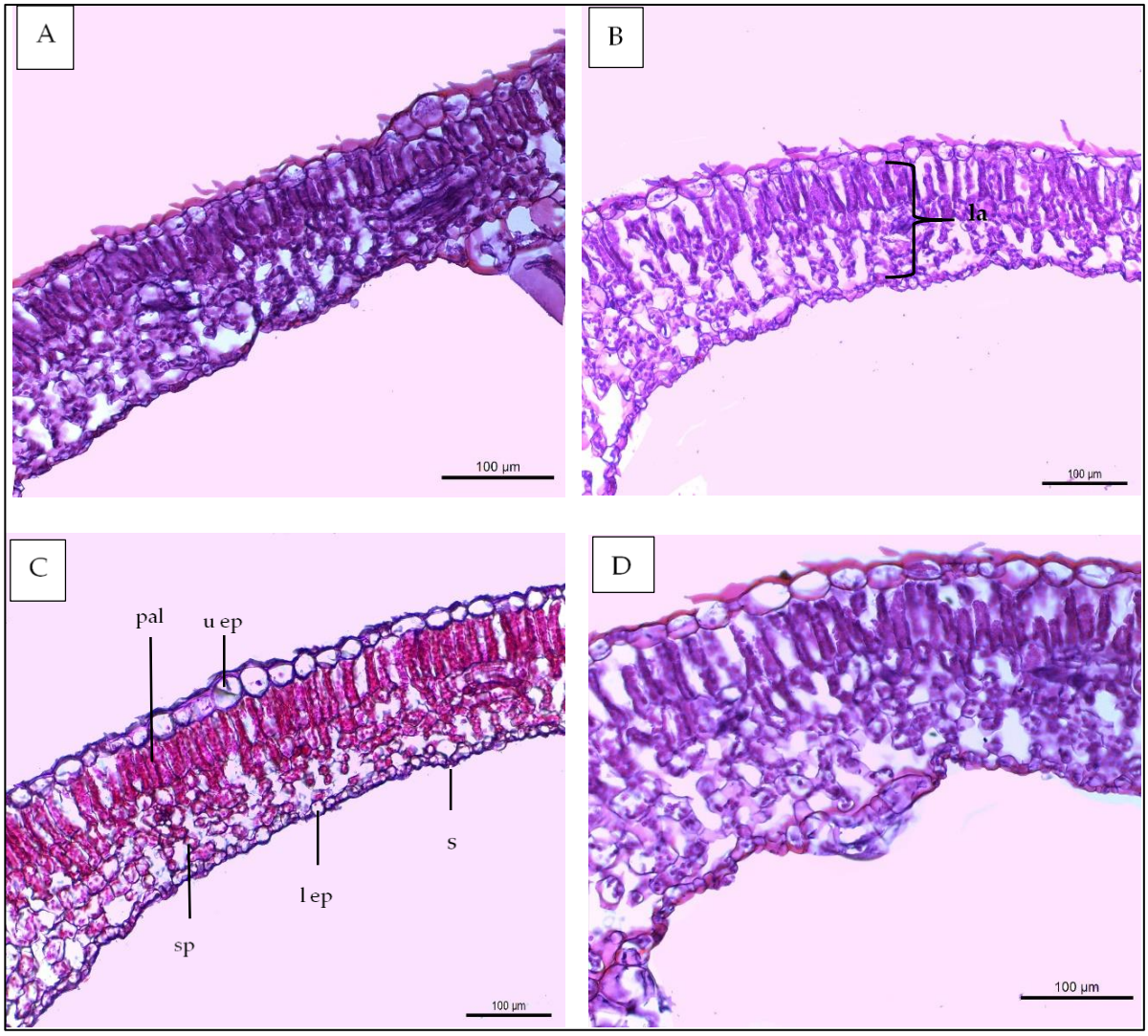

Figure 10. Magnified portions of the leaf blade lamina of summer squash (cv. Eskandarani), aged 50 days. Scale bars = 100 µm. (**A**) Plants affected by powdery mildew (control). (**B**) Plants treated by 40 ppm shikimic acid (SA). (**C**) Plants treated by *S. rochei* + *T. Asperullum* (SR + TS). (**D**) Plants treated by mix between 40 ppm SA and (SR + TS). Abbreviations: la—lamina; l ep—lower epidermis; spo—spongy tissue; pal—palisade tissue; u ep—upper epidermis; and s—stomata.

The data in Table 7 and Figures 9 and 10 reveal that treatment with shikimic acid (40 ppm), bioagents *S. rochei* and *T. asperellum*, and a mixture of the two improved all histological aspects under study. The values for thickness of midvein, dimension of main midvein bundles, number of xylem rows in the main midvein bundle, mean diameter of vessels, and mean diameter of parenchyma cells in the ground tissue of plants treated with a combination of shikimic acid (40 ppm) and *S. rochei* + *T. asperellum* were increased by 39.17%, 23.09%, 9.37%, 1.00%, and 14.75% compared to the control, respectively (Figure 9A,D). A combination of shikimic acid (40 ppm) and *S. rochei* + *T. asperellum* yielded an increase in thickness of the upper epidermis, lower epidermis, lamina, palisade tissue, and spongy tissue of 27.11%, 28.10%, 21.42%, 25.08%, and 29.76% compared to the control, respectively (Figure 10A,D). Plants that were treated with *S. rochei* + *T. asperellum* showed an increase in the thickness of the midvein, upper epidermis, lower epidermis, lamina, spongy tissue, number of vascular bundles, dimension of the main midvein bundle, and mean diameter of vessels of 4.02%, 8.38%, 19.96%, 3.52%, 8.81%, 25%, 4.54%, and 0.61% compared to those treated with shikimic acid (40 ppm) alone. In contrast, treatment with shikimic acid (40 ppm) showed increases of 4.75% and 2.7% in the thickness of palisade tissue and mean

diameter of parenchyma cells in the ground tissues, respectively, compared with plants treated with *S. rochei* + *T. asperellum* (Figures 9 and 10B,C). This treatment caused recovery of the reduction in all examined tissues of the leaf blade that were affected by powdery mildew. It is clear from Figure 11A and B that plants affected by powdery mildew had an accumulation of conidia on the epidermal cells, while those treated with the bioagents (*S. rochei* + *T. asperellum*) and shikimic acid (40 ppm) appeared to be largely free of conidia on the epidermal cells. In addition, the cutical layer appeared thicker in the plants treated with the bioagents (*S. rochei* + *T. asperellum)* with shikimic acid 40 ppm compared to the control. The cells of the palisade tissue appeared closely packed in two rows in the plants treated with the bioagents (*S. rochei* + *T. asperellum*), while the cells of the palisade tissue of the control plants were dilapidated and undefined.

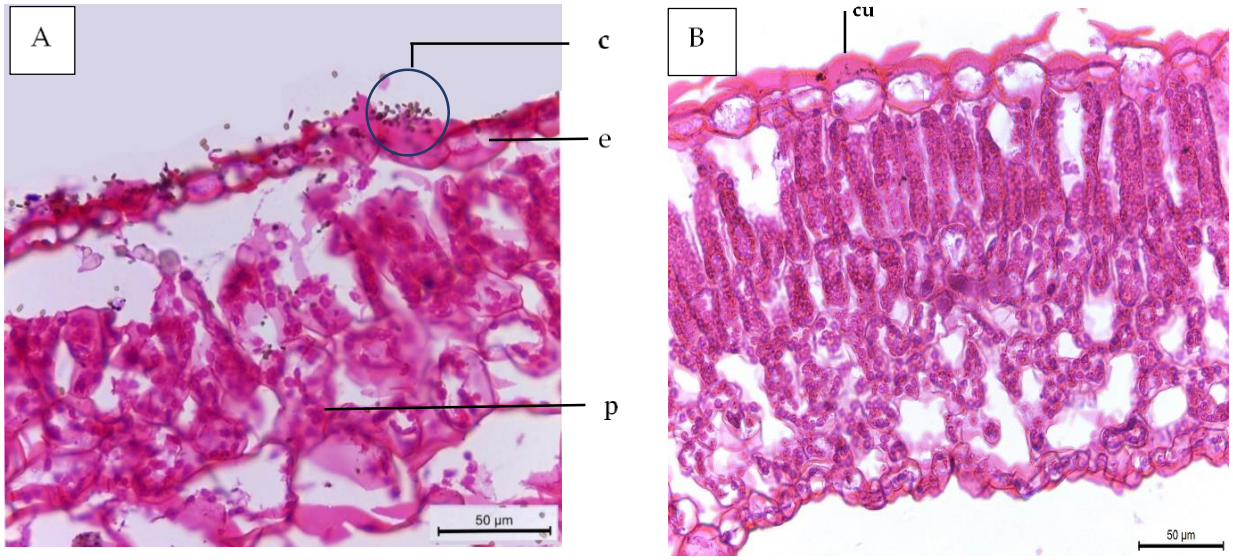

Figure 11. Magnified portions of the leaf blade lamina of summer squash (cv. Eskandarani), aged 50 days. Scale bars = 50 µm. (**A**) Plants affected by powdery mildew (control). (**B**) Plants treated by mix between 40 ppm SA and (SR + TS). Abbreviations: cu—cuticle; e—epidermis; c—conidia; and p—plastids.

4. Discussion

In crop protection against powdery mildew, restrictions on fungicides have encouraged research into biological management as an alternative. Recent discoveries support the idea that some BCAs may have beneficial impacts on plants, including disease control, plant growth stimulation, increased yield, and improved nutrient uptake and crop quality [34]. In particular, plant growth-promoting fungi such as *Trichoderma* spp. are capable of producing a variety of bioactive secondary metabolites that stimulate plant growth and protect plants from numerous phytopathogens [35]. The stimulatory effect of *Trichoderma* spp. on plants is probably related to their participation in the crosstalk between the growth hormones synthesized by these fungi and the defense hormones they induce in the plant [36]

In this study, *T. asperellum* alone reduced powdery mildew severity under controlled conditions by 18.01% and 16.34% in the first and second experiments, respectively. Moreover, *S. rochei* alone was less effective than *T. asperellum*, as it reduced the percentage of powdery mildew by 14.01% and 10.22% in the first and second experiments, respectively. Surprisingly, when applied together, they activated one another; this positive effect was reflected in an increase in resistance to the disease, with a decrease in powdery mildew severity of 22.00% and 28.57% in the first and second pot experiments, respectively. Accordingly, we can infer that a combination of *S. rochei* and *T. asperellum* as a foliar spray may have a synergistic effect against powdery mildew. This observation led us to quantify the components of *T. asperellum* and *S. rochei* culture filtrates by GC-MS analysis. An analysis

of *T. asperellum* culture filtrate revealed 28 compounds. Hemin cation, a naturally occurring metalloporphyrin, was identified during GC-MS analysis of the *T. asperellum* culture filtrate (area percentages). This compound is primarily indicated as a basis for the new fungicides owing to its recognized pro-oxidant capabilities, which also likely contribute to its antibacterial activity [37]. The antifungal effect of myeloperoxidase is due to the synthesis of ROS [38]. Therefore, the presence of hemin may be responsible for the antifungal activity of *T. asperellum* culture filtrate against the powdery mildew pathogen observed in this study.

On the other hand, our GC-MS analysis of the *S. rochei* culture filtrate (area percentages) revealed the presence of 54 compounds. In peak 5, the volatile phenolic compound 2,4-di-tert-butylphenol (1.04%), often a major component of violates that exhibits potent antifungal, antibacterial, antiviral, antimicrobial, antioxidant, and phytotoxic activities. The second most abundant compound, in peak 16 (0.96%), was the fatty acid hexadecanoic acid methyl ester which has a strong odor and which acts as an enzyme inhibitor [39]. Fatty acids such as 9-heptadecenoic acid and 6-methyl-9-heptadecenoic acid have anti-powdery mildew activity [40]. Antifungal fatty acids enter powdery mildew cells and disrupt the membrane, causing the release of intracellular components, cytoplasmic disorder, and, eventually, cell disintegration [40]. Therefore, a possible explanation for the synergistic effect between *S. rochei* and *T. asperellum* found in this study may be the interaction between hemin cations from *T. asperellum* and the antifungal compounds, hexadecanoic acid methyl ester and 2,4-di-tert-butylphenol from *S. rochei*. This finding coincides with that of [41], who found that a combination of *Streptomyces* spp. and *Trichoderma* spp. could suppress the intensity of leaf spot disease by up to 16.2% in chili fruits and enhance plant growth compared to either one as a single treatment.

We further hypothesized that seed priming with shikimic acid, a natural organic acid, in combination with the tested BCAs as a foliar spray would increase plant resistance to powdery mildew infection and promote plant growth. When applied as a seed primer under controlled conditions, shikimic acid alone at 20, 40, and 60 ppm reduced powdery mildew severity by 24.00%, 24.00%, and 5.99% in the first experiment and by 32.67%, 32.67%, and 16.34%, in the second experiment, respectively. It was noted that by increasing shikimic acid concentrations to 60 ppm, the disease severity increased while the plant growth parameters (plant height, number of leaves, chlorophyll content), photosynthesis, transpiration rate, and stomatal conductance decreased. The increased severity of the infection may be attributed to the decrease in chlorophyll content and the efficiency of photosynthesis, which leads to a decrease in disease resistance

In most cases, the improvement in powdery mildew control, plant height, number of leaves, and chlorophyll reading (SPAD) was due to a combination of seed priming with shikimic acid at 40 ppm and a BCA mixture (*T. asperellum* + *S. rochei*) as a foliar application treatment. Throughout the two trials, this effect was consistent. The additive effects were most likely due to a combination of mechanisms involved in disease control by the combined treatment, as opposed to the modes of action provided by shikimic acid or individual antagonists alone [7,11]. According to [42], shikimic acid is effective as a seed soak to cure chocolate spot disease in faba beans. Similarly, various authors have reported that exogenous shikimic acid application can improve plant growth, improve antioxidant defense, and reduce oxidative damage [13].

Another finding was that untreated squash leaves showed a significant reduction in plant growth and productivity with powdery mildew infection. This finding is consistent with that of [43], who found that the powdery mildew fungal infection resulted in a significant reduction in photosynthetic rate, leaf pigments, transpiration rate, and stomatal conductance. A possible explanation is that mildew colonies on the leaf surface limit the amount of light reaching the mesophyll cells, resulting in a considerable drop in leaf chlorophyll and carotenoids [44]. Photosynthetic pigment reduction is proportional to the leaf surface's photosynthetic efficiency [43]. As a result, the photosynthesis rate is significantly decreased in untreated infected plants. Furthermore, the photosynthesis rate is inversely proportional to the transpiration rate and stomatal conductance [45].

Fungal colonization results in partial coverage of the stomata cavity or hole by the hyphae and/or spores of the fungus. As a result, transpiration rates in fungus-infected plants are consistently low [44]. In addition, an increase in chlorophyll content, which is a reliable indicator of plant health, improves the efficiency of the photosynthetic system and increases disease resistance [46].

This study examined, for the first time, the effect of seed priming with shikimic acid and the foliar application of BCAs to improve the growth and productivity of squash. Improved growth of squash plants in response to shikimic acid might be due to improvement in leaf survival by maintaining photosynthesis and increasing mineral content, which may increase plant growth [13]. Plant growth parameters are a perfect indicator for evaluating abiotic and biotic stresses. *Trichoderma* spp. root colonization has been linked to increased plant nutrient uptake as a result of more efficient macro- and micronutrient solubilization [47]. These results are in agreement with those obtained by [48] in squash. Plant growth-promoting streptomycetes promote a variety of direct and indirect biosynthetic processes in plants, including inorganic phosphate solubilization, chemical biosynthesis chelation, phytohormone production, pathogen suppression, and abiotic stress reduction [49]. In this study, priming seeds of squash plants with 40 ppm of shikimic acid combined with the foliar application of *T. asperellum* and *S. rochei* yielded significantly higher fruit numbers, fruit weight, and total yield per plant.

We further studied the responses of squash plants to the treatments by determining the level of plant hormones in the leaves. SA is a plant hormone that plays a pivotal role in plant defense against biotrophic and semi-biotrophic pathogens [23]. When comparing treated and non-treated plants under natural infection with powdery mildew, lower amounts of JA and SA were observed in the water-treated control. Surprisingly, in plants grown after seed priming with 40 ppm of shikimic acid combined with foliar spray of *S. rochei* + *T. asperellum*, the levels of JA and SA were much higher than in the water-treated control. We also observed that because JA is often negatively correlated with SA, the SA level was decreased. Elevated levels of JA and SA in treated plants compared to the control were to be expected, because both hormones are usually a part of resistance mechanisms. The JA and SA contents increase in infected tissues, and the application of shikimic acid through seed priming combined with foliar spray of BCAs reduces disease symptoms. The work conducted by [50] studied some of the physiological mechanisms resulting from powdery mildew inoculation of the sensitive Ingrid barley cultivar and near-isogenic lines carrying various resistant genes (Mla, Mlg, and mlo). They found that in the leaves of the cultivar Ingrid, the JA content was significantly decreased after inoculation compared to the non-inoculated control. Meanwhile, powdery mildew inoculation of Mla and Mlg barley that showed hypersensitive reactions led to notably elevated levels of JA and SA compared to the Ingrid cultivar.

Phenolic compounds are formed by plants primarily for growth, protection, and development. These aromatic benzene ring components are very important during the interactions between plants and abiotic stresses. They represent an essential component of phytochemicals and play an important role in various mechanical and physiological activities [51]. In the present study, the total phenol content in squash leaves was increased in all treatments compared with control plants, whereas plants primed with 40 ppm of shikimic acid combined with foliar spray of *S. rochei* + *T. asperellum* showed a greater accumulation of phenolics, which enhance resistance against invasion of P. xanthii. This finding is in line with that obtained by [52], who found an increase in total phenols, yield, and powdery mildew resistance in squash plants treated with certain BCAs. Furthermore, disease resistance induction is a powerful strategy to enhance the defense mechanisms of plants. This mechanism could be linked to the activities of defense-associated enzymes such as PPO and POD, which are key proteins related to pathogenesis in plant tissues owing to their ability to disrupt the cell wall structure of pathogens and contribute to resistance to pathogen invasion [53]. PPO and POD are terminal enzymes; the former can oxidize phenolic substances and produce toxic quinines that can restrict and kill invading

pathogens, while the latter is linked to the synthesis of lignin [54]. SOD is essential to plant stress resistance; it provides the first line of defense against the harmful effects of elevated levels of ROS [55]. In addition, catalase is an oxygen-binding enzyme that protects cells from the harmful effects of H_2O_2 and acts as a signaling molecule to increase plant defense genes, which helps protect plants from infection [56].

Our results are in line with earlier studies that demonstrated enhanced activities of POD, SOD, and CAT under different treatments. Shikimic acid, when combined with the foliar application of a BCA mixture (*T. asperellum* + *S. rochei*), increased the activity of antioxidant enzymes such as POD, SOD, and CAT, thereby protecting against powdery mildew [20,42]. Antioxidant enzymes are proteins involved in the catalytic conversion of ROS and their byproducts into stable, non-toxic molecules. Therefore, they represent the main defense mechanism against cellular damage caused by oxidative stress. The authors of [57] found that bioagents *Bacillus* spp. and *Trichoderma* spp. could significantly reduce the severity of squash powdery mildew (*P. axanthii*). However, the role of these bioagents was attributed to the upregulation of defense-related enzymes such as CAT, POD, and PPO, which stimulate growth and yield characteristics.

The interrelationship among the evaluated parameters (Figures 7 and 8) indicates that the yield parameters were positively associated with plant height and the mineral content of leaves, including N%, P%, and K%. We speculate that the high levels of antioxidant activity, including SOD, POD, and CAT, were associated with the greater total yield and its related traits, especially after seed priming with 40 ppm of shikimic acid combined with foliar spray of *S. rochei* + *T. asperellum*. In accordance with these results, it is important to note that specific biochemical and physiological traits are closely associated with yield-related traits under natural infection with powdery mildew.

With regard to the anatomy of squash leaves, this study demonstrated that plants infected with *P. xanthii* that received no treatment showed a reduction in the thickness of the upper epidermis, lower epidermis, and midvein, dimension of the main midvein bundle, number of xylem rows in main midvein bundle, mean diameter of vessels, and mean diameter of parenchyma cells in the ground tissues. A possible explanation for this might be that the powdery mildew mycelium grows on the surface of the plant, with nutrients being obtained via the haustoria in the plant epidermal cells, leading to nutrient uptake. In addition, powdery mildew colonies on the leaf surface limit the amount of light reaching the mesophyll cells (palisade and spongy), resulting in a considerable drop in leaf chlorophyll and carotenoids, reduced photosynthesis, increased respiration and transpiration, and impaired growth [44]. In general, the alteration of anatomical traits of squash leaves with the applied treatments is of great interest, because these alterations included increases in the thickness of upper epidermis, lower epidermis, and midvein, dimension of the main midvein bundle, number of xylem rows in main midvein bundle, mean diameter of vessels, and mean diameter of parenchyma cells in the ground tissues. The treatments improved the process of photosynthesis by increasing the area of epidermis and mesophyll tissues exposed to the sunlight. They also reflect those of [42], who found that seed priming with shikimic acid improved the growth parameters of cowpea plants by simulating effects on leaf expansion and photosynthetic pigments as well as on the transpiration rate of cowpea plants during growth periods.

On the other hand, shikimic acid might exert effects on the photosynthetic machinery at the mesophyll and chloroplast level by increasing plastid biogenesis via an increase in the biosynthesis of indole acetic acid from tryptophan. Furthermore, some reports have indicated that a combination of antagonistic bacteria and antagonistic fungi, especially *Trichoderma* spp., provides greater plant protection than their use individually [58]. Additionally, the authors of [59] reported that application of a mixture of *T. harzianum* and *S. rochei* is more effective at controlling Phytophthora root rot in pepper. In the present study, a combination of shikimic acid (40 ppm) seed priming and *S. rochei* + *T. asperellum* as a foliar application yielded the best results in terms of the anatomical features of leaves. Additionally, the upper surface of the plant showed a reduction of powdery mildew symptoms.

This improved the plants' ability to receive sunlight and improved the mechanism of chloroplast function, thereby enhancing the process of photosynthesis. To our knowledge, this study is the first to report the effects of shikimic acid (40 ppm) and *S. rochei* + *T. asperellum* as a foliar application on the anatomical structure of squash leaves.

5. Conclusions

This study showed the effects of *T. asperellum*, *S. rochei*, and shikimic acid-primed seeds, and their combination treatments on squash productivity under artificial and natural infection with powdery mildew. The use of *T. asperellum* + *S. rochei* with shikimic acid primed seeds treatment was more effective than using each treatment separately, while *T. asperellum* outperformed *S. rochei*. The use of *T. asperellum* + *S. rochei* with shikimic acid primed seeds treatment showed an improvement in terms of plant growth, total yield, mineral components, physiological traits, and antioxidant activity, as well as in all histological aspects of the studied squash plants.Moreever, maximum disease severity reduction and a reduction in AUDPC values were observed following treatment of *T. asperellum* + *S. rochei* with 40 ppm shikimic acid primed seeds. Therefore, this treatment could be used as an alternative method to lessen powdery mildew infection in squash plants.

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