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Weaponizing Plants

Biocontrol and Biosecurity in Plant Protection

Edited by
Francesca Degola, Giovanna Visioli, Agnieszka Jamiołkowska and Izabela Kot

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Article

Antifungal Potency of Amaranth Leaf Extract: An In Vitro Study

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Abstract: Plant diseases are a serious problem for agricultural crops, the food industry and human health. Significant efforts have been made in recent years to find natural products that could reduce the growth of plant pathogens and improve food quality. At present, there is an increased interest in plants as a source of biological active compounds that can protect crops from diseases. Important sources of these phytochemicals are lesser-known pseudocereals such as amaranth. The objective of this study was to determine the antifungal activity of leaf extracts of four amaranth species (*A. cruentus*, *A. hypochondriacus* × *hybridus*, *A. retroflexus* and *A. hybridus*). The antifungal potency of amaranth extracts was analyzed against selected strains of fungi. The results suggested that the antimicrobial properties of the tested extracts varied depending on the amaranth species and the fungal strain. The studied extracts inhibited the growth of *Fusarium equiseti*, *Rhizoctonia solani*, *Trichoderma harzianum* and *Alternaria alternata*. A lower inhibitory effect of the extracts was recorded against *F. solani*, while no inhibitory effect was observed against *F. oxysporum* and *Colletotrichum coccodes*.

Keywords: Amaranthus cruentus; Amaranthus hypochondriacus × hybridus; Amaranthus retroflexus; Amaranthus hybridus; plant extract; fungal diseases

1. Introduction

Food-borne illnesses are a major concern for consumers, the food industry and food safety authorities. Pathogenic microorganisms present on plants cause diseases, as well as deterioration in the quality of stored food products. Among these microorganisms are many polyphagous phytopathogenic fungi that contribute to food spoilage. These include, among others, fungi of the genus *Fusarium*, *Botrytis* or *Alternaria*. In addition to the losses they cause in crops, they are also a source of mycotoxins that accumulate in food. The genus *Fusarium* produces specific toxins whose profile and harmfulness depend on the species and even strain of the fungus. Fusarium species are dangerous pathogens with a high toxicity potential. Secondary metabolites of these fungi, such as deoxynivalenol, zearalenone and fumonisin B1, are among the five most important mycotoxins in Europe and the world [1–3]. Similarly, the genus *Alternaria* can produce under certain conditions secondary metabolites with toxic properties, known as *Alternaria* toxins [4]. Currently, scientists are emphasizing the importance of several toxins, including alternariol (AOH), alternariol monomethyl ether (AME), altertoxin (ATX), altenuene (ALT), tenuazonic acid (TeA), tentoxin (TEN) and AAL toxins that can be found as contaminants in agricultural crops [5]. Mycotoxins can spread from rotten plant tissue to surrounding areas; thus, they are also present in processed foods [6]. Therefore, many pathogenic microorganisms remain an important problem for agriculture and the food industry.

Consumers are concerned about synthetic preservatives used in food. As a result, there is a growing demand for natural products that can serve as an alternative to food

preservatives. The current concept of sustainable agriculture assumes a reduction in the use of synthetic pesticides and a wider use of natural products for plant protection and food preservation [7]. Therefore, currently, natural preparations, which are as effective as chemical preparations, are increasingly used in integrated and organic production to protect plants against diseases [8,9]. Many of them are based on plant extracts enriched with minerals and beneficial microorganisms [10]. Numerous studies have been conducted on plants containing biologically active compounds that inhibit the growth of pathogenic microorganisms [11–13]. Many plant species, mainly herbs, are already promising sources of bioactive substances such as phenols, anthocyanins, flavonoids or carotenoids, which are used to extend the shelf life of meals and processed foods [14–17]. The action of these natural compounds is not specific, and their effects on pathogens vary. The natural bioactive compounds used in plant protection destroy pathogens (fungicides) or limit their development (fungistatics), as well as induce plant defense reactions by acting as elicitors [9]. Many studies have carried out systematic screenings of plants and the compounds contained in them with antimicrobial and antiviral properties. Europe is an area extremely rich in medicinal plants that contain a variety of antimicrobial components. In recent decades, the antimicrobial and antifungal properties of various extracts and their components, including essential oils, have been tested and attention has been drawn to the application of these natural raw materials as alternative plant protection products, because they are biodegradable and non-toxic to the environment [18]. Some of them contain natural products from secondary metabolic pathways that allow plants to protect themselves from their natural enemies [13]. In addition to many herbal plants occurring in the natural environment, certain pseudo-cereals are also rich in a wide range of compounds, e.g., flavonoids and phenolic acids, present not only in grains, but also in other aerial plant parts (leaves, stems) [19,20]. One such plant is *Amaranthus* spp. It is characterized by a high resistance to pathogens, and infection by pathogenic fungi does not pose a significant threat to plantations of this plant [21,22]. Plants exposed to stress, both biotic and abiotic, produce secondary metabolites such as phytohormones, betaine compounds, phenolic compounds, polysaccharides and oligosaccharides, fatty acids, sterols, humic acids and carotenoids. Literature reports indicate that these compounds can also act as plant growth stimulants [9,23].

The aim of the conducted research was a laboratory evaluation of the effect of amaranth leaf extracts on selected filamentous fungi occurring plants and the soil environment. The study used four species of amaranth from Poland and Turkey (*A. cruentus*, *A. hypochondriacus* × *hybridus*, *A. retroflexus*, *A. hybridus*). The biochemical composition of the extracts obtained from the leaves was analyzed, and their fungistatic activity against selected fungal species important in phytopathology and the food industry was assessed.

2. Results

2.1. Polyphenol Content

The research object was the leaves of *Amaranthus cruentus* (PC), *A. hypochondricus* × *hybridus* (PH), *A. retroflexus* (TR) and *A. hybridus* (TH). Dried amaranth leaves were used as a material for the production of plant extracts, in which the total polyphenol content and antioxidant activity were determined using the synthetic DPPH radical. Table 1 presents the results of the total polyphenol content in the tested extracts obtained from amaranth leaves and the respective antioxidant activity values.

The highest concentration of total polyphenols in the initial extracts from amaranth leaves was recorded for *A. hybridus* (TH)—6.75 mg GAE/mL, while the lowest concentration of polyphenols was determined in the extract from *A. cruentus* (PC)—4.31 mg GAE/mL, which had an approx. 36% lower concentration of the tested active substances. It should be noted that extracts from plant material obtained from Turkish crops were characterized by significantly higher concentrations of polyphenols compared with amaranth extracts from Poland. The content of biologically active ingredients in plants is influenced by varietal factors, as well as cultivation factors (growing season, fertilization, soil, temperature, rainfall, etc.) [24]. Species of the genus *Amaranthus* are thermophilic plants. Turkey has

more optimal growing conditions for this type of plant compared with Poland [25]. Optimal cultivation conditions promote more efficient synthesis of biologically active substances, which was confirmed by higher concentrations of polyphenolic compounds in the extracts of amaranth species cultivated in Turkey. The results of the antioxidant activity of the tested extracts corresponded with polyphenol levels. The highest antioxidant activity was observed in the extract obtained from the leaves of the species *A. hybridus* (TH) from Turkey (21.04 mM TE/mL), while extracts from Polish raw materials showed significantly lower antioxidant activity (13.84 mM TE/mL (PH) and 8.77 mM TE/mL (PC).

Table 1. Total polyphenol content and antioxidant activity of amaranth extracts.

Plant Extract	Polyphenols (mg GAE/mL) ± SD	Antioxidant Activity, Free Radical Scavenging Ability	
		% Inhibition ± SD	mM TE/mL ± SD
A. cruentus (PC)	4.31 d ± 0.308	30.42 d ± 2.553	8.77 d ± 0.805
A. hypochondriacus × *A. hybridus L.* (PH)	5.39 c ± 0.259	46.51 c ± 0.225	13.84 c ± 0.071
A. retroflexus (TR)	5.81 b ± 0.082	56.59 b ± 1.137	17.02 b ± 0.359
A. hybridus (TH)	6.75 a ± 0.162	69.34 a ± 1.240	21.04 a ± 0.391

GAE—Gallic acid equivalent; TE—Trolox equivalent; SD—Standard deviation; a–d—Values in rows marked with the same letter do not differ significantly at the significance level of $p \leq 0.05$.

2.2. Fungistatic Activity

Amaranth extracts exerted different effects on the growth of the tested fungal strains, depending on the fungal species, the type and concentration of the extract, and the duration of action of the biologically active ingredients (Tables 2 and 3, Figure 1).

Table 2. Fungal colony diameter (mm) after application of amaranth extracts of *Alternaria alternata*, *Colletotrichum coccodes* and *Trichoderma harzianum*.

Experimental Combination	Concentration (%)	Number of Days ± SD		
		4	8	12
Alternaria alternata				
PC	10	23.0 ± 0.81 ef	51.0 ± 0.0 b	75.7 ± 1.62 ab
	15	20.3 ± 0.47 f	44.7 ± 2.05 d	71.7 ± 1.24 bc
PH	10	24.7 ± 0.47 de	49.7 ± 0.47 bc	76.7 ± 0.47 ab
	15	28.3 ± 0.81 bc	60.0 ± 0.0a	76.0 ± 0.47 ab
TR	10	30.0 ± 0.47 b	62.7 ± 0.47a	79.3 ± 0.47 ab
	15	26.3 ± 1.24 cd	60.7 ± 0.47 a	80.7 ± 0.47 a
TH	10	20.3 ± 0.47 f	47.7 ± 1.24 cd	67.0 ± +1.63 c
	15	15.3 ± 0.47 g	39.0 ± 0.81 e	59.0 ± 2.16 d
CE	10	34.3 ± 1.24 a	51.0 ± 0.81 b	68.0 ± 2.16 c
	15	34.3 ± 1.24 a	51.0 ± 0.81 b	68.0 ± 2.16 c
F		108.8507	132.152	37.9273
p		5.27×10^{-15}	7.94×10^{-16}	1.23×10^{-10}
LSD		2.9	3.3	5.3
Colletotrichum coccodes				
PC	10	23.0 ± 1.24 cd	51.0 ± 0.0 bc	76.0 ± 0.47 ab
	15	20.3 ± 0.00 de	44.7 ± 0.47 d	71.7 ± 0.81 ab

Table 2. *Cont.*

Experimental Combination	Concentration (%)	Number of Days ± SD		
		4	**8**	**12**
PH	10	24.7 ± 0.81 bc	49.7 ± 0.47cd	76.7 ± 0.47 ab
	15	25.0 ± 0.00 bc	57.7 ± 0.47 a	80.0 ± 0.00 a
TR	10	28.3 ± 1.24 a	59.7 ± 0.47 a	78.7 ± 1.88 a
	15	26.7 ± 1.24 ab	55.0 ± 2.44 ab	79.3 ± 1.69 a
TH	10	20.7 ± 0.47 de	45.7 ± 2.86 de	72.7 ± 1.88 abc
	15	19.0 ± 0.81 e	44.0 ± 2.44 e	64.0 ± 7.78 c
CE	10	26.0 ± 0.81 abc	50.0 ± 0.00 bcd	67.3 ± 2.05 bc
	15	26.0 ± 0.81 abc	50.0 ± 0.00 bcd	67.3 ± 2.05 bc
F	29.8333	29.8333	32.84678	10.3675
p	1.12×10^{-9}	1.12×10^{-9}	4.64×10^{-10}	8.72×10^{-6}
LSD	0.7	3.1	5.1	10.0
Trichoderma harzianum				
PC	10	81.3 ± 0.47 c	* No measurements	
	15	77.0 ± 0.81 d		
PH	10	56.3 ± 0.47 g		
	15	56.0 ± 0.81 g		
TR	10	86.0 ± 0.81 b		
	15	72.3 ± 2.05 e		
TH	10	69.0 ± 0.81 f		
	15	51.0 ± 0.81 h		
CE	10	90.0 ± 0.00 a		
	15	90.0 ± 0.00 a		
F		527.6574	No correlations	
p		9.09×10^{-22}		
LSD		3.2		

PC—*A. cruentus* extract, PH—*A. hypochondriacus* × *hybridus* extract; TR—*A. retroflexus* extract, TH—*A. hybridus* extract; CE—control; a–h—values in the rows marked with the same letter do not differ significantly at a significance level of $p \leq 0.05$; LSD—the least significant difference; *—no measurements, fungal colony diameter exceeded plate diameter.

Table 3. Fungal colony diameter (mm) after application of amaranth extracts of *Fusarium equiseti*, *Fusarium oxysporum*, *Fusarium solani* and *Rhizoctonia solani*.

Experimental Combination	Concentration (%)	Number of Days ± SD		
		4	**8**	**12**
Fusarium equiseti				
PC	10	13.3 ± 1.24 c	68.0 ± 2.16 a	86.7 ± 0.47 a
	15	10.0 ± 0.00 cd	28.0 ± 1.63 cde	47.3 ± 3.68 d
PH	10	8.7 ± 0.94 d	19.7 ± 0.47 e	30.0 ± 0.00 e
	15	12.0 ± 0.81 cd	30.7 ± 047 c	57.3 ± 2.05 c
TR	10	20.3 ± 1.24 b	43.7 ± 2.05 b	67.7 ± 6.12 b
	15	10.7 ± 0.47 cd	22.0 ± 1.63 de	41.0 ± 0.81 d

Table 3. *Cont.*

Experimental Combination	Concentration (%)	Number of Days ± SD		
		4	**8**	**12**
TH	10	28.0 ± 2.94 a	45.0 ± 5.31 b	85.0 ± 4.08 a
	15	20.0 ± 0.81 b	44.7 ± 0.47 b	70.7 ± 0.94 b
CE	10	28.0 ± 0.81 a	71.0 ± 2.94 a	89.0 ± 1.41 a
	15	28.0 ± 0.81 a	71.0 ± 2.94 a	89.0 ± 1.41 a
F		102.3873	140.2651	120.3965
P		9.56×10^{-15}	4.43×10^{-16}	1.97×10^{-15}
LSD		4.4	8.7	9.9
Fusarium oxysporum				
PC	10	26.7 ± 2.35 b	60.0 ± 4.08 bc	75.0 ± 0.00 bc
	15	26.0 ± 0.00 b	59.7 ± 0.47 bcd	67.7 ± 2.05 d
PH	10	27.7 ± 1.69 ab	67.0 ± 0.00 a	75.3 ± 0.47 bc
	15	27.0 ± 2.16 b	60.0 ± 0.00 bc	68.0 ± 0.81 d
TR	10	25.3 ± 0.47 b	51.0 ± 0.00 d	71.7 ± 0.47 cd
	15	27.3 ± 0.81 b	53.7 ± 1.24 cd	77.0 ± 0.81 b
TH	10	32.2 ± 0.41 a	61.7 ± 3.68 ab	82.7 ± 2.05 a
	15	25.0 ± 0.00 b	57.0 ± 1.63 bcd	78.7 ± 1.24 ab
CE	10	24.7 ± 1.24 b	53.7 ± 1.24 cd	70.0 ± 1.36 d
	15	24.7 ± 1.24 b	53.7 ± 1.24 cd	70.0 ± 1.36 d
F		5.942576	12.40261	28.65224
P		0.000453	2.14×10^{-6}	1.61×10^{-9}
LSD		4.8	6.9	4.6
Fusarium solani				
PC	10	15.0 ± 0.00 e	52.7 ± 0.47 cd	77.7 ± 1.24 d
	15	22.3 ± 1.24 cd	55.7 ± 1.24 bc	79.7 ± 0.47 cd
PH	10	24.3 ± 0.47 bc	55.0 ± 2.44 bc	80.0 ± 0.00 cd
	15	25.7 ± 0.47 b	59.3 ± 0.94 ab	80.0 ± 0.00 cd
TR	10	30.3 ± 0.47 a	64.7 ± 0.47 a	81.7 ± 0.47 bc
	15	29.3 ± 0.47 a	64.7 ± 2.05 a	83.0 ± 1.63 ab
TH	10	24.0 ± 0.81 bc	41.3 ± 2.44 e	79.7 ± 0.47 cd
	15	21.7 ± 0.47 d	49.0 ± 0.00 d	75.0 ± 0.00 e
CE	10	29.7 ± 0.47 a	62.7 ± 1.69 a	85.0 ± 0.00 a
	15	29.7 ± 0.47 a	62.7 ± 1.69 a	85.0 ± 0.00 a
F		122.9346	22.87798	40.0
P		1.61×10^{-15}	1.21×10^{-8}	7.52×10^{-11}
LSD		2.2	5.6	2.5

Table 3. *Cont.*

Experimental Combination	Concentration (%)	Number of Days ± SD		
		4	8	12
Rhizoctonia solani				
PC	10	39.0 ± 0.81 c	*—No measurements	
	15	30.3 ± 1.24 c		
PH	10	50.3 ± 0.47 b		
	15	51.0 ± 0.47 b		
TR	10	56.0 ± 3.26 b		
	15	49.7 ± 6.12 b		
TH	10	30.3 ± 0.47 c		
	15	17.3 ± 2.05 d		
CE	10	90.0 ± 0.00 a		
	15	90.0 ± 0.00 a		
F		158.4078	No correlation	
P		1.34×10^{-16}		
LSD		9.5		

PC—*A. cruentus* extract; PH—*A. hypochondriacus* × *hybridus* extract; TR—*A. retroflexus* extract, TH—*A. hybridus* extract; CE—control; a–e—values in the rows marked with the same letter do not differ significantly at a significance level of $p \leq 0.05$; LSD—the least significant difference; *—no measurements, fungal colony diameter exceeded plate diameter.

The fungistatic effects of amaranth leaf extracts largely depended on the fungus species tested (Tables 2 and 3). The best results were recorded for *F. equiseti*, regardless of the concentration and type of extract (Table 3). Extracts from species cultivated in Poland such as *A. cruentus* (PC) and *A. hypochondriacus* × *hybridus* (PH) showed the strongest fungistatic effect, significantly inhibiting the surface growth of *F. equiseti* throughout the experiment (35.6–72.2%) (Figure 1). A strong fungistatic effect was also exhibited by the 15% extract from the species cultivated in Turkey, such as *A. retroflexus* (TR15), and the inhibition of fungal growth compared with the control remained at a high level throughout the experiment, i.e., 53.9–61.9% (Figures 1 and 2). The extracts tested inhibited the growth of other species of the genus *Fusarium* to a low extent. The superficial growth of *F. solani* was most strongly inhibited by the 10% extract of *A. cruentus* (PC10), but only on day 4 of the experiment, and reached 49.4%. The remaining types and concentrations of extracts had no significant effect on *F. solani* growth inhibition; their antifungal activity in the first days of the experiment ranged from 1.3 to 26.5% and quickly decreased in the following days to a statistically insignificant level (2.4–11.8%), even contributing to a slight stimulation of the surface mycelium growth (TR10, TR15) (Figure 1). Amaranth extracts did not show any fungistatic activity against *F. oxysporum*, and the highest recorded level of fungal growth inhibition was 2.9–5.0% (TR10). Extracts, mainly from *A. hybridus* (TH10), stimulated superficial colony growth by up to 30% compared with the control sample.

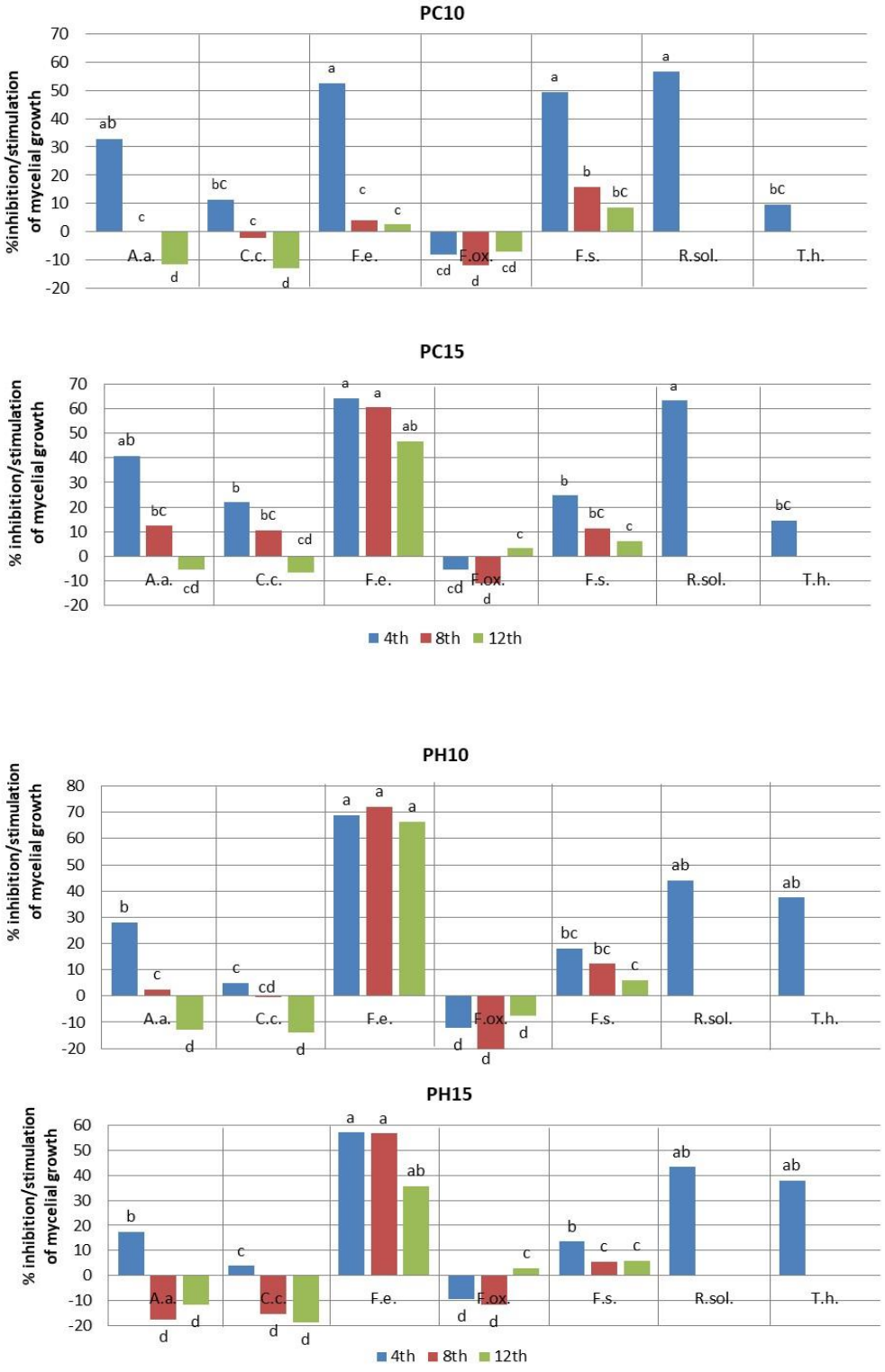

Figure 1. *Cont.*

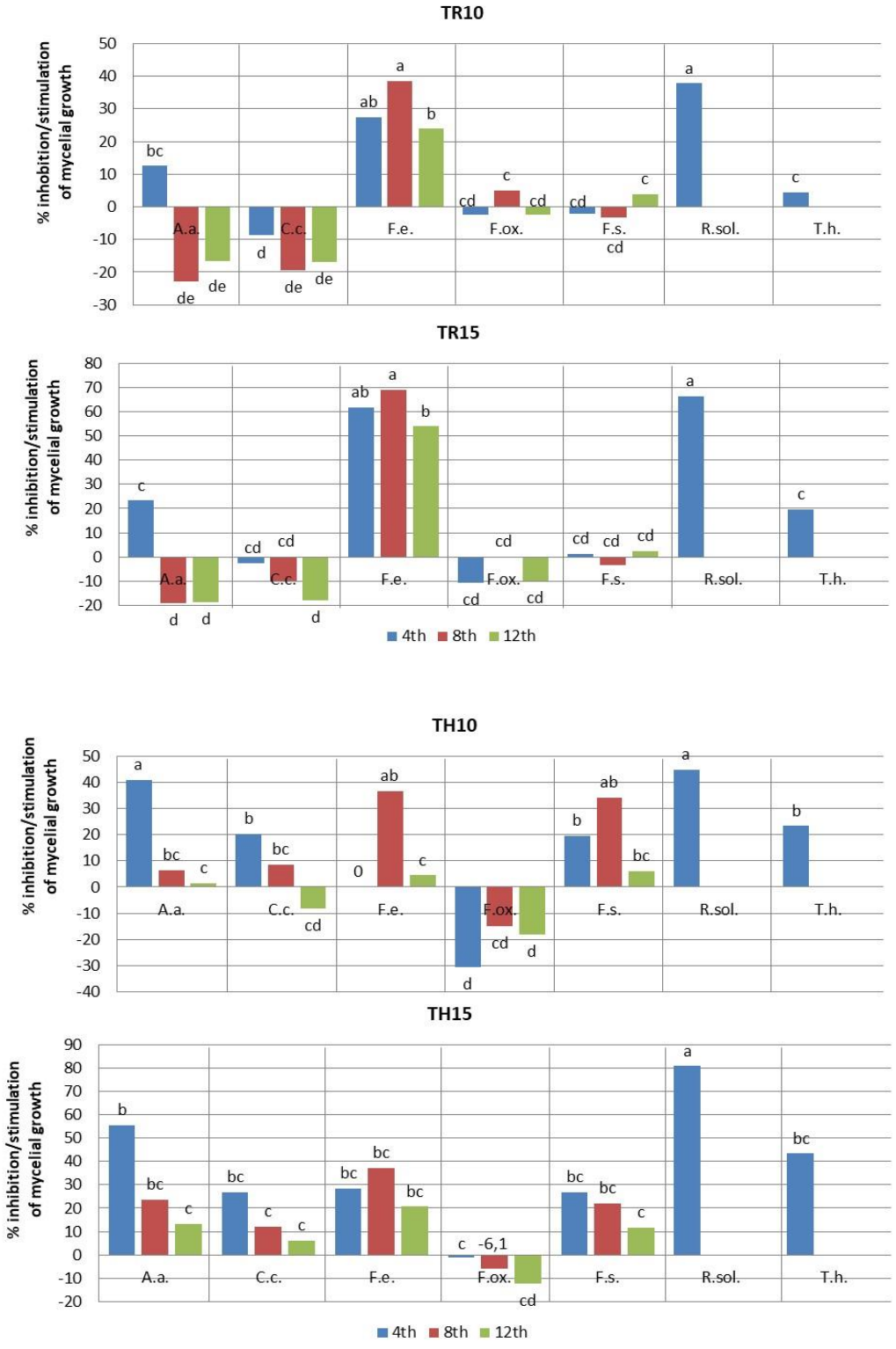

Figure 1. Inhibition/stimulation (%) of the mycelial growth after application of amaranth leaf extracts; PC—*A. cruentus* extract; PH—*A. hypochondriacus* × *hybridus* extract; TR—*A. retroflexus* extract, TH—*A. hybridus* extract; 10—extract concentration—10%; 15—extract concentration—15%; A.a.—*A. alternata*, C.c.—*C. coccodes*, F.e.—*F. equiseti*, F.ox.—*F. oxysporum*, F.s.—*F. solani*, R.sol.—*Rhizoctonia solani*, T.h.—*T. harzianum;* a–d—values marked with the same letter do not differ significantly at a significance level of $p \leq 0.05$.

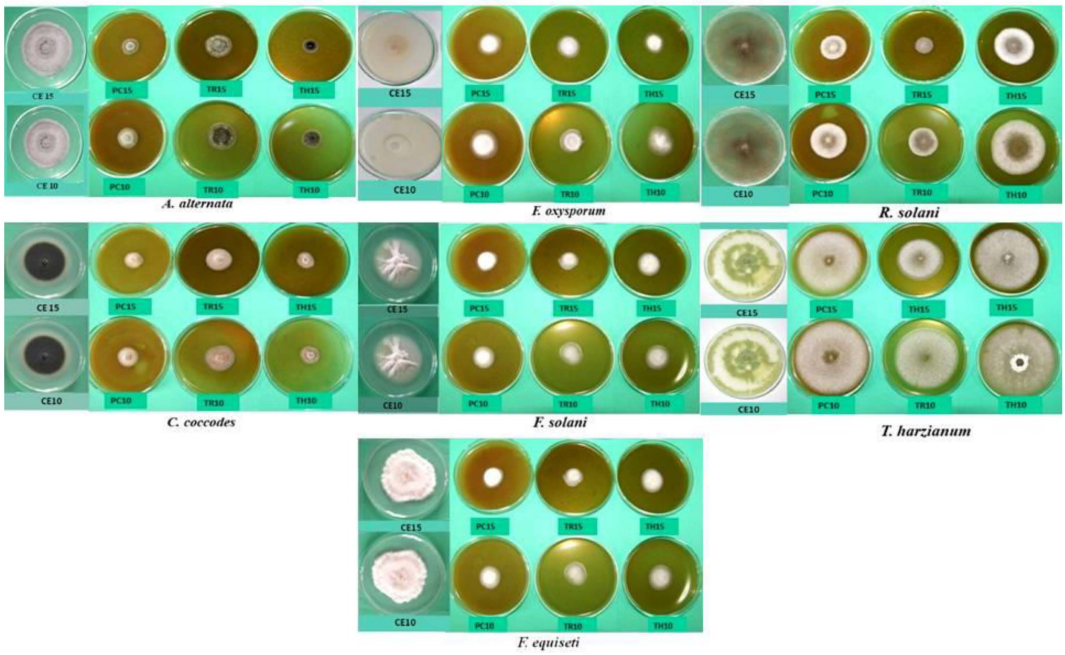

Figure 2. Linear growth of fungal colonies on PDA medium with the addition of amaranth leaf extracts (day 4). PC—*A. cruentus* extract; PH—*A. hypochondricus* × *hybridus* extract; TR—*A. retroflexus* extract, TH—*A. hybridus* extract; CE- control; 10—extract concentration—10%; 15—extract concentration—15%.

A strong fungistatic effect of amaranth extracts was recorded against *Rhizoctonia solani*, but only in the first days of the experiment (day 4) for all tested experimental combinations. Extracts from *A. cruentus* (PC15), *A. hybridus* and *A. retroflexus* (TH15, TR15) strongly reduced the surface growth of *R. solani* at the level of 66.3–80.8% (Figures 1 and 2). The remaining extracts inhibited the growth of *R. solani* by only 37.8–44.7%. On subsequent days, the size of *R. solani* colonies exceeded the plate diameter (90.0 mm); therefore, measurements for individual experimental combinations were not performed (Figure 1). Amaranth extracts inhibited the growth of *A. alternata* only in the first days of the experiment, while they stimulated the growth of the fungus in the subsequent days. The strongest antifungal effect was recorded on day 4 of the experiment for extracts from species cultivated in Turkey: *A. retroflexus* (TH10—40.8%; TH15—55.4%) and *A. cruentus* (PC15—40.8%). Other concentrations of these extracts (PH, TR) inhibited *A. alternata* growth, but at a statistically insignificant level, i.e., 12.5–27.9%. In the consecutive days, the antifungal activity of the extracts decreased, and the surface growth of *A. alternata* was stimulated by more than 20% compared with the control sample (TR10) (Figure 1). Amaranth extracts, from species cultivated in both Poland and Turkey, showed very weak antifungal activity against *C. coccodes*. The highest degree of fungistatic effect was recorded only for the 15% *A. cruentus* extract at the beginning of the experiment (PC15—21.9%) and for the *A. hybridus* extract (TH10, TH15—20.4 and 26.9%, respectively), while their fungistatic effect in the following days rapidly decreased, even contributing to the stimulation of fungus growth. On the other hand, Amaranth extracts inhibited the surface growth of *T. harzianum*. Significant antifungal activity in the first days of the experiment was recorded for the following extracts: *A. hypochondriacus* × *hybridus* (PH10, PH15—37.4%; 37.8%) and *A. hybridus* (TH15—43.3%). In the following days of the experiment, the *T. harzianum* colony diameter exceeded the diameter of the plate (90.00 mm); therefore, measurements for individual experimental combinations were not performed (Figure 1)

The conducted experiment also focused on changes in the morphology of the fungi under the influence of plant extracts (Figure 2, Table 4). Most common were changes in the mycelial structure and the coloration of the obverse and reverse of the colony. The addition

of extracts to the medium caused changes in the mycelial structure. Fungi growing on the medium with the addition of extracts formed less fluffy aerial mycelium with more relaxed growth. In some species, the disappearance of aerial mycelium and the growth of substrate mycelium were even observed (*C. coccodes*, *T. harzianum*). The reverse of the test fungi had a more intense color than in the control samples (*F. equiseti*, *F. oxysporum*) (Table 4). The addition of the extract to the medium caused deformation of conidia (*A. alternata*) and impaired sporulation.

Table 4. Selected features of fungal morphology under the influence of plant extracts (day 8 of the experiment).

Fungus Species	Experimental Combination	Mycelium Surface and Structure	Obverse	Reverse	Presence of Spores
A. alternata	PC10, PC15	Aerial, regular	White and gray	Black	No aleuroconidia
	PH10, PH15	Aerial, regular	White and gray	Black	No aleuroconidia
	TH10, TH15	Aerial, regular, fluffy	Gray	Black	Sparse or no aleuroconidia
	TR10, TR15	Poor growth, low mycelium slightly compacted, regular	Grey and black; gray	Black	Numerous deformed aleuroconidia
	CE10, CE15	fluffy, regular growth	Gray	Black	Sparse aleuroconidia
C. coccodes	PC10, PC15	Substrate, regular	White and salmon; microsclerotia in the center	Colorless	Sparse conidia
	PH10, PH15	Substrate, regular	White and salmon; no microsclerotia	Colorless	No conidia
	TH10, TH15	Aerial, regular	White and orange; sparse black microsclerotia in the center	Colorless	No conidia
	TR10, TR15	Aerial, regular	White and orange; sparse black microsclerotia in the center	Colorless	No conidia
	CE10, CE15	Substrate	Light white; sparse black microsclerotia	Colorless	Sparse conidia
T. harzianum	PC10, PC15	Substrate, restricted aerial	White; green sporulation on the edge	Colorless	Very numerous conidia
	PH10, PH15	Substrate, restricted aerial	White; green sporulation in the center	Colorless	Very numerous conidia
	TH10, TH15	Substrate, restricted aerial	White; high green sporulation	Colorless	Very numerous conidia
	TR10, TR15	Substrate, restricted aerial	White; high dark green sporulation	Colorless	Very numerous conidia
	CE10, CE15	Aerial, regular	White; light green sporulation	Colorless	Sparse conidia
F. equiseti	PC10, PC15	Aerial, regular	White and creamy; creamy and beige	Colorless; creamy and brown	Moderately numerous conidia
	PH10, PH15	Aerial, regular	White and creamy; creamy and beige	Colorless; creamy and brown	Moderately numerous conidia
	TH10, TH15	Abundant aerial mycelium	Creamy and white	Colorless	Sparse conidia
	TR10, TR15	Mainly substrate, restricted aerial	Creamy and white	Colorless	Sparse conidia
	CE10, CE15	Aerial, regular	Creamy and white	Colorless	Numerous microconidia and sparse macroconidia
F. oxysporum	PC10, PC15	Regular growth	White	Colorless	Medium-sized microconidia
	PH10, PH15	Regular growth	White; pink and white	Colorless	Medium-sized microconidia
	TH10, TH15	Regular growth	White	Purple and red	Microconidia
	TR10, TR15	Regular growth	White	Purple and red	Microconidia
	CE10, CE15	Regular growth	White	Colorless	Macro- and microconidia

Table 4. *Cont.*

Fungus Species	Experimental Combination	Mycelium Surface and Structure	Obverse	Reverse	Presence of Spores
F. solani	PC10, PC15	Aerial, regular, abundant	White	Colorless	No conidia
	PH10, PH15	Aerial, regular, abundant	White	Colorless	No conidia
	TH10, TH15	Aerial fine, substrate	White	Creamy	Sparse conidia
	TR10, TR15	Aerial fine, substrate	White	Creamy	Sparse conidia
	CE10, CE15	Aerial regular	White	Colorless	Very numerous conidia
R. solani	PC10, PC15	Aerial, fluffy, abundant	White	Colorless	-
	PH10, PH15	Aerial, fluffy, abundant	White and creamy	Colorless	-
	TH10, TH15	Aerial, fluffy, abundant	White and creamy	Colorless	-
	TR10, TR15	Aerial, fluffy, abundant	White and creamy	Colorless	-
	CE10, CE15	Aerial, fluffy, abundant	Creamy and brown	Creamy	-

PC—*A. cruentus* extract; PH—*A. hypochondriacus* × *hybridus* extract; TR—*A. retroflexus* extract, TH—*A. hybridus* extract; 10—extract concentration 10%; 15—extract concentration 15%; day 8.

3. Discussion

Amaranth is a plant originating from Central America and is now widely cultivated in the countries of the tropics. In many countries, including India and tropical countries, it is still used in folk medicine as a laxative, to heal purulent lesions, boils and burns, and as an anti-malarial agent [26]. It has been proven that *Amaranthus*, thanks to its rich chemical composition, also shows antibacterial and antioxidant properties [27]. The plant is described in the literature as an important source of bioactive compounds such as lectins, phenols and flavonoids [28–30]. Flavonoids are an important group of biologically active compounds commonly present in many plant species. These compounds, contained in green plan parts, are characterized by, e.g., antimicrobial, anti-inflammatory or antioxidant activity [31,32]. The presence of twenty-five flavonoid and phenolic acids, such as protocatechuic acid, vanillic acid, gallic acid, salicylic acid, gentisic acid, *p*-hydroxybenzoic acid, β-resorcylic acid, syringic acid, ellagic acid, *m*-coumaric acid, *trans*-cinnamic acid, caffeic acid, chlorogenic acid, ferulic acid, sinapic acid, *p*-coumaric acid, rutin, naringenin, kaempferol, myricetin, catechin, isoquercetin, apigenin, hyperoside and quercetin, was detected in the leaves of *A. gangeticus*. Among the compounds, seven were identified as cinnamic acids, nine as benzoic acids and nine as flavonoid compounds. With respect to the three major classes of phenolics, the most prominent compounds were identified in four advanced lines of *A. gangeticus* genotypes in the following order: benzoic acids, cinnamic acids, flavonoids [33]. Not only flavonoids, but also phenolic acids determine the antimicrobial effect of amaranth extracts. The chemical composition of extracts varies and depends, among others, on the geographical location, as well as the composition and quality of the soil during plant growth [24]. The high content of polyphenolic compounds in alcoholic plant extracts was shown to be correlated with their high antioxidant capacity [34]. This was confirmed in our study involving amaranth leaf extracts carried out using the DPPH free radical method.

The biologically active compounds contained in amaranth plants are characterized by significant antimicrobial activity and cytotoxicity [35]. The conducted research showed the varied fungistatic effects of amaranth extracts. The strongest antifungal activity was recorded for extracts from *A. cruentus* (PC), *A. hypohondriacus* (PH) and *A. hybridus* (TH), of which TH was characterized by the highest polyphenol contents. The extracts strongly inhibited the growth of *F. equiseti*, *R. solani* and *T. harzianum*, and their highest fungistatic effect was observed at the beginning of the experiment. Jadhav and Biradar [20] conducted a similar study investigating the effect of *A. spinosus* on *Fusarium* spp. and *Aspergillus*

spp. They showed that ethanol extract of amaranth leaves, applied at a concentration of 1000 μg/mL, strongly inhibited the surface growth of *F. oxysporum*, while its lower concentrations in the medium (100 μg/mL and 500 μg/mL) had a fungistatic effect only against *A. flavus* and *A. niger*. According to the studies conducted by Akbar et al. [36], *Amaranthus viridis* leaf extract demonstrated potent antifungal activity against *F. oxysporum,* because they reduced the fungal growth up to 48% in *F. oxysporum*. Our research did not confirm these reports, as it indicated not only a lack of inhibition of the surface growth of *F. oxysporum* (regardless of extract concentration), but even its stimulation. Studies on the antifungal effect of various plant extracts against *F. oxysporum* have been conducted by many researchers [12,17] who showed that *F. oxysporum* was not very sensitive to the effects of extracts from medicinal plants. Kursa et al. [13] showed that 20% ethanol extracts of tansy, yarrow and horseradish, considered potent against many phytopathogens, inhibited the surface growth of *F. oxysporum* only at the level of 24.7–26.56%, and only in the first days of the experiment. The strong inhibitory effect of the amaranth extract was recorded against *F. equiseti*, mainly in relation to the extracts of *A. retroflexus* (TR) and *A. hypochondriacus* × *hybrisus* (PH). *F. equiseti* is an important pathogen of crops, recorded increasingly often in horticultural plants that have been shown to be highly pathogenic to seedlings, causing root rot and decay in horticultural plants [8,37–39]. *F. equiseti*, as other species of the genus, produces toxins and antibiotics, especially trichothecenes and equisetin [1,40]. Therefore, in this regard, *A. cruentus* (PC), *A. hypochondriacus* × *hybrisus* (PH) and *A. retroflexus* (TR) can be considered as plants with significant antifungal potential. A study of Carminate et al. [41] confirmed the antifungal efficacy of *Amaranthus viridis* against *Colletotrichum musae* causing banana anthracnose. The present study showed no antifungal effect against *C. coccodes*, and a stimulating effect on mycelial surface growth was even observed. The growth of this species was also poorly inhibited by other plant extracts, including extracts from plants with high biocidal potential [13]. Carminate et al. [41] also indicated the great potential of *Amaranthus viridis* in controlling *F. solani* f. sp. *piperis*, responsible for fusariosis in black pepper. Similarly, our research demonstrated the possibility of using amaranth extracts (PC, PH, TH) to limit the development of *F. solani*. The strongest antifungal activity against the species was recorded for the *A. cruentus* (PC) extract at the beginning of the experiment. This raises the prospect of obtaining more interesting results using higher amaranth extract concentrations in the preventive plant protection against *F. solani*. Promising results were also obtained against *Rhizoctonia solani*. All the tested extracts strongly limited the surface growth of the fungus, but only in the first days of the experiment. Shirazi et al. [42] showed that *Amaranthus viridis* extract at concentrations of 1, 3 and 5% exhibited maximum inhibitory potential activity in the disc diffusion method against soil-borne pathogens *R. solani, F. oxysporum* and *M. phaseolina*. The authors reported that *A. viridis* could be used for the control of fungal diseases, particularly those caused by *R. solani*; promising results have also been obtained with *A. alternata*. Fungi of the genus *Alternaria* are pathogens of stored fruits and vegetables. They penetrate the plant through mechanical and enzymatic degradation of the cell wall, causing rotting in the stored crops. The destructive effect on the host plant is caused by the production of enzymes and specific (HST) and non-specific toxins (NHST) [6]. Higher concentrations of amaranth extracts (mainly TH and PC) already significantly inhibited the surface growth of *A. alternata* in the first days of the experiment, but their antifungal activity decreased over time. The obtained results were consistent with the study of Akbar et al. [36]. These authors showed that all organic solvent extracts from *Amaranthus viridis* significantly reduced the biomass of the tested fungi with increasing extract concentrations (5, 10, 15, 20 and 25 mg mL^{-1}); strong activity was shown especially by ethyl acetate leaf fraction, resulting in reduced *A. alternata* growth by up to 44%.

Due to their cytotoxic properties, the biologically active compounds contained in plants have a direct effect on pathogen cells [35]. The antimicrobial activity of plant extracts rich in polyphenols causes many changes at the cellular level, e.g., damage of the microbial cell membrane through increased cell membrane permeability and, consequently, the leakage of cell contents [10]. Plant extracts from *Amaranthus* spp., in addition to

inhibiting the growth of the tested fungi, caused changes in the color and structure of aerial mycelium, as well as sporulation. Kursa et al. [17] reported that the addition of plant extracts from tansy, sage and wormwood also caused changes in the color and structure of the aerial mycelium of the tested fungi. The results of the present experiment on amaranth extracts are a valuable source of information for further field research in plant protection against pathogens, and as an ingredient of biological products protecting agricultural crops from rotting.

4. Materials and Methods

4.1. Plant Materials

The leaves of fully matured *Amaranthus* spp. (BBCH19), *A. cruentus* (PC) and *A. hypochondriacus* × *hybridus* (PH) species from a cultivation located in Bodaczów near Zamość (south-eastern Poland) and *A. retroflexus* (TR) and *A. hybridus* (TH) from plantations located in the province of Düzce (north-western Turkey) were collected in 2019. The collected materials were dried at room temperature (in the shade) and subsequently stored in polyethylene bags at 4 °C.

4.2. Biochemical Characteristics of Extracts

4.2.1. Extract preparation

Dried amaranth leaves were ground to a homogeneous fraction using an A11 Basic laboratory mill (IKA).

4.2.2. Extraction

Crushed plant material (100.0 g) was weighed into round bottom flasks and then 1000 mL of 70% ethanol was added. Extraction was carried out for 6 h under reflux condenser at the boiling point of ethanol. The obtained extract was filtered through a sterile 22 µm filter (AlfaChem) and concentrated to 100 mL (1:1 extract) using a rotary evaporator (Heidolph Instruments, Schwabach, Germany). The final extract did not contain ethyl alcohol.

4.2.3. Total Polyphenol Analysis

The concentration of total polyphenols in the tested extracts was determined using the spectrophotometric method (λ = 725 nm) with the Folin –Ciocalteau reagent, according to the modified method of Singelton and Rossi [43]. Phenol content results are expressed in gallic acid equivalents (GAE) (Sigma-Aldrich, St. Louis, MO, USA, ACS reagent $\geq$ 98.00%). The results were calculated based on the equation of the calibration curve prepared for gallic acid standards in the concentration range of 0.01–1 mg/mL (0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 1.00 mg/mL). Each sample, depending on the initial concentration, was diluted according to the range on the standard curve [17]. All analyses were performed in triplicate.

4.2.4. Assessment of Extract Antioxidant Activities

The antioxidant activity of plant extracts was determined using the modified method of Brand-Willams et al. [44] using the synthetic radical DPPH (1,1-diphenyl-2-picrylhydrase1 Sigma) converted to mM Trolox [45]. The inhibition of the DPPH radical by the extract sample was calculated according to the following formula: inhibition % = 100 $(A_0 - A_1)/A_0$, where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample. Each extract sample was diluted appropriately to the range of the standard curve prepared for Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standards. All analyses were performed in triplicate. Concentration values were based on the standard Trolox curve (0.2–1.2 mM) and expressed as millimoles of Trolox equivalents (TE) per ml of extract.

4.3. Fungal Cultures

Amaranth leaf extracts were individually tested against pathogenic fungi, such as *Fusarium solani* (ARIR14), *Fusarium oxysporum* (ECER4), *Fusarium equiseti* (ERIS8), *Alternaria alternata* (PCL10), *Colletotrichum coccodes* (P74/2), *Rhizoctonia solani* (TB71) and *Trichoderma harzianum* (A8/10). Fungal cultures were obtained in 2017–2020 as a result of plant mycological analyses (roots and aerial parts) of tomato (*Lycopersicon esculantum* L.) and pepper (*Capsicum annuum* L.) grown in the field. The fungal inoculum was derived from 10-day-old single-spore colonies grown on glucose-potato agar (PDA Difco, Becton, Dickinson and C., France), stored in the fungal collection of the Department of Plant Protection, University of Life Sciences in Lublin. Confirmation of strain species was carried out on the basis of microscopic analysis of each isolate/strain (spore structure and size, colony color) using appropriate mycological keys.

The study evaluated the effects of ethanol extracts of amaranth leaves (Polish and Turkish species: PC, PH, TR, TH) at concentrations of 10% and 15% on the linear growth of the test fungi. The method of poisoned substrates was used in the study, which is recommended to test chemical agents under laboratory conditions [13]. The method consisted of adding the test substance to sterile potato dextrose agar (PDA) medium cooled to 50 °C and inoculating the fungus species on the solidified medium. Medium and *Amaranthus* spp. extracts were poured into sterile Petri dishes of 90 mm and, subsequently, medium surface was inoculated with fungi colonies with a diameter of 3 mm. The control (CE) consisted of fungal colonies growing on potato dextrose agar (Difco PDA) with 10 and 15% residue after evaporation of the extractant used in the experiment (70% ethanol; a total volume of 1000 mL was evaporated to 100 mL in a rotary evaporator under the same conditions as in plant extract preparation). Five replicates of the tested extracts prepared at specific concentrations and added to each fungus were considered as objects. The plates prepared in this way were kept in an incubator for 12 days at 25°C. After 4, 8 and 12 days, the diameter of fungal colonies was measured. The measure of antifungal activity was the inhibition of mycelial growth on medium enriched with *Amaranthus* spp. extract relative to growth on control medium. The antifungal efficacy of *Amaranthus* spp. extract was calculated from the Abbott formula:

$$I = [(C - T)/C] \times 100\%$$

where: I—linear growth inhibition index of the fungus (percentage), C—diameter of the fungus colony in the control combination, T—diameter of the fungus colony in the combination containing the test substance concentration in the agar [13].

4.4. Statistical Analysis

Values are given as means ± standard deviation (SD) of each measurement. Where appropriate, the data were analyzed by analysis of variance (Duncan's test) at the 5% significance level using the SAS statistical software (SAS Version 9.1, SAS Inst., Cary, NC, USA).

5. Conclusions

The use of natural compounds to control pathogens is very attractive and creates new opportunities for biological plant protection. The results of the present experiment demonstrated a very diverse effect of amaranth leaf extracts (*A. cruentus*, *A. hypochondriacus* × *hybridus*, *A. retroflexus*, *A. hybridus*) on selected phytopathogenic and antagonistic fungi. The extracts were fungistatic only against *F. equiseti*, *R. solani* and *A. alternata*, and showed activity only during the first days of the experiment, which proved their selective and short-term antifungal effect. The strongest fungicidal effect was recorded for species cultivated in Turkey such as *A. hybridus* (TH), which, due to having the highest content of polyphenols and high antioxidant activity, can be recommended for limiting the growth of some phytopathogenic fungi, also causing spoilage of agricultural crops. Due to the lack of

a strong fungistatic effect, it can be used as an ingredient in plant preparations. In addition to direct antifungal activity, plant extracts act as elicitors of defense reactions in the plant and as biostimulants. The effect of amaranth leaf extracts as plant biostimulants requires further in-depth laboratory and field studies.

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Biocontrol Effect of *Clonostachys rosea* on *Fusarium graminearum* Infection and Mycotoxin Detoxification in Oat (*Avena sativa*)

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Abstract: Oat (*Avena sativa*) is susceptible to Fusarium head blight (FHB). The quality of oat grain is threatened by the accumulation of mycotoxins, particularly the trichothecene deoxynivalenol (DON), which also acts as a virulence factor for the main pathogen *Fusarium graminearum*. The plant can defend itself, e.g., by DON detoxification by UGT-glycosyltransferases (UTGs) and accumulation of PR-proteins, even though these mechanisms do not deliver effective levels of resistance. We studied the ability of the fungal biocontrol agent (BCA) *Clonostachys rosea* to reduce FHB and mycotoxin accumulation. Greenhouse trials showed that *C. rosea*-inoculation of oat spikelets at anthesis 3 days prior to *F. graminearum* inoculation reduced both the amount of *Fusarium* DNA (79%) and DON level (80%) in mature oat kernels substantially. DON applied to *C. rosea*-treated spikelets resulted in higher conversion of DON to DON-3-Glc than in mock treated plants. Moreover, there was a significant enhancement of expression of two oat UGT-glycosyltransferase genes in *C. rosea*-treated oat. In addition, *C. rosea* treatment activated expression of genes encoding four PR-proteins and a WRKY23-like transcription factor, suggesting that *C. rosea* may induce resistance in oat. Thus, *C. rosea* IK726 has strong potential to be used as a BCA against FHB in oat as it inhibits *F. graminearum* infection effectively, whilst detoxifying DON mycotoxin rapidly.

Keywords: oat; biocontrol; Fusarium head blight; deoxynivalenol; mycotoxins; *Clonostachys rosea*

1. Introduction

Oat (*Avena sativa*) is an important food and fodder crop in Northern Europe and Northern America [1,2]. The majority of the oats produced is used for livestock feed, but human consumption of oats has increased during the past decade due to its high nutritional value, good taste, recognised health benefits and use in new food products such as oat milk [3–5]. Thus, oat is emerging as a valuable crop in the transition towards a plant-based diet. However, oats are vulnerable to the disease Fusarium head blight (FHB) caused by a complex of *Fusarium*-species including *F. graminearum*, *F. avenaceum*, *F. poae*, *F. langsethiae* and *F. culmorum*. Although these fungal pathogens can cause substantial yield losses, their main threat lies in their production of contaminating mycotoxins, which compromise food and feed safety [6–9].

During the last decade, FHB infection in oat has been high with *F. graminearum* as the dominating species and deoxynivalenol (DON) as the prevalent mycotoxin [7,10–12]. DON is a type B trichothecene, which inhibits protein biosynthesis [13,14] and can cause both acute and chronic toxicoses in humans and livestock. Another important mycotoxin produced by *F. graminearum* is zearalenone (ZEA), a mycoestrogen causing reproductive disorders in mammals [15]. To protect human and animal health, the European Food Safety Authority (EFSA) established maximum limits for DON and ZEA in cereals and cereal

products. Thus, maximum EU levels for DON and ZEA in unprocessed oats are 1750 μg/kg and 100 μg/kg, respectively [16,17].

Fungicides sprayed at anthesis can reduce FHB and DON levels in cereals [18–20]. However, there are rising concerns about their use due to the increase of fungicide resistance and accumulation of fungicide residues in the environment. Moreover, as it takes over a week for an oat panicle and about a month for the whole plant to go through anthesis [21], finding optimal fungicide application times for the treatment of FHB in oat is a difficult task. Disease management using microbial biological control agents (BCAs) has gained momentum recently as it is considered to offer a sustainable and environmentally friendly path to crop production [22]. For the control of FHB in cereals, the fungus *Clonostachys rosea* has been tested in several trials. In Canadian field trials, isolate ACM941 of *C. rosea* showed a significant reduction of FHB in wheat as well as a 22–33% reduction in the DON content following spray treatments at flowering [23,24]. In addition, spraying an oil-based formulation of *C. rosea* isolate SHA77.3 reduced FHB in Swiss field trials in one of two tested wheat cultivars whereas the DON content was reduced in both cultivars by 45–69% [25]. However, despite problems with FHB and mycotoxin accumulation in oats, there are, to the best of our knowledge, no reports on the use of BCAs to combat the disease in oat production.

DON plays an important role for the pathogen, by acting as a virulence factor during *Fusarium*-infection [26,27]. However, plants can reduce the phytotoxic effect of DON by detoxification reactions, with conjugation into the much less acutely toxic DON-3-glucoside (DON-3-Glc) by UDP-glucosyltransferases (UTGs) as the main known mechanism [28–30]. Thus, the overexpression of the barley gene *HvUGT13248* in wheat reduced levels of DON (and nivalenol) and simultaneously decreased disease severity of both FHB and Fusarium crown rot [31,32]. DON-3-Glc is categorised as a 'masked mycotoxin', a term used to describe plant metabolites of mycotoxins, which can potentially be toxic after ingestion by mammals [33]. For example, DON-3-Glc was shown to be hydrolysed in rats by their gut microflora to release DON [34]. Cereals, including oat, can also detoxify DON by conjugating with other sugars, glutathione and other substances, but to a much lower extent [29,35].

Recently, we identified and characterised two oat DON-detoxifying UGTs, *AsUGT1* and *AsUGT2*, orthologous to barley *HvUGT13248* [36]. As increased DON detoxification has been linked directly to increased resistance to FHB in cereals, finding ways to enhance DON-detoxifying capacity in plants could contribute to developing resistance against FHB in the field. In addition to using cultivars with confirmed high DON-detoxifying ability, an equivalent DON-detoxifying response in a plant could, hypothetically, be induced by the application of BCAs.

It has been widely demonstrated that fungal BCAs can activate plant defence genes that play important roles in hampering pathogen infection, such as those encoding phytoalexin production and pathogenesis-related proteins (PR-proteins) [37]. The BCA *C. rosea* can induce expression of PR-genes encoding PR-proteins in wheat [38] and tomato [39]. These proteins are synthesised in plants in response to pathogen infection or exposure to abiotic stress. Several PR-proteins have antifungal properties and their induction is observed in plants exhibiting a high level of disease resistance. In common with most pathogens, *F. graminearum* is known to upregulate PR-genes in cereals [40–42], suggesting their role in plant defence against the pathogen.

WRKY transcription factors have likewise been reported to play a major role in plant disease resistance [43]. WRKY transcription factors were upregulated in wheat and barley tissues in response to *F. graminearum* infection [44,45] and specifically, WRKY23 and WRKY70 were shown to participate in activating defence against *F. graminearum*, correlating with resistance [46,47]. It was demonstrated that *C. rosea* upregulates genes in the WRKY family and other transcription factors in tomato [48–50], but, to our knowledge, no such transcription factor activation by *C. rosea* has been demonstrated in cereals.

In the current study, we used the *C. rosea* strain IK726, isolated from barley roots and reported to act as an effective BCA to control several plant pathogens [51,52], including pathogenic *Fusarium*-species causing seedling blight in wheat and barley. Recently, it has also been demonstrated that *C. rosea* IK726 reduced FHB symptoms as well as DON content in wheat both in greenhouse tests and in the field [53,54]. We show that application of *C. rosea* IK726 to oat spikelets: (i) reduces both *F. graminearum* biomass and mycotoxin content in the spikelets, (ii) enhances expression of two oat UGT genes and DON-glucosylation in response to DON application and (iii) activates the expression of genes encoding four PR-proteins and a WRKY transcription factor.

2. Results

2.1. C. rosea Reduces F. graminearum Biomass and Mycotoxin Content in Mature Oat Kernels

In order to elucidate a potential biocontrol ability of *C. rosea* strain IK726 against FHB in oat, three independent greenhouse experiments were performed where oat spikelets were inoculated first with spores of *C. rosea* and subsequently with *F. graminearum* spores under conditions favouring *Fusarium*-infection. Quantifying *F. graminearum* DNA after harvest showed that *C. rosea* significantly reduced the amount of *F. graminearum* DNA in mature grain (Figure 1A). The amount of *F. graminearum* DNA in three trials was reduced in the range of 69–97%, with an average reduction of 79% in *C. rosea*-treated spikelets. Special care was taken to avoid contamination by droplets of fungal conidial suspension; no *F. graminearum* DNA was detected in control treatments (mock (no *F. graminearum*)/mock and *C. rosea*/mock, results not shown).

We quantified 3-ADON, DON-3-Glc and DON in the infected oat kernels using LC-MS/MS. This analysis showed that DON accumulation (sum of DON, 3ADON and DON-3-Glc) was significantly reduced by 62–93% in three trials in *C. rosea*-treated compared to mock-treated spikelets (Figure 1B and Table 1). Taken separately, DON was reduced by 64–93% (significantly in all three trials), 3ADON was reduced by 53–95% (significantly in two trials), and DON-3-Glc was reduced by 37–78% (significantly in one trial). Zearalenone (ZEA) was found in the samples in much lower quantities (10–30 pg/mg) compared to DON, but *C. rosea* treatment did not have a significant effect on the level of this mycotoxin (data not shown).

Table 1. Percent decrease of mycotoxins and amount of *F. graminearum* DNA (pg *Fusarium* DNA/ng oat DNA) in *C. rosea*-treated compared to non-treated spikelets and percent increase of DON3G relative to total level of DON.

Trial	*F. graminearum* DNA	Sum of DONs	DON	3-ADON	DON-3-Glc	DON-3-Glc/Sum of DONs [1]
1	69.3	61.9	64.0	64.0	40.5	30.9
2	78.4	61.4	62.7	62.7	37.3	68.0
3	97.1	92.8	93.3	93.3	77.7	195.0

[1] Percent DON3G relative to total level of DON (DON+3ADON+DON-3-Glc).

The calculated percentage of DON-3-Glc relative to the total level of DON showed a tendency of *C. rosea* treatment to increase the conversion of DON into DON-3-Glc, but a significant increase was observed only in trial 3 (Figure 1F).

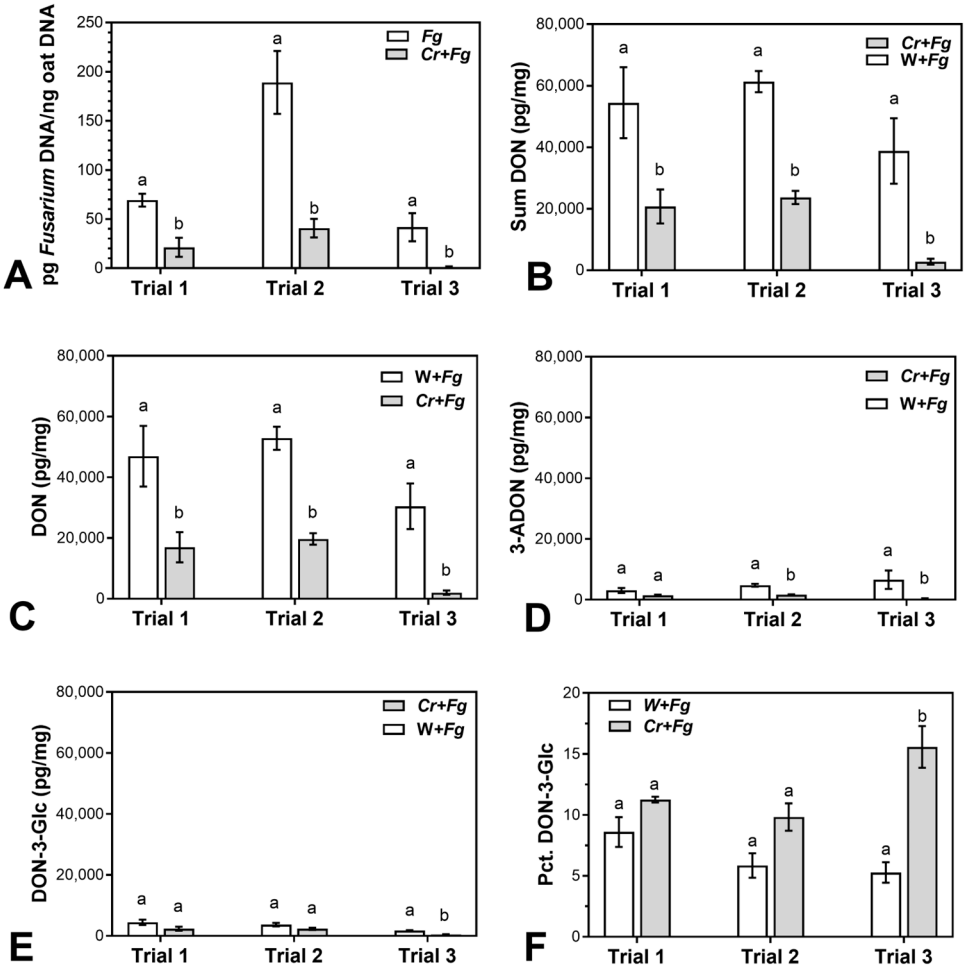

Figure 1. *Fusarium graminearum* biomass and mycotoxin levels in three independent experiments. (**A**) *F. graminearum* DNA amount (pg *Fusarium* DNA/ng oat DNA) in three independent trials. (**B**–**F**): Levels of three mycotoxins or masked mycotoxins in three independent trials: (**B**) sum of DON, 3-ADON, and DON-3-Glc, (**C**) DON, (**D**) 3-ADON, (**E**) DON-3-Glc, (**F**) Percentage of DON-3-Glc relative to the sum of DON, 3ADON and DON-3-Glc. Error bars represent standard error of the mean. Within each part of the figure, means marked with different letters are significantly different ($p < 0.05$).

2.2. C. rosea Enhances Conversion of DON into DON-3-Glc in Oat Spikelets

In the experiment with infected oats, it was difficult to assess the effect of *C. rosea* on glucosylation of DON, as the suppression of the infection led to variable amounts of DON in the samples. To obtain a more reliable comparison of levels of conversion of DON into DON-3-Glc in *C. rosea*-treated and mock-treated oat spikelets, DON was applied to oat spikelets and the compounds were quantified at 24, 48, 72 and 96 h after application. LC-MS/MS analysis showed that, in spikelets treated with *C. rosea*, DON was conjugated into DON-3-Glc at a much higher level by 24 h compared to non-treated spikelets (Figure 2) and remained high through the course of the experiment. In addition to DON-3-Glc, compounds with molecular masses corresponding to three more DON conjugates were detected: DON-diglucoside, DON-hexitol and 15-acetyl-DON-glucoside that are all described DON-detoxification products [29]. Due to lack of standards, these metabolites could not be quantified, but judging from the areas of the chromatogram peaks (Supplementary Figure S1) higher amounts of DON-diglucoside and 15-acetyl-DON-glucoside were formed in *C. rosea*-treated spikelets at early time points compared to the water treated control. The peaks were small compared to those of DON and DON-3-Glc; as all compounds are glycosidic derivatives of DON, their ionization efficiencies in the

LC-MS analysis can be assumed to be roughly similar, indicating that DON-3-Glc is the main DON detoxification product. DON-glutathione conjugates were previously identified in *Fusarium*-infected mature oat grain (unpublished data), but not detected here in the DON treated spikelets.

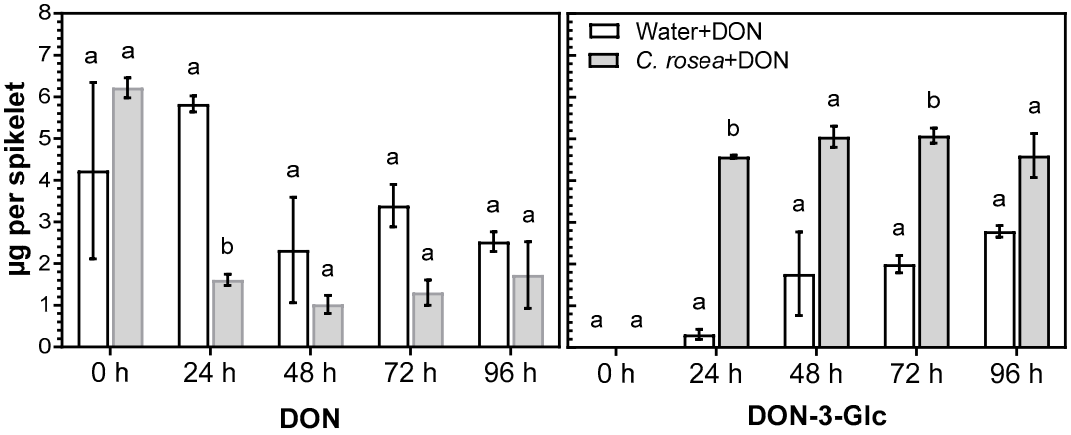

Figure 2. DON and DON-3-Glc in *C. rosea*-treated and mock treated oat spikelets. Spikelets were treated either with *C. rosea* followed by DON (*C. rosea* + DON), water+DON, *C. rosea*+water or water + water. DON and DON-3-Glc were only detected in *C. rosea*+DON and water + DON treated samples and the quantification of these two compounds is shown in the figure. Error bars represent standard error of the mean. Within each part of the figure, means marked with different letters are significantly different ($p < 0.05$).

2.3. C. rosea Enhances Expression of Oat UDP-glucosyltransferases in DON-Treated Oat Spikelets

Samples from the same experiment as described above, where oat panicles were inoculated with *C. rosea* and treated with DON after 3 days, were used to study the effect of *C. rosea* on the expression of the two previously identified UGT genes [36] in the treated oat spikelets. Spikelets for qPCR analysis were collected at 0, 2, 4, 8, 12 and 24 h after DON treatment. Quantification of the UGT transcripts showed that the accumulation of both *AsUGT1* and *AsUGT2* transcripts at all time points was significantly higher in *C. rosea* than in mock-treated spikelets (Figure 3).

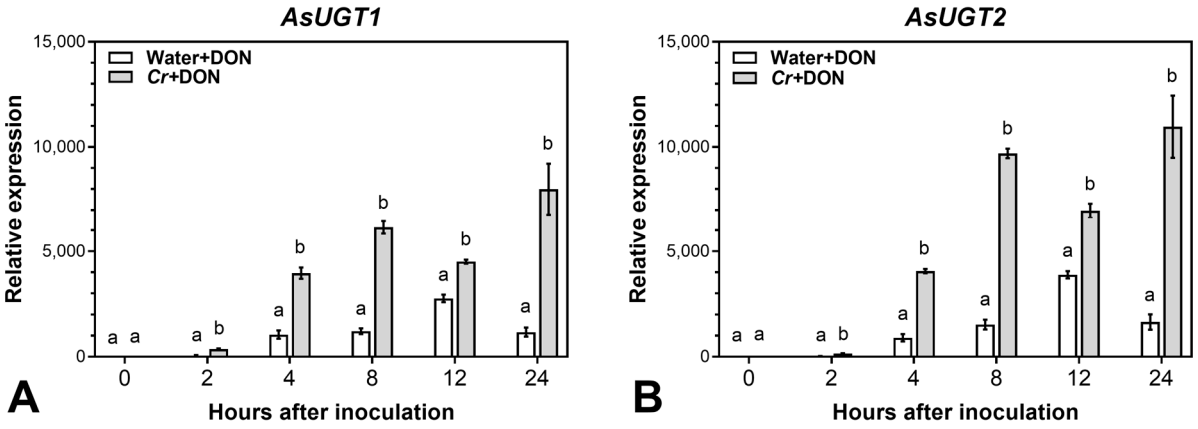

Figure 3. Relative expression of DON-induced (**A**) *AsUGT1* and (**B**) *AsUGT2* transcripts in *C. rosea*-treated and mock-treated oat spikelets. Error bars represent standard error of the mean. Bars marked with same letter are not significantly different ($p \leq 0.05$).

Interestingly, already at 2 h after DON application, the expression of *AsUGT1* was 6 fold higher and the expression of *AsUGT2* 7 fold higher in *C. rosea*-treated compared to mock-treated spikelets. Induction of UGTs in *C. rosea*-treated spikelets at 4 h after DON application was as high as the induction of UGTs at 12 h in water-treated spikelets.

2.4. C. rosea Induces Expression of Genes of Oat PR-Proteins and WRKY23 Transcription Factor

To shed light on the mode of biocontrol activity of *C. rosea* against *F. graminearum* in oat, the expression of genes coding for four PR-proteins and two WRKY transcription factors were analysed in oat spikelets inoculated with *C. rosea* or left untreated (mock-treatment). *C. rosea* treatment significantly induced expression of the genes *PR1* (anti-fungal) by 58 fold, *PR3* (chitinase) by 30 fold, *PR4* (wheatwin) by 69 fold and *PR5* (thaumatin-like) by 27 fold compared to mock treatment (Figure 4). Expression of the *WRKY23-like* gene was significantly induced by 7 fold, while expression of the *WRKY70-like* gene did not change significantly.

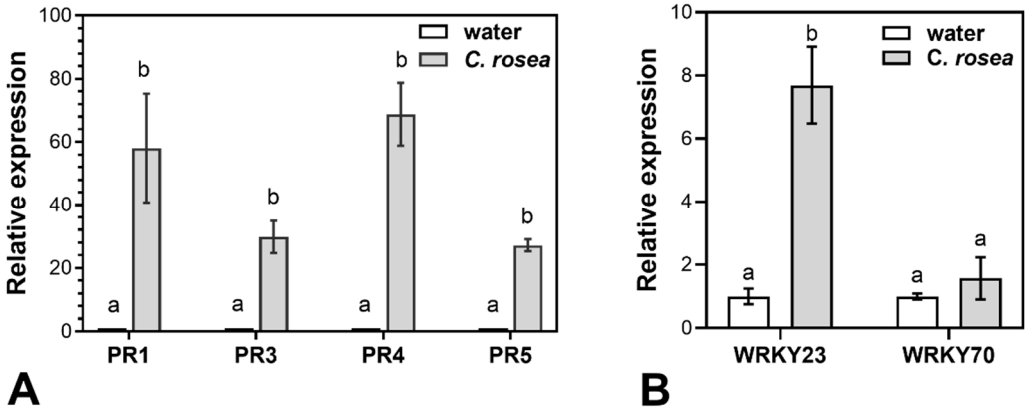

Figure 4. Expression of genes of four PR-proteins (**A**) and two WRKY transcription factors (**B**) in *C. rosea*-treated and water-treated oat spikelets 3 days after the inoculation. Error bars represent standard error of the mean. Bars marked with same letter are not significantly different ($p \leq 0.05$).

3. Discussion

While oat is affected by FHB to a similar degree as other cereals in terms of yield losses and mycotoxin accumulation, biological control of FHB in oat has been neglected. Previously, *C. rosea* has found to control *Fusarium* infection in several crops, including wheat [23,24,38] and maize [55,56]. Here, we studied the ability of *C. rosea* IK726 to control FHB and reduce mycotoxin content in oat and investigated the effect of *C. rosea* on DON glucosylation. Additionally, we examined the ability of *C. rosea* to induce the expression of certain PR proteins and WRKY transcription factors, indicating that mechanisms of control could involve induced resistance.

3.1. C. rosea Reduces F. graminearum Biomass and Mycotoxin Content in Mature Oat Kernels

Application of *C. rosea* IK726 to oat spikelets at anthesis at 3 days prior to *F. graminearum* inoculation reduced *Fusarium* biomass in three trials substantially (69–97% reduction). In wheat, where the biocontrol ability of *C. rosea* strain ACM941 on FHB has been studied previously, the effect was quantified as the percentage of *Fusarium* damaged kernels (FDK) [23,24]. In greenhouse trials, reductions of FDK at 65% and 68–92% were found, respectively. In wheat, there is a high correlation between FDK and *F. graminearum* biomass [25,57]. However, symptoms of FHB in oat are often quite cryptic due to the hulls covering the seeds, which makes a visual quantification of FDK unreliable. Therefore, in the present work, we quantified the level of *Fusarium* infection by amount of *Fusarium* DNA in oat spikelets. *C. rosea*-mediated biocontrol efficacy against FHB in oat is potentially at the same level as the efficacy obtained with *C. rosea* in wheat.

Treatment with *C. rosea* also reduced levels of DON considerably (64–93%) in mature oat kernels. This is in line with DON reduction observed in wheat [23–25] and further strengthens the potential of *C. rosea* for biocontrol of FHB in cereals. *F. graminearum* strains initially synthesise an acetylated form of DON, i.e., 3-ADON or 15-ADON depending on the chemotype [58,59], which are later deacetylated into DON in the plant [60]. Strain

LS G2 is a 3-ADON producer and DON-3-Glc was the main DON detoxification product identified when oat spikelets were inoculated with DON (Figure 3). Therefore, we consider that DON, 3-ADON and DON-3-Glc, roughly represent the bulk of all DON and DON derivatives produced by *F. graminearum* in the inoculated spikelets. Collectively, the sum of DON, 3-ADON and DON-3-Glc produced during the infection was reduced by 62–93% in *C. rosea*-inoculated compared to mock-treated spikelets.

Whereas reduction of DON accumulation is the main goal of biocontrol of *Fusarium* spp. in cereals, the potential ability of *C. rosea* to affect the detoxification of DON is also relevant. The percentage of DON-3-Glc relative to the sum of DON, 3ADON and DON-3-Glc was higher in *C. rosea*-treated compared to mock treated spikelets, although differences were significant only in one of three trials (Figure 1E). The higher percentage of DON-3-Glc in the *C. rosea* treated spikelets is an indicator of enhanced DON-detoxification occurring in the plant cells. This could occur either by *C. rosea*-mediated upregulation of plant UGTs as demonstrated in this study, the involvement of *C. rosea* glucosylation enzymes or a combination of both. As for the ability of *C. rosea* to detoxify DON, recently, 15-acetyl-DON-3-Glc was reported in the interaction zone between *C. rosea* and *F. graminearum* in a dual culture assay [61]. Whether such mechanisms are active *in planta* remains to be determined.

In a previous study, Gimeno et al. [25] found that *C. rosea* either did not have any effect on ZEA accumulation in wheat grain or in some cases increased it. In our three trials, ZEA was detected in low quantities and *C. rosea* did not have any effect on ZEA accumulation. However, ZEA production appears to be highly dependent on climate conditions, especially the amount of rainfall [62,63]. Since we performed our trials in the greenhouse, the conditions were most probably more favourable for DON than for ZEA production.

3.2. Treatment of C. rosea-Inoculated Spikelets with DON Increased Conversion of DON into DON-3-Glc and Enhanced Expression of Two Glucosyltransferase Genes

To investigate the fate of DON and expression of DON-detoxifying genes in green oat spikelets, we applied a high concentration of DON to spikelets pre-treated with *C. rosea*. By applying only the mycotoxin DON rather than *F. graminearum* itself, the time needed for the pathogen to produce large amounts of DON (up to 144 h) [44] was eliminated. At the same time, the possibility of natural senescence in the oat spikelet tissues occurring during the infection period was excluded.

Conjugation of DON into DON-3-Glc at all time points occurred to a much higher degree in *C. rosea*-treated spikelets compared to mock-treated spikelets. The largest difference was observed at the earliest time point, 24h post-DON application, when the level of DON-3-Glc was 14 times higher in *C. rosea*-treated spikelets than in mock-treated.

Demissie et al. [61] found that *C. rosea* induces formation of 15-ADON-3-Glc in confrontation assays with *F. graminearum*, which suggests an upregulation of glucosyltransferases in *C. rosea*. In the current experiment, we cannot exclude the possibility that *C. rosea* enzymes contribute directly to DON-glycosylation in plant tissues, although this contribution is unlikely to be as pronounced as that of a plant's own detoxifying machinery. *C. rosea* possesses a very effective mechanism for removing toxic substances from the cells into the apoplast in form of numerous drug membrane transporters [52,61,64]. Therefore, this fungus is probably less dependent on DON-detoxification mechanisms than plants.

High levels of DON-3-Glc are most probably the result of induction of UGT genes in plant tissues, as we observed a significantly enhanced expression of the two oat UTG genes (*AsUTG1* and *AsUTG2*) in *C. rosea*-treated samples. These two UGTs were previously characterised and found to be highly inducible by both DON-treatment and *F. graminearum* infection [36]. The presence of *C. rosea* led to significantly higher induction of the oat UGTs in DON-treated spikelets at all time points after DON-application. Effective and fast DON-detoxification, especially in earlier stages of infection, is an important component of resistance against *F. graminearum* infection [29]. Such a considerable and rapid response in *C. rosea*-treated tissues could better prevent protein biosynthesis damage caused by binding

DON to the ribosomes. To the best of our knowledge, we demonstrated for the first time the activation of cereal UTGs by a fungal BCA.

The two oat UGT genes were not directly induced by *C. rosea*. Thus, 3 days after *C. rosea* inoculation and immediately before DON application (Figure 3A,B, 0 h), expression of the genes was low and did not differ between *C. rosea* and mock treatments. Enhanced DON-induced expression of these genes in *C. rosea*-treated spikelets could be the result of *C. rosea* upregulating the expression of various transcription factors, preceding the expression of the defence genes.

Furthermore, *C. rosea* might directly or indirectly upregulate genes of other plant DON-detoxifying enzymes. While no glutathione (GSH) conjugates were detected, we detected compounds with molecular masses corresponding to DON-diglucoside, 15-acetyl-DON-glucoside and DON-hexitol, which were previously found in DON-treated wheat spikes among several other conjugates [29]. These compounds could not be quantified, but the two first are glucosides and likely derivatives of DON-3-Glc, underscoring the importance of the UGT-mediated pathway for DON detoxification in oat.

3.3. C. rosea Inoculation Induces Expression of PR-Genes and a WRKY Transcription Factor

Induction of PR-genes by BCAs as a first line of plant resistance is well known [65], but has not been studied previously in oat. In a hexaploid species like oat, there are numerous gene copies within each PR-protein family, which could be expressed in addition to those that we have quantified. Transcripts of genes corresponding to the four oat PR-proteins accumulated to high levels (27–69 fold increase) in spikelets treated with *C. rosea* at 3 days prior to sampling as compared to the expression of these genes in spikelets treated with water only. In the biocontrol trials (Figure 1), *F. graminearum* inoculum was applied at 3 days after inoculation with *C. rosea* and therefore we assume that genes coding for these PR-proteins were already expressed at that time point. Previously, transcripts for PR1, PR4 and PR5 were found to accumulate in coleoptiles and roots of wheat when these tissues were inoculated with *C. rosea* [38]. Homologues of the four PR-proteins studied here in response to *C. rosea* treatment are known to play important roles during *F. graminearum* infection in other cereals. Thus, genes belonging to the PR1-protein family were specifically induced in *F. graminearum*-inoculated maize [66]; transgenic wheat, expressing chitinase II (PR3), exhibited increased resistance against *F. graminearum* [67]; PR4-protein was shown to inhibit *F. graminearum* development in wheat kernels [68] and PR5-protein was expressed in a resistant barley cultivar after *F. graminearum* inoculation [69]. Substantial accumulation of these four PR-protein transcripts in oat spikelets after treatment with *C. rosea* suggests that protection against *F. graminearum* in spikes may at least partly include induced resistance. However, whether *C. rosea* can induce resistance against *F. graminearum* would need to be tested. This would be the case if rapid and enhanced expression of defence-related processes occur after pathogen arrival [65]. To the best of our knowledge, such a strong response in PR-genes, mediated by the presence of *C. rosea* alone, has not been demonstrated for BCAs previously.

WRKY transcription factors are known to play an important role in plant defence responses as they regulate expression of various defence genes [43]. WRKY70 was shown to have a positive effect on the resistance against *F. graminearum* in wheat [46]. Recently, WRKY23 was found to be involved in modulating defence responses against FHB in barley by regulating genes important for reinforcing cell walls against the invasion of pathogen, including a glucosyltransferase gene [47]. To determine the ability of *C. rosea* to upregulate WRKY transcription factors, relevant in defence against *F. graminearum*, we examined expression of the *WRKY23-like* and *WRKY70-like* genes in oat spikelets treated with *C. rosea*. While we did not observe any significant increase in transcript accumulation in *C. rosea*-treated oat spikelets for the *WRKY70-like* gene, WRKY23-*like* transcript accumulation was significantly increased by 7 fold. This suggests that one of the modes of biocontrol action of *C. rosea* could be an upregulation of transcription factors involved in the expression of defence genes.

4. Conclusions

We showed that treatment with *C. rosea* IK726 substantially reduced both *F. graminearum* biomass and mycotoxin content in oat kernels. *C. rosea* appears to upregulate different types of defence in plant tissues. While defence genes, such as those encoding four PR-proteins and WRKY23-like transcription factors were activated directly, induction of UGT encoding genes was indirect and could occur due to *C. rosea*-mediated pre-activation of certain transcription factors. *C. rosea*-mediated induced resistance in oat spikelets helps the plant to not only effectively inhibit the *F. graminearum* infection, but also to detoxify mycotoxins produced by the pathogen more rapidly, demonstrating the versatile potential of treatment with this BCA. Hence, this strain has a strong potential to be used as a BCA against FHB in oat. Future experiments should investigate the durability of *C. rosea* for reduction of FHB and mycotoxin content in oat under field conditions.

5. Materials and Methods

5.1. Plant and Fungal Material

Oat cv Belinda was cultivated as follows: two seeds per pot (16 cm in diameter) were planted in a mix containing soil (*Krukväxtjord med lera och kisel*, SW Horto, Sweden), 9% (*v*/*v*) perlite and 0.3% (*v*/*v*) Basacote plus 3M granulated fertilizer (N-P-K[+Mg+S] 16–8-12 [+2+5], Compo Expert, Münster, Germany). Plants were grown in a greenhouse with the light intensity set at 300 µE m^{-2} s^{-1} at the panicle level and relative humidity 65%. The temperature was maintained at 22 °C during the 16 h day and 18 °C during the 8 h night.

C. rosea strain IK726 [51] was cultured on potato dextrose agar (PDA, Scharlau Microbiology, Barcelona, Spain) plates for 7 days. Spores were harvested by flooding the plates with sterile MilliQ water with 0.02% (*v*/*v*) Tween 20 added. The suspension was filtered through a 100-µm cell strainer (Sarstedt, Nümbrecht, Germany) and the concentration of the spores was adjusted to 10^7 spores/mL.

F. graminearum strain LS_G2 (3-ADON chemotype) was previously isolated from a farm sample of *Fusarium*-damaged oat seeds grown in Sweden. The attribution of this strain to *F. graminearum* species was determined by sequencing of *EF-1α* and *RPB2* genes fragments according to O'Donnel et al., 2010 [70] and the chemotype was determined by PCR according to Quarta et al., 2006 [71]. Inoculum was obtained by culturing the fungus on PDA plates for 5 days and subsequently growth in mung bean broth [72] for another 5 days at 25 °C with shaking at 150 rpm. Macroconidia were filtered through a 100-µm cell strainer (Sarstedt, Nümbrecht, Germany), harvested by centrifugation at 3800 RCF for 10 min and washed once with sterile distilled water. Spores were resuspended in sterile water with added Tween 20 (0.02%, *v*/*v*). The concentration of macroconidia was adjusted to 10^6 spores/mL.

5.2. Application of C. rosea, F. graminearum and DON to Flowering Spikelets

To evaluate the biocontrol effect of *C. rosea* and the corresponding effect on DON accumulation in mature grains, plants were inoculated at anthesis (GS 65), either with a spore suspension or with water containing 0.02% (*v*/*v*) Tween 20 (mock). Twenty-µL *C. rosea* spore suspension (10^6 spores/mL) or water was pipetted in the space between two adjacent florets in a spikelet. Inoculated spikelets were marked with a waterproof marker. Panicles were covered with 3-L plastic bags, misted with distilled water beforehand. After 48 h, the bags were removed and panicles were sprayed with distilled water three times per day. At 72 h after inoculation with *C. rosea*, spikelets were inoculated with 15 µL *F. graminearum* spore suspension (10^5 spores/mL) or with water containing 0.02% (*v*/*v*) Tween 20 (mock), in the same way as above. Treated oat panicles were handled in the same way as above, except that bags were removed after 72 h. A total of 15 spikelets were inoculated per panicle with 4 panicles per treatment. Spikelets were harvested at grain maturity (100 days after sowing). Thus, 15 spikelets from each panicle were pooled and represented one replicate. Thus, there were four replicates per treatment. Three separate greenhouse trials were performed over a six-month period of the same year.

To study the DON-induced expression of oat UDP genes in *C. rosea*-treated spikelets, plants were inoculated with *C. rosea* as described above. At 72 h after inoculation, spikelets were treated with an aqueous DON solution (0.6 mg/mL) or with distilled water (mock). At time point zero, 10 µL DON solution was pipetted into the space between two adjacent florets in a spikelet. Treated spikelets were marked with a waterproof marker. Inoculated panicles were covered with 3-L plastic bags, sprayed with distilled water beforehand. A total of 10 spikelets were treated per panicle. At 0, 2, 4, 8, 12, 24 and 48 h after treatment, spikelets were sampled and immediately frozen in liquid nitrogen.

5.3. Quantification of F. graminearum DNA

Marked spikelets from each panicle were harvested and hand-threshed (glumes were removed) and milled into a fine flour using a "Pulverisette 23" ball mill (Fritsch, Idar-Oberstein, Germany) set at 50 1/s oscillation for 4 min. DNA was extracted using the Bead Beat micro AX Gravity kit for genomic DNA purification (A&A Biotechnology, Gdansk, Poland). The quality and quantity of the extracted DNA were analysed spectrophotometrically and with Qubit Fluorometric Quantification (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Primers and the probe 5′ 6-FAM/ZEN/3′IMFG (Integrated DNA Technologies, Leuven, Belgium) were designed to amplify a fragment of the *F. graminearum* TRI5 gene, based on the fragment from Waalwijk et al. [73]. A pair of primers with the probe 5′HEX/ZEN/3′IBFQ (Integrated DNA Technologies, Leuven, Belgium) were designed to amplify a fragment of the oat α-tubulin gene, which was used as a plant DNA reference gene (for primer sequences, see Supplementary Table). Duplex qPCR reactions targeting *F. graminearum* and the oat α-tubulin gene were performed in a final volume of 15 µL, containing 40 ng DNA, 750 pmol of each primer, 225 pmol of each probe and 7.5 µL PrimeTime™ Gene Expression Master Mix (Integrated DNA Technologies, Leuven, Belgium). Reactions were carried out with technical duplicates using a CFX96 Real-Time qPCR system (Bio-Rad Laboratories, Hercules, CA, USA). The thermocycler programme was set for 3 min at 95 °C followed by 42 cycles of 15 s at 95 °C, 20 s at 62 °C and 20 s at 72 °C. Default parameters of the Bio-Rad system were used to determine Ct-values for each reaction. Standard curves for DNA quantification were made using six 10-fold dilutions starting from 10 ng pure *F. graminearum* DNA and 50 ng pure oat DNA. For *F. graminearum* primers and probe, the sensitivity and efficiency of the assay were 1.5 pg and 97.3% and for oat α-tubulin primers and probes 5 pg and 102.3%, respectively. The ratio between the total amount of *F. graminearum* DNA and the total amount of oat DNA was calculated as the DNA load of *F. graminearum* in the samples.

5.4. Analysis of DON-Induced UGT Expression in Oat

Experiments were performed in three biological repetitions. In each, RNA was extracted from two spikelets. Separate spikelets were ground under frozen conditions in pre-cooled (liquid nitrogen) plastic tubes with screwcaps (Sarstedt, Nümbrecht, Germany) and two 5 mm stainless steel beads (Qiagen, Hilden, Germany) using a Precellys Evolution homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) as follows: 25 s homogenisation at 5500 rpm, cooling in liquid nitrogen for 10 s and repeating the homogenisation for 25 s at 5500 rpm. RNA was extracted from the pool of two homogenised spikelets using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany). The quality and quantity of the extracted RNA were analysed by agarose electrophoresis and spectrophotometrically. DNA was removed from the samples with DNA free Kit (Invitrogen, Waltham, MA, USA) and cDNA synthesis was performed using the iScript cDNA synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). cDNA samples were diluted 1:20 (*v*/*v*) with TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA), aliquoted and stored at −20 °C. Primers and the probe 5′ 6-FAM/ZEN/3′IMFG (Integrated DNA Technologies, Leuven, Belgium) were designed to amplify fragments of the *AsUGT1* and *AsUGT2* oat genes (for primer sequences, see Supplementary Table S1). The oat α-tubulin gene was used as a reference target and a pair of primers together with the probe 5′HEX/ZEN/3′IBFQ (Integrated DNA Technologies,

Leuven, Belgium), amplifying a fragment of this gene, were used. The efficiency and sensitivity of the primers and probes in duplex reactions were previously tested [36]. The efficiency of the primers and probes was in range of 97–103% and the sensitivity was 0.1 ng of total oat RNA. Duplex qPCR reactions targeting one of the UGTs and the reference gene were performed in a final volume of 15 µL, containing 2 µL of the diluted cDNA product, 750 pmol of each primer, 225 pmol of each probe and 7.5 µL PrimeTime™ Gene Expression Master Mix (Integrated DNA Technologies, Leuven, Belgium). Reactions were carried out in technical duplicates using a CFX96 Real-Time qPCR system (Bio-Rad Laboratories, Hercules, CA, USA). The following thermocycler programme was used: 3 min at 95 °C followed by 42 cycles of 15 sec at 95 °C, 20 sec at 62 °C and 30 sec at 72 °C. The Ct-values were automatically determined for each reaction by the Bio-Rad system set with default parameters. The comparative ΔΔCt method was used to evaluate the relative quantities of each amplified product in the samples [74].

5.5. Analysis of the Expression of Oat PR-Proteins Genes and WRKY-Transcription Factors

Oat PR-protein and WRKY-transcription factor sequences were selected based on the sequence homology with previously characterised barley proteins: PR1 (X74939.1), PR3 (X78672.1), PR4 (Y10814.1), PR5 (AJ001268.1), WRKY23 (KT962219.1) and WRKY70 (MLOC_66134). We used the Sequenceserver v2.0.0 [75] tool to perform BLASTP [76] searches (E-value < 1.0×10^{-5}) of the barley proteins against the oat cv. Sang v1.1 proteins. Additionally, we checked that the oat proteins and their corresponding barley homologues belong to the same orthogroups, previously reported by Kamal et al. [77].

Primers and the probe 5′ 6-FAM/ZEN/3′IMFG (Integrated DNA Technologies, Leuven, Belgium) were designed using the PrimerQuest Tool (Integrated DNA Technologies, Leuven, Belgium) to amplify gene fragments, corresponding to 4 oat PR-proteins and 2 WRKY transcription factors. Each primer pair and the probe were specific to two PR-gene copies and three WRKY gene copies in the hexaploid oat Sang genome. For primer sequences and the corresponding oat genes, see Supplementary Table S1. The oat α-tubulin gene was used as a reference target. qPCR reactions were performed as described above.

5.6. UPLC-MS/MS Analysis of DON, 3-ADON and DON-3-Glc in Mature Oat Kernels

The LC-MS/MS analyses were performed by the facility of the Swedish Metabolomics Centre, Umeå, Sweden. The following solvents were used: acetonitrile (ACN) and 2-propanol (IPA) hypergrade for LC-MS LiChrosolv (Merck, Darmstadt, Germany), formic acid, HiPerSolv Chromanorm for LC-MS (VWR Chemicals), and MilliQ gradient system purified H_2O.

Mycotoxins were extracted by adding 1 mL solution containing 80% ACN (v/v), 0.1 ng/µL caffeine-13C3 (as an internal standard) to approximately 50 mg milled flour. The samples were shaken with one tungsten bead each at 30 Hz for 3 min in a mixer mill (MM 400, Retsch) and then centrifuged at 18,620× g) for 10 min at +4 °C. Fifty-µL supernatant was transferred to vials, dried under nitrogen gas flow and stored at −20 °C until analysis. Small aliquots of the remaining supernatants were pooled and used to make quality control (QC) samples. Blank samples, i.e., samples without starting material, were prepared the same way as the samples.

The analyses were conducted using an Agilent UHPLC system (Infinity 1290) coupled with an electrospray ionization source (ESI) to an Agilent 6495 triple quadrupole system equipped with iFunnel Technology (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was performed on a Waters UPLC HSS T3 column (2.1 mm × 50 mm, 1.8-µm particle size). The mobile phase consisted of 0.1% (v/v) formic acid in MQ-water (A) and 0.1% (v/v) formic acid in ACN:IPA (75:25) (B).

Gradient: the flow rate was set to 500 µL/min and the column was heated to 40 °C. 100% A was run the first min followed by a linear increase of B to 70% over 2 min. A linear increase to 85% B under 1.5 min was followed by isocratic 85% B for 1.5 min. B was linearly increased to 99% for 0.5 min and held isocratic 1.5 min. The column was

returned to its initial conditions in 0.3 min and re-equilibrated at 800 µL/min flow, for 1.5 min. Analysis parameters: sample injection volumes were 2 µL. The mass spectrometer was operated in both negative and positive ESI mode with gas temperature set at 150 °C; gas flow 16 L/min; nebulizer pressure 35 psi; sheath gas temperature 350 °C; sheath gas flow 11 L/min; capillary voltage 3000 V (neg) and 4000 V (pos); nozzle voltage 1500 V (neg) and 300 V (pos); iFunnel high pressure RF 90 V (neg) and 200 V (pos); iFunnel low pressure RF 60 V (neg) and 100 V (pos). The fragmentor voltage 380 V and cell acceleration voltage 5 V. For a list of dMRM transitions, see Supplementary Table S2. Data was processed using MassHunter Qualitative Analysis and Quantitative Analysis (QqQ; Agilent Technologies, Atlanta, GA, USA) and Excel software (Microsoft, Redmond, WA, USA). The following standards were used: the internal standard caffeine-13C3 (Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA) and the mycotoxin specific internal standards deoxynivalenol-13C15 (DON-13C15), 3-acetyl-deoxynovalenol-13C17 (3-AcDON-13C17), zearalenone-13C18 (ZEA-13C18), all purchased from Romer Labs, Austria. Internal standards were introduced to the samples at a final concentration of 0.5 ng/µL (caffeine-13C3 and ZEA-13C18) and 0.1 ng/µL, (DON-13C15 and 3-AcDON-13C17). A 13-level calibration curve of pure standards, comprising deoxynivalenol-3-glucoside, zearalenone and deoxynivalenol all purchased from Sigma/Merck, was prepared by serial dilution (range: 2.5 fg/µL–1500 pg/µL) and spiked with the internal standards (0.1 or 0.5 ng/µL).

5.7. UHPLC-MS Analysis of DON and its Conjugates in Green Oat Spikelets

Metabolites were extracted as follows: from each time point (of individual treatments), one spikelet was extracted in 200 µL 85% MeOH + 0,5% HCOOH (*v*/*v*), in 1.5 mL Eppendorf tubes, containing two steel balls and fitted in a tissue-lyzer for 2 min at 30 shakes/sec. The homogenates were centrifuged at 20,000× *g* for 10 min at 4 °C. The supernatant (methanol extract) was collected and stored at −20 °C until LC-MS analysis.

For UHPLC-MS analyses, methanol extracts were diluted 2.5x with MilliQ + 0.5% (*v*/*v*) HCOOH. All samples were filtered using 0.22 µM PVDF multiwall filters at 3000 rpm for 5 min at 20 °C. Linamarin (Sigma Aldrich) was added as an internal standard to a final concentration of 20 µM.

UHPLC-MS analyses were performed using a Dionex Ultimate 3000RS UHPLC system (Thermo Fisher Scientific) coupled to a CompactTM (QqToF) mass spectrometer (Bruker Daltonics) equipped with a temperature controlled auto-sampler (10 °C) and column oven (set to 40 °C). Five-µL aliquots were injected onto a Kinetex XB-C18 UHPLC column (150 × 2.1 mm, 1.7 µm, 100 Å pore size; Phenomenex) and eluted with a flow rate of 0.3 mL/min. The mobile phase comprised solvent A (0.05% (*v*/*v*) HCOOH in water) and solvent B (0.05% (*v*/*v*) HCOOH in acetonitrile), eluted at an initial composition of 98% A and 2% B for 1 min, before a linear increase to 70% solvent B over 6 min and subsequent increase to 100% B over 2 min. The column was washed with 100% solvent B for 2 min, returned to 2% B over 1 min and subsequently re-equilibrated at 2% B for 4 min. The QqToF mass spectrometer was operated in electrospray full scan negative ion mode with the following instrument settings: m/z 50–1200: nebulizer gas (nitrogen), 2.5 bar; drying gas (nitrogen), 8 L/min; drying gas temperature, 220 °C; capillary voltage, 4500 V; spectra acquisition rate, 5 Hz. Data dependent MS/MS acquisition (collision energy 25 eV) was triggered for the 3 most intense ions in the full scan MS spectra. All LC-MS data were analysed using Compass DataAnalysis 4.3 software (Bruker Daltonik GmbH, Bremen, Germany).

5.8. Statistics Analyses

Data for *F. graminearum* biomass, mycotoxin accumulation, gene expression and LC-MSMS analyses were analysed by a mixed effect-model analysis of variance, with treatment as fixed effect and replication as random effect, assuming a normal distribution. If necessary, variances were stabilised by appropriate transformation. Hypotheses were rejected at $P < 0.05$. All analyses were performed in PC-SAS (release 9.4, SAS Institute, Cary, NC, USA).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants12030500/s1, Figure S1: Additional DON metabolites; Figure S2: Symptoms of *F. graminearum* infection in mature oat; Table S1: Primers and probes; Table S2: MRM transitions and retention times.

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Phyto-Beneficial Traits of Rhizosphere Bacteria: In Vitro Exploration of Plant Growth Promoting and Phytopathogen Biocontrol Ability of Selected Strains Isolated from Harsh Environments

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Abstract: Beneficial interactions between plants and some bacterial species have been long recognized, as they proved to exert various growth-promoting and health-protective activities on economically relevant crops. In this study, the growth promoting and antifungal activity of six bacterial strains, *Paenarthrobacter ureafaciens*, *Beijerinckia fluminensis*, *Pseudomonas protegens*, *Arthrobacter* sp., *Arthrobacter defluii*, and *Arthrobacter nicotinovorans*, were investigated. The tested strains resulted positive for some plant growth promoting (PGP) traits, such as indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylate-deaminase (ACC-deaminase), siderophore production, and solubilization of phosphates. The effect of the selected bacteria on *Arabidopsis thaliana* seedlings growth was assessed using different morphological parameters. Bacterial activity against the phytopathogenic fungal species *Aspergillus flavus*, *Fusarium proliferatum*, and *Fusarium verticillioides* was also assessed, since these cause major yield losses in cereal crops and are well-known mycotoxin producers. Strains Pvr_9 (*B. fluminensis*) and PHA_1 (*P. protegens*) showed an important growth-promoting effect on *A. thaliana* coupled with a high antifungal activity on all the three fungal species. The analysis of bacterial broths through ultra performance liquid chromatography–mass spectrometry (UPLC–MS) and liquid chromatography–electrospray ionization–mass spectrometry (LC–ESI–MS/MS) confirmed the presence of potential PGP-compounds, among these are desferrioxamine B, aminochelin, asperchrome B, quinolobactin siderophores, and salicylic acid.

Keywords: antifungal metabolites; biocontrol agents; plant growth promoting rhizobacteria; phytopathogen antagonists; siderophore production; stressful soils

1. Introduction

The rhizosphere is a complex ecosystem in which many relationships are established between bacteria, fungi, and plant root apparatus, and represents the main source of nutrients for plant growth [1]. In particular, many soil microbes have established good relationships with plants, supporting their growth and health, for example helping plants to manage both biotic and abiotic stress [2–4]. In particular, plant growth promoting rhizobacteria (PGPR) are microorganisms, which form symbiotic interactions with plant roots, promoting plant health and productivity through different mechanisms such as

production of plant hormones (auxins, cytokinin, and gibberellins); inhibition of plant senescence; N_2 fixation; phosphate solubilization and mineralization of other nutrients; and siderophores production [5]. In addition, being present in the rhizosphere, PGPR may also be endophytic (PGPE) (for example, by colonizing the plant's tissues), symbiotic (for example, by colonizing the interior of the roots of specific plants by forming nodules), or phyllospheric (i.e., they can be found on the surfaces of plant leaves and stems) [6].

The majority of the most known PGPR belong to the genera *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Rhizobium*, and *Serratia* [7]. PGPR beneficial effects on plants include an increase in root growth and shoot biomass, chlorophyll content, nutrient uptake, total protein content, hydraulic activity, abiotic stress tolerance, shoot and root weights, and a delayed senescence. PGPR are, thus, often employed as biofertilizers [8].

Besides being determinant for plant health and soil fertility, the interactions between beneficial microbes and plant rhizosphere can also exert direct, positive effects against phytopathies. PGPR can suppress diseases by directly synthesizing pathogen-antagonizing compounds, as well as by triggering plant immune responses [9]. Some PGPR have been found to possess several chemotypical traits that make them potential antifungal agents for biocontrol purposes. They can produce siderophores, antimicrobials, lytic enzymes, and various extracellular metabolites which can interfere with, if not completely inhibit, the growth of different, devastating phytopathogenic fungal species with a broad host range [10]. For example, *Pseudomonas* spp. strains isolated from the rhizosphere of alfalfa and clover plants growing on extremely poor pseudogley soil showed interesting antifungal activity against *Trichoderma viride*, *Aspergillus fumigatus*, and *Aspergillus niger* [11], while plant-promoting *Pseudomonas fluorescens* and *Bacillus* spp. strains from a PGPR collection were found to effectively inhibit three spore-forming genera (*Alternaria* spp., *Fusarium* spp., *Bipolaris* spp.) [12]. Again, *Phytophthora capsici*, a cucumber pathogen, was successfully suppressed by specific isolates of *Pseudomonas stutzeri* and *B. amyloliquefaciens* [13]. Recently, a battery of bacteria isolated from the rhizosphere of crops cultivated in different agroecosystems of Pakistan was screened for their biocontrol potential against a range of fungal phytopathogens, showing antagonistic activity against *Fusarium oxysporum*, *F. moniliforme*, *Rhizoctonia solani*, *Colletotrichum gloeosporioides*, *C. falcatum*, *Aspergillus niger*, and *A. flavus* [14]; the antimicrobial effect, which was ascribed to the individuation of antifungal metabolites such as specific antibiotics and cell wall degrading enzymes, was accompanied by the production of a number of compounds recognized as plant growth promoters (hormones and siderophores), suggesting that these PGPR can be exploited for dual-purpose strategies based on the application of a single formulation acting as biopesticide and biofertilizer [15]. It is worthy of consideration that specific bacterial siderophores have been demonstrated to possess direct antifungal activity (often affecting spore germination) against phytopathogens such as *F. oxysporum*, *F. udum*, *A. niger*, *A. flavus*, and *Sclerotium rolfsii* [16–18]; pyoverdine and pyocheline in particular, produced by *P. aeruginosa* and *Burkholderia* spp., have been attributed the most relevant antifungal activities of these bacterial species [19].

Interestingly, other molecules produced by some rhizosphere bacteria and also involved in the plant disease resistance show antifungal properties, as it is the case of salicylic acid (SA) and its derivatives [20–24].

In this panorama, the aim of this work was (i) to characterize bacterial isolates—derived from different soil and rhizosphere environments—for their capacity to improve *Arabidopsis thaliana* growth, (ii) to test their potential activity as biocontrol agents against phytopathogenic fungi species, and (iii) to identify possible molecules involved in plant mineral nutrition or with antimicrobial activity.

2. Results

2.1. Evaluation of the Bacterial Strains Properties

In this study, a deeper characterization of six different bacterial strains previously isolated from different environments was performed. As reported in Table 1, the selected strains Pvr_5, and Pvr_9, Bioch_2, Bioch_7, NCr-1 showed some features of PGPR as high in vitro IAA production and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity (Table 1) [25–27], as well as the production of siderophores, which was diagnosed by using a qualitative method [25–27]. In this work, a liquid chrome azurol S (CAS) assay was used to evaluate the siderophore production in a semiquantitative manner. To this purpose, the bacteria selected were cultured in two different media: succinate minimal salt (SMS) medium, containing a low amount of iron, and succinic medium (SM), completely deprived of iron. The CAS assay revealed that all bacterial strains are able to produce, in both growth media, molecules involved in chelating ferric ions, as indicated by the elevated percent siderophore units (PSU) values observed (Table 1).

The capacities to solubilize phosphate and bacterial protease activity were also tested with qualitative test assays (Figure S1); PHA_1 [28] represents the only strain able to solubilize phosphate while Pvr_5, NCr-1, Bioch_2 and Bioch_7 showed protease activity. Microbial peptidases play a central role in the nitrogen cycle in the soils as they make nitrogen available for plants [29]. Finally, Bioch_7 and Pvr_9 showed an in vitro capacity to form biofilm.

Table 1. Characteristics of PGPR bacteria strains isolated from different soil types, rhizosphere, and endosphere samples.

Strains	Homology	Siderophore Production (PSU) [a]		IAA Production (mg L^{-1})	ACC Deaminase Activity [b]	Phosphate Solubilization Ability [c]	Protease Activity [d]	Biofilm Formation (Abs Units)	References
		SMS	Succinic						
Pvr_5	*Paenarthrobacter ureafaciens* (98.16%)	88.64 ± 0.74	91.5 ± 1.05	62.48 ± 6.3	+	-	+	0.037 ± 0.010	[25]
Prv_9	*Beijerinckia fluminensis* (100%)	91.90 ± 0.11	70.7 ± 2.60	82.08 ± 1.7	+	-	-	1.048 ± 0.141	[25]
Bioch_2	*Arthrobacter defluii* (98%)	91.29 ± 0.56	85.91 ± 4.70	44.02 ± 2.3	+	-	+	0.1 ± 0.007	[26]
Bioch_7	*Arthrobacter nicotinovorans* (99%)	92.33 ± 0.70	89.02 ± 1.12	58.65 ± 4.2	+	-	+	0.216 ± 0.032	[26]
NCr-1	*Arthrobacter* sp. (99%)	93.04 ± 0.08	58.78 ± 2.78	25.6 ± 1.3	+	-	+	0.059 ± 0.003	[27]
PHA_1	*Pseudomonas protegens* (98%)	90.38 ± 0.09	76.89 ± 4.94	n.d.	n.d.	+	-	0.134 ± 0.007	[28]

[a] Siderophore production on SMS and succinic media (see Material and Methods for media composition). [b] ACC deaminase activity: (-) no bacterial growth on medium containing 1-aminocyclopropane-1-carboxylate as the only N source; (+) bacterial growth on medium containing 1-aminocyclopropane-1-carboxylate as the only N source. [c] Phosphate solubilization: (-) absence of solubilization halo; (+) presence of solubilization halo. [d] Protease activity. (-) absence of solubilization halo; (+) presence of solubilization halo. Data are average of three independent experiments ± S.D.

2.2. Effect of Bacterial Inoculation on A. thaliana Morphological Features

The primary root length measurement was carried out on 14-day-old *A. thaliana* seedlings after single bacterial strains inoculation on seeds. As reported in Figure 1A, Pvr_9 proved to be the only strain able to significantly increase ($p < 0.05$) the primary root length of seedlings. On the contrary, plantlets grown in the presence of PHA_1 and Bioch_2 showed a slight although significant reduction ($p < 0.05$) of the primary root length with

respect to the control condition, while no significant differences were observed for plants grown in association with Pvr_5, NCr-1, and Bioch_7 (Figure S2).

The density of the seedlings secondary roots treated with bacteria was also evaluated: in PHA_1-inoculated plantlets the value was about three times significantly higher ($p < 0.05$) than in the control condition, and a significant increase ($p < 0.05$) was also observed for NCr-1 and Bioch_2-treated seedlings. No significant differences were detected in the case of the application of the other strains (Figure 1B).

The last parameter measured was the leaf projection area: seeds inoculation with Pvr_9 and NCr-1 were found to be the only condition that determined a significant increase ($p < 0.05$) of values in treated plantlets (Figure 1C).

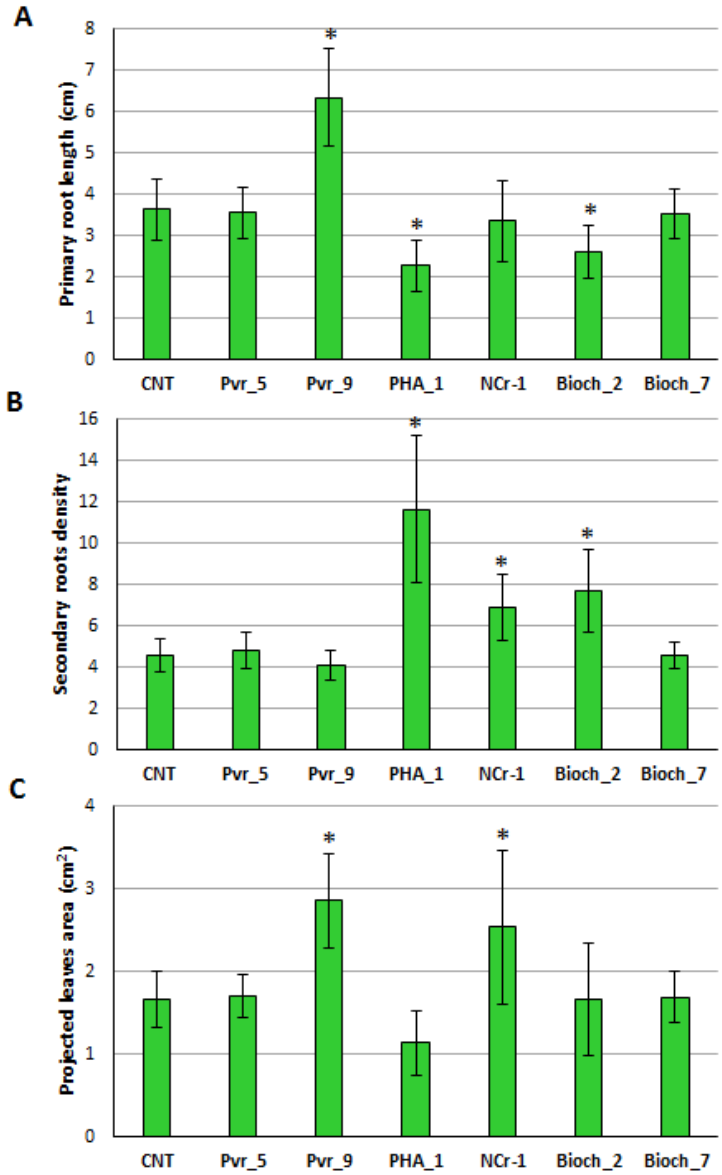

Figure 1. Effect of bacterial inoculation on *Arabidopsis* 14-day-old seedling morphological traits. (**A**) Primary root length, expressed in cm; (**B**) secondary roots density, expressed as number of secondary roots per cm of primary root; (**C**) projected leaves area, expressed in square cm. Data presented are means of 30 biological replicates ± standard deviation (S.D.). Asterisks indicate statistically significant differences between control condition (not inoculated) and treatments (inoculated), according to ANOVA and Tukey's test ($p < 0.05$).

2.3. Antifungal Activity against Selected Phytopathogenic Fungi

Bacterial strains were tested for their direct activity against the phytopathogenic species *A. flavus*, *F. verticillioides*, and *F. proliferatum*: the antifungal potential was assayed by both co-inoculating bacteria cells and fungal conidia and cultivating fungal strains in presence of bacterial filtrated culture medium (Figures 2 and 3). Bacteria were tested at two different concentrations (2.5 and 5 $\times$ 10^3 cells/well). The highest antifungal activity was obtained against *A. flavus* with the co-inoculation, at both the bacterial concentration of Pvr_9 and PHA_1, which reached 100% inhibition (Figure 2A). The same effectiveness was observed against the other two fungal species investigated, *F. verticillioides* and *F. proliferatum* (Figure 2B,C). Co-inoculation with Bioch_7 determined approximately a 40% inhibition in *A. flavus* and 20% in *F. verticillioides* growth at the highest cell concentration (5 $\times$ 10^3), while *F. proliferatum* was subjected to a 40% inhibition at both the concentrations used (Figure 2).

When inoculating 5 $\times$ 10^3 cells of NCr-1 and Bioch_2, a 20% and 35% reduction of growth in *F. verticillioides* was observed, respectively; an inhibition at both the concentrations used against *F. proliferatum* was also detected (Figure 2B,C). On the contrary, no antifungal activity against *A. flavus* was found (Figure 2A). Bacterial strain Pvr_5 was able to impair the growth of *F. proliferatum* but not of the other two fungal species (Figure 2).

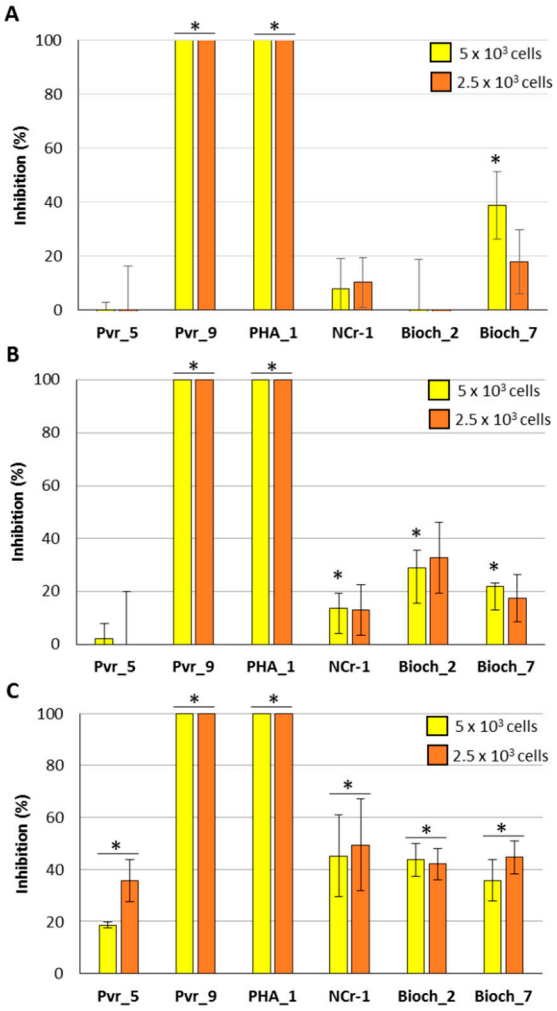

Figure 2. Antifungal activity of bacteria co-inoculum against *A. flavus* (**A**), *F. verticillioides* (**B**), and *F. proliferatum* (**C**). Concentrations of 5 or 2.5 $\times$ 10^3 bacterial cells were co-inoculated with 5 $\times$ 10^3 of fungal conidia. Data are presented as percentage inhibition with respect to the control (only fungal cultures) and are the means of six biological replicates $\pm$ standard deviation (S.D.). Asterisks indicate statistically significant differences between control and co-inoculated cultures according to ANOVA and Tukey's test ($p < 0.05$).

The antifungal effect of the bacterial broths was assayed by adding 25 and 50% (*v*/*v*) to the fungal culture medium, where 5×10^3 conidia/well was inoculated. Through the administration of the filtered bacterial broth, only Pvr_9 was shown able to interfere with the growth of *A. flavus* (Figure 3A).

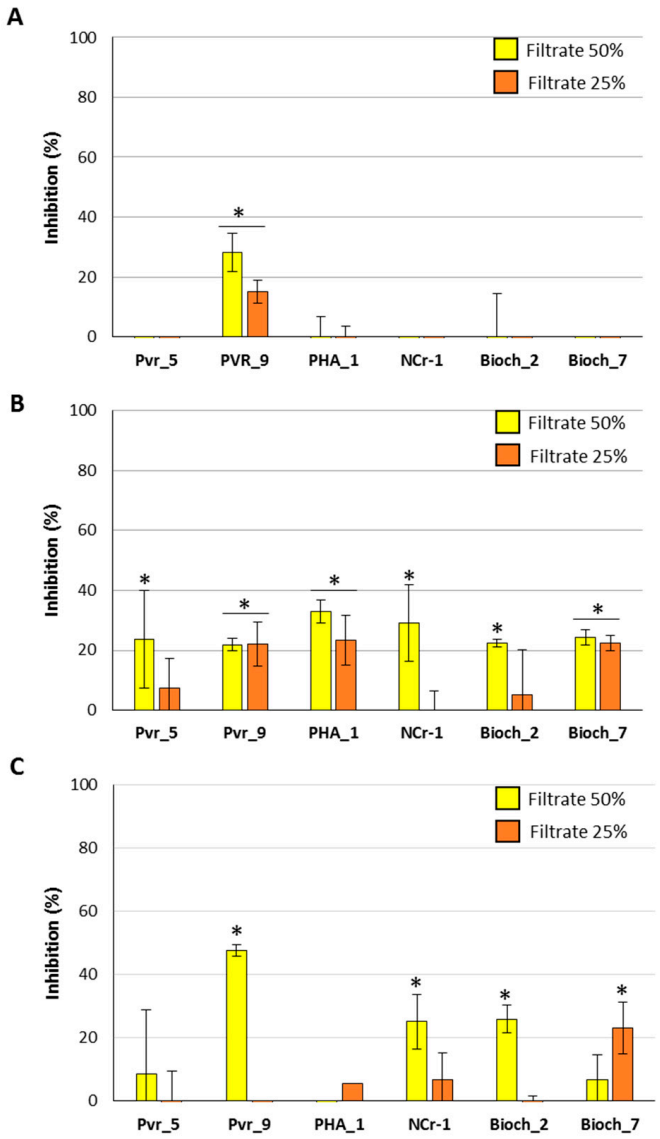

Figure 3. Antifungal activity of filtered bacterial culture broth against *A. flavus* (**A**), *F. verticillioides* (**B**), and *F. proliferatum* (**C**). Cultures of 5×10^3 of fungal conidia/well were amended with 25 or 50% bacterial broth. Data are presented as percentage inhibition with respect to the control (fungal cultures only) and are the means of six biological replicates ± standard deviation (S.D.). Asterisks indicate statistically significant differences between control and co-inoculated cultures according to ANOVA and Tukey's test ($p < 0.05$).

The inhibition was about 30 and 15% by amending the medium with 50 and 25% of filtered culture broth, respectively. Pvr_9 broth was also effective in reducing the mycelium growth of the other two fungal species: 20% inhibition was observed against *F. verticilliodes* at both broth concentrations used while around 50% inhibition was obtained when 50% of broth was added to *F. proliferatum* (Figure 3B,C). Administration of 50 and 25% of PHA_1 broth determined 35 and 20% inhibition on *F. verticillioides*, respectively (Figure 3B); on the contrary, no effects were achieved on *F. proliferatum*, which instead resulted in inhibition (50%) by NCr-1 and Bioch_2 filtrates (Figure 3C). Interestingly, the same fungal

species resulted in more effect by 25 than 50% of Bioch_7 broth, a peculiarity that might be attributed to a combined effect of specific and nonspecific inhibitors that differentially act on mycelium development (Figure 3C).

Finally, 20% inhibition of *F. verticilliodes* growth was recorded when using Bioch_7 filtered culture broth at every percentage, and 50% of Pvr_5, NCr-1, and Bioch_2.

2.4. Identification of Potentially Beneficial Molecules for Plant in Bacterial Broths

UPLC–MS and LC–ESI–MS analyses of SMS and SM culture broths from bacteria were conducted in order to identify compounds possibly linked to the plant-promoting and/or fungal-inhibitory activities observed. Salicylic acid was found in the culture broth of Pvr_5 and Bioch_7 grown in SMS medium, and in Pvr_9, PHA_1, and NCr-1 grown in SMS and SM media. Among the molecules identified, UPLC–MS analysis revealed the presence of the hydroxamate desferrioxamine B in the SMS broth of Pvr_5, while LC–ESI–MS/MS analysis was able to detect the presence of the catecholate aminochelin in the SM broth of Pvr_9. In SM medium, NCr-1 was found to produce the hydroxamate siderophore asperchrome B, and Bioch_2 the carboxylate quinolobactin (Table 2).

Table 2. Identified molecules produced by bacteria and their relative functional groups, along with the growth medium and the technique used for the analysis (n.d., not detected).

Isolates	Functional Group	SMS Medium	Succinic Medium
Pvr_5	Carboxylate Hydroxamate	Salicylic Acid (UPLC–MS) Desferrioxamine B (UPLC–MS)	n.d. n.d.
Pvr_9	Carboxylate Catecholate	Salicylic Acid (UPLC–MS; LC–ESI–MS/MS) n.d.	Salicylic Acid (UPLC–MS; LC–ESI–MS/MS) Aminochelin (LC–ESI–MS/MS)
PHA_1	Carboxylate	Salicylic Acid (UPLC–MS; LC–ESI–MS/MS)	Salicylic Acid (UPLC–MS; LC–ESI–MS/MS)
NCr-1	Carboxylate Hydroxamate	Salicylic Acid (UPLC–MS; LC–ESI–MS/MS) n.d.	Salicylic Acid (UPLC–MS; LC–ESI–MS/MS) Asperchrome B (UPLC–MS)
Bioch_2	Carboxylate	Quinolobactin (UPLC–MS)	n.d.
Bioch_7	Carboxylate	Salicylic Acid (UPLC–MS)	n.d.

3. Discussion

The beneficial interaction between plants and some rhizobacteria has been long recognized, as they proved to exert various growth-promoting and health-protective activities on economically relevant crops. However, although many of them express similar PGPR activity, some typically possess more than one beneficial trait, facilitating in different ways the interfacing of their plant symbionts with the environment. Thus, since the nature and the mechanism of such positive biological interactions have still to be completely clarified, and because PGPR species from same genus often exhibit different interactions with the phytosphere [30,31], the exploration and characterization of new, potentially beneficial strains is highly desirable as well. With this purpose, the bacterial strains analyzed in this study were chosen amongst a previously described panel, containing isolates recovered from the rhizosphere—or the surrounding soil—of plants grown in stressful environments; the mining of harsh ecosystems is in fact considered particularly promising for seeking plant-beneficial bacteria, having the microbiota from these areas subjected to evolutionary pressures that have, in turn, led to adaptive features related to a more effective stress response of their hosts than plants (and the relevant rhizosphere) found in cultivated land [32]. Recently, the screening of rhizobacteria associated with halophytes and drought-tolerant plants inhabiting salty and arid areas of the Mediterranean basin successfully allowed for individualizing isolates that showed interesting abiotic stress-contrasting and biocontrol traits [33], validating the exploration of similar, extreme environments as a rewarding strategy for the individuation of PGP strains.

Identified at the genera and species level by 16S rDNA sequencing, and only partially characterized for their putative PGPR properties, bacteria strains elected for this work belonged to differently demanding environments: Pvr_5 and Pvr_9 were isolated from the rhizosphere of the As-hyperaccumulating fern *Pteris vittata* [27], PHA_1 from a soil rich in hydrocarbons [28], NCr-1 was found to be an endophyte of the Ni-hyperaccumulator *Noccaea caerulescens* [25], and Bioch_2 and Bioch_7 were isolated from a third-year biochar-amended soil [26]. In this work, for this purpose, a deeper characterization of the selected bacterial strains was performed. In particular, a study on their plant-protective/promoting characteristics and potentials was carried out, performing observations of the direct effects on the growth parameters of the model plant species *A. thaliana* and on selected phytopathogenic fungi.

Among the tested strains, Pvr_9 was considered the most interesting, due to the important effects shown as both plant growth promoter and biocontrol agent against some phytopathogenic fungi. The molecular characterization previously conducted showed a homology with the bacterial species *Beijerinckia fluminensis* [27], belonging to a genus that is still poorly characterized for its putative PGPR properties.

On the contrary, strain PHA_1, which shows a significant increase in *Arabidopsis* secondary root formation and interesting features as a biocontrol agent against phytopathogenic fungi tested, belong to the well-known *Pseudomonas* genus, which group includes various interesting species that show microbial biocontrol features and PGP traits, and that has proven to be very versatile, with great potential from an agronomic point of view. Many works described *P. protegens* as an effective antimicrobial agent. Cesa-Luna and collaborators [34] evaluated the ability of *P. protegens* strain EMM-1 against different fungal species, reporting significant activity against *Aspergillus* spp. and *Fusarium* spp. *P. protegens* strain AS15 was shown to be an effective biocontrol agent against *A. flavus*, whose growth and aflatoxin production were lowered on rice grains after the bacterial co-inoculation [35]. The powerful antifungal activity of this species was confirmed by our results; in fact, PHA_1 proved to be highly inhibitory on the fungal growth, especially when the conidia were forced to germinate in the presence of the bacterial cells in co-inoculation assays; in fact, the inhibition reached 100%, independent of the bacterial cell concentration. In addition to the production of antimicrobial compounds, which have also been suggested by the presence, in the genome of the strain FD6, of 12 putative gene clusters for secondary metabolites production, including the antibiotics 2,4-diacetylphloroglucinol (2,4-DAPG), pyoluteorin (PLT), and pyrrolnitrin (PRN) [21], various PGP traits were also reported for some *P. protegens* strains, as the production of siderophores, ammonia, and IAA, the phosphate solubilization [36]. Here, the evaluation of the association of PHA_1 with *A. thaliana* showed a significant increase in the number of secondary roots per cm of primary root, in accordance with what has been recently observed on maize roots inoculated with *Pseudomonas* PS01 strain [37].

Bioch_2, Bioch_7, and NCr-1 belong to the *Arthrobacter* genus and Pvr_5 to the *Paenarthrobacter* genus, in which many plant endophytes are grouped. The plant growth promoting traits of the genus *Arthrobacter* is well documented; their capabilities to produce auxins, siderophores, and ACC deaminase, as well as to exert a P-solubilizing activity, are widely reported, and often associated with a reduction of plant stress when *Arthrobacter* is inoculated. Safdarian et al. [38] showed that *A. nitroguajacolicus* was able to act as a plant growth promoting bacterium on maize under salt stress condition; Tchuisseu Tchakounté et al. [39] recovered, from the maize rhizosphere, 29 isolates belonging to the *Arthrobacter* genus and showed that many possessed at least one of the tested PGP traits. The presence of PGP trait within the *Arthrobacter* genus was also confirmed by Kumar et al. [40]. In his work *A. chlorophenolicus* showed NH_3 production, HCN production, N_2 fixation, IAA production, and P-solubilizing capabilities.

All the bacteria tested were siderophore producers and, with the exclusion of Pvr_5, all the strains were more or less able to interfere with the mycelium growth of *Fusarium*. As

previously reported, siderophores can mitigate the toxic effect of fusaric acid produced by the genus *Fusarium* on *Pseudomonas protegens* Pf-5 [41].

In addition, all the bacterial strains selected showed high siderophore activity. There is increasing interest on siderophore-producing bacteria and siderophore molecules, not only for their possible role in iron bioavailability for plant nutrition, but also to their suppressive activity against fungal phytopathogens. Jin et al. showed that IAA and soil microbial siderophores are both important for Fe uptake by plants [42]. The siderophore pyoverdine produced by *P. fluorescens* was shown to have an important role in the iron uptake of *A. thaliana* [43]. Masalha et al. [44] showed the importance of microbial activity for the iron acquisition in *Zea mays* and in *Helianthus annuus*. Siderophores produced by *Pseudomonas syringae* are biologically active against *Fusarium oxysporum* and other plant pathogenic fungi, through suppression of sporulation and of fungal growth [45].

For this purpose, as an objective of this study, the identification of the siderophores produced by bacterial strains could help to better investigate possible molecules involved not only in plant nutrition, but also in bacterial antimicrobial activity against the phytopathogenic fungi tested. Among the molecules with hydroxamate functional group, asperchrome B and desferrioxamine B are well-known siderophores, which are produced by various species of bacteria and fungi. Desferrioxamine B in particular is a linear trihydroxamic acid siderophore [46]. In addition to chelating Fe (III), desferrioxamine B is also able to bind, for instance, Cu (II), Se (II), Pb (II), Co (III), Mn (III), and Bi (III) [47]. Desferrioxamine B and its chemical derivatives have received much attention because of their particular biological activity. The applications in the medical field of this molecule concerns its use in antimalarial prophylaxis, in a strategy based on the use of antibiotics linked to siderophores to facilitate their entry into cells (Trojan horse strategy), its use as a fluorescent sensor, and in treatment in cases of patients suffering from metal poisoning and iron overload [48].

Among the molecules with catecholate functional groups, we find aminochelin produced by Pvr_9. A characterization of the chemical properties of aminochelin was carried out by [49]. Aminochelin is a triprotic acid with two chatecol protons and one amine proton, with a simple bidentate structure and a high hydrophobicity. This structure enables Fe (III) chelation and to solubilize ferric hydroxides. The carboxylate quinolobactin, an 8-hydroxy-4-methoxy-2-quinoline carboxylic acid, was identified as a siderophore for *Pseudomonas fluorescens* ATCC 17400 [50].

Finally, the carboxylate containing salicylic acid (SA) was found to be produced by most of the bacterial strains tested. In addition to its use by bacteria to maintain iron-limiting growth conditions [20], SA production was reported to also exert an inhibitory potential against several postharvest pathogens, including *Botrytis cinerea* [21], *F. oxysporum* [22], *Penicillium expansum* [23], and *Rhizopus stolonifer* [24].

4. Materials and Methods

4.1. Microorganisms Used in This Study and Growth Conditions

Six bacterial strains isolated from different sources were selected for this work from a collection of PGPR present in our laboratory: Bioch_2 (homologous to *Arthrobacter defluvii*) and Bioch_7 (homologous to *Arthrobacter nicotinovorans*) strains were previously isolated by a maize-derived biochar utilized as amendment in a three year poplar short rotation coppice plantation [26]; Ncr-1 (homologous to *Arthrobacter* sp.) is an endophyte strain isolated from the roots of the Ni-hyperaccumulator *Noccaea caerulescens* [27]; PHA_1 (homologous to *Pseudomonas protegens*) was isolated from a soil contaminated with hydrocarbons [28]; Pvr_5 (homologous to *Paenarthrobacter ureafaciens*); and Pvr_9 (homologous to *Beijerinckia fluminensis*) were isolated from the rhizosphere of the As-hyperaccumulator *Pteris vittata* fern [25]. PGPR characteristics were reported in Table 1.

The aflatoxigenic *A. flavus* strain CR10 and two strains of *F. verticilloides* and *F. proliferatum* were used to assess the antifungal activity of bacteria. All the fungal strains were maintained on potato dextrose agar medium (PDA; Oxoid, Thermo Fisher Scientific

Waltham, MA, USA). For conidia production, *A. flavus* was cultured on PDA for 14 days at 28 °C, while *Fusarium* strains were cultured on nutrient synthetic medium (SNA; KH_2PO_4 1.0 g L^{-1}, KNO_3 1.0 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.5 g L^{-1}, KCl 0.5 g L^{-1} Glucose 0.2 g L^{-1}, Sucrose 0.2 g L^{-1}, Agar 15.0 g L^{-1}) for 20 days.

4.2. Assessment of PGP Traits of Bacterial Strains

Inorganic phosphate solubilization activity of the selected bacteria was assessed using Pikovskaya (PVK) medium (dextrose 10 g L^{-1}, yeast extract 0.5 g L^{-1}, calcium phosphate 5 g L^{-1}, ammonium sulfate 0.5 g L^{-1}, potassium chloride 0.2 g L^{-1}, magnesium sulphate 0.1 g L^{-1}, manganese sulfate 0.0001 g L^{-1}, ferrous sulfate 0.0001 g L^{-1}, agar 10 g L^{-1}) [51]. Bacterial strains were streaked on PVK agar medium and incubated for 5 days at 28 °C. The phosphate solubilization was assessed by the visualization of a clear halo around the bacterial colony.

Protease activity was evaluated in skim milk agar plate medium (casein hydrolysate 10 g L^{-1}, yeast extract 5 g L^{-1}, NaCl 4 g L^{-1}, skim milk powder 20 g L^{-1}, agar 10 g L^{-1}). Bacterial strains were streaked and incubated for 5 days at 28 °C. Protease production was determined by the presence of a clear halo surrounding the bacterial colony [52].

Biofilm formation was assessed following the protocol described by O'Toole, with some modifications [53]. An overnight bacterial culture in plate count agar (PCA) medium was diluted 1:100 in fresh PCA liquid medium and 100 µL was inoculated in a well of a 96-well plate and then placed in static growth for 5 days at 28 °C. After incubation, the medium was discarded and the plate submerged in water two times. Then, 125 µL of a 0.1% solution of crystal violet for each well was added and the plate incubated for 15 min at room temperature. The plate was rinsed 3 times with water and, after water removal, dried for 2 h. A volume of 125 µL of 30% acetic acid solution was added; after 15 min of incubation, absorbance was quantified at 595 nm wavelength.

To measure siderophore activity, bacteria were grown in either SMS (sucrose 1% (w/v), $(NH_4)_2SO_4$ 0.1%, K_2HPO_4 0.2%, $MgSO_4$ 0.05%, NaCl 0.01%, yeast extract 0.05%, $CaCO_3$ 0.05%, tryptophan 0.5 mg mL^{-1}) or SM (succinic acid 4%, $(NH_4)_2SO_4$ 1%, KH_2PO_4 3%, K_2HPO_4 0.1%, $MgSO_4$ 0.2%) for three days; cultures were then centrifuged to remove the cells and 500 µL of supernatant was added to the same volume of CAS solution, then incubated for 20 min at RT. The CAS assay solution contained 6 mL of 10 mM hexadecyltrimethylammonium bromide (HDTMA), 1.5 mL of 1 mM $FeCl_3$, 7.5 mL of 2 mM CAS, 4.307 g of piperazine, and 6.25 mL of 12 M HCl, then diluted to 100 mL with double-distilled water according to Jeong et al. 2014 [54]. To quantify the activity of siderophores produced by each strain, absorbance at 630 nm was determined, and the result was expressed as siderophore unit (percentage) [55]. Three replicates per bacterial colony were analyzed. The results are expressed as mean ± S.D.

4.3. Seed Bacterial Inoculation and Plants Growth Parameters

Arabidopsis thaliana (L.) Heynh. Columbia-0 seeds were used. Seeds were surface sterilized for 5 min with 40% NaClO solution, then washed four times with double-distilled sterile water. After washing, seeds were kept three days in the dark at 4 °C to allow the synchronization of germination. Bacterial strains were grown in 3 mL of Luria and Bertani medium on shaking (130 rpm) at 28 °C for 24 h. Seed inoculation with the different strains was performed as follows: seeds were kept for 1.5 h in a bacterial solution (1×10^8 cells mL^{-1}) on shaking, then recovered and plated on half strength MS [56] + 1% w/v sucrose agar medium. Plates were incubated in a vertical position in an environmentally controlled chamber growth (24 °C; 16/8 h light/dark photoperiod; 120 µmol m^{-2} s^{-1} photosynthetically active radiation, 75% relative humidity (RH)) for germination and root elongation. Plantlets were collected after 14 days for growth measurements. Primary root length, rosette area, and number of lateral roots were measured on 14-day-old plants inoculated or not with bacteria isolates. The number of total lateral roots was normalized for the total length of the primary root. All the measures were performed using ImageJ

software (available at http://rsb.info.nih.gov/ij/ accessed on 20 September 2021; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). The results are expressed as mean ± S.D. A total of 30 plants per treatment were analyzed.

4.4. Direct Antifungal Activity Assay

Antifungal activity of bacteria was assessed through a 96-multiwell plate cultivation system. In the first assay, bacteria were grown for three days in PCA (enzymatic digest of casein 10.0 g L^{-1}, yeast extract 2.5 g L^{-1}, dextrose 1.0 g L^{-1}) liquid medium on shaking at 28 °C, then aliquots of cells were recovered and washed twice in bidistilled water; bacterial cells were then properly diluted and co-inoculated in 96-multiwell plates, in a final volume of 200 µL of PCA liquid medium, with fungal conidia suspensions at the same concentration. Plates were incubated in the dark in static growth at 28 °C.

A second assay was performed to assess the antifungal activity of bacteria broth: bacteria were grown for three days in PCA liquid medium on shaking at 28 °C; cultures were then centrifuged at 4000 rpm for 20 min and the cells discarded. Each broth was filtered with a 0.22 µm filter. Then, spores of each fungal species (5×10^3) were inoculated in 96-multiwell plates with 50 or 100 µL of filtered broth to a final volume of 200 µL/well of PCA medium, corresponding to the 25 and 50% (*v*/*v*) of the culture, respectively.

In both assays, biomass production was assessed after ten days of incubation for *A. flavus*, while *F. verticilloides* and *F. proliferatum* were evaluated after 14 days; mycelia from single wells were recovered, slightly dried on paper, and weighted. Values were expressed as percentage of inhibition with respect to the control. Inocula were performed in quadruplicate, and experiments were performed in triplicate.

4.5. Identification of Potential Plant Growth Beneficial Molecules by Bacterial Strains

Bacterial broths obtained from a three-day culture were centrifuged and the supernatant was recovered and added with methanol at a 3:1 volume ratio. Then, four volumes of ethanol were added and the samples were left undisturbed overnight at 4 °C [44]. The supernatant was recovered and concentrated at 45 °C with a vacuum rotary evaporator and utilized for the following analyses.

4.5.1. Detection of Functional Groups

Each sample was subjected to two different tests for the detection of the iron-chelating functional groups. The tetrazolium test was employed to verify the presence of hydroxamate type of siderophore [57]. Briefly, a pinch of tetrazolium salt was added in a test tube to which 1–2 drops of 2 N NaOH was added and subsequently 1 mL of test sample. Immediate development of a deep red color was taken as a positive reaction by hydroxamate-type siderophore. Moreover, Arnow's test was used to determine functional groups belonging to the catecholate type of siderophores [58]. This method is based on the reaction between catechol and nitrite–molybdate reagent, in acidic conditions, producing a yellow color. The color changes to an intense orange-red in alkaline conditions. For this purpose, 1.0 mL of culture filtrate was combined with 1.0 mL of HCl 0.5 mol·L^{-1}. Subsequently, 1.0 mL of nitrite–molybdate reagent was added and then 1.0 mL of NaOH 1.0 mol·L^{-1}. The assay was incubated at room temperature for approximately 5 min to allow full color development. As a blank control sample, 1.0 mL of deionized water was used. Nitrite–molybdate reagent was prepared by dissolving 10 g of sodium nitrite and 10 g of sodium molybdate in 100 mL of deionized water. The presence of an orange-red color solution detects the catecholate type siderophore. The color intensity depends on the amount of catechol present [58,59].

4.5.2. UPLC Determination

To better identify the siderophore, the solutions were also tested by means of ultra-performance liquid chromatography (UPLC) (Waters S.p.A. Sesto San Giovanni (MI), Italy) associated with electrospray ionization mass spectrometry (Waters Acquity UPLC/ESI–MS, single quadrupoles detector) (Waters S.p.A. Sesto San Giovanni (MI), Italy). To separate

active components, each sample was injected and separated on a C18 column (Waters Acquity UPLC BEH300 C18 1.7 µm, 2.1·50 mm) using a gradient of 0.1% aqueous formic acid (A) and acetonitrile (B) as mobile phase (0–5 min 1.5–45% B, 5–16 min 45–100% B and then 16–19 min 100% B; flow rate 0.2 mL·min^{-1}; temperature 30 °C). The capillary and cone voltages in ESI mode were 3.8 kV and 25 V, respectively [59,60]. Ion transfer capillary was heated at 300 °C. Cone and desolvation gas flow was, respectively, at 100 and 480 L·h^{-1}. Positive-ion full-scan mass spectra were recorded from m/z 50 to 2000.

4.5.3. LC–ESI–MS/MS Determination

High resolution mass spectrometry was performed on the samples using a HPLC DIONEX Ultimate3000 interfaced with a LTQ-Orbitrap XL Thermo Fisher Scientific (Waltham, MA, USA). Samples were injected on an Aeris Peptide 3.6u XB-C18 2.1 mm × 15 cm (Phenomenex; Via M. Serenari, 15/D, 40013 Castel Maggiore (BO), Italy). The mobile phase consisted of water with 0.1% formic acid (solvent A) and methanol with 0.1% formic acid (solvent B); gradient: 0–5 min 99% A, 5–35 min from 99% A to 5% A, 35–40 min 5% A, 40–41 min from 5% A to 99% A, 41–50 min 99% A; flow rate was 0.2 mL/min; column temperature 35 °C; injection volume 5 µL. Samples were acquired in positive and negative mode. Electrospray ionization at positive (spray voltage 3 kV; capillary voltage 13 V; source temperature 275 °C; tube lens 100 V; sheath gas flow rate 40; aux gas flow rate 10; and sweep gas flow rate 5) and negative (spray voltage 3.2 kV; capillary voltage −35 V; source temperature 275 °C; tube lens −110 V; sheath gas flow rate 40; aux gas flow rate 10; and sweep gas flow rate 5) ion modes. The mass data acquisition was performed by four scan events. Data were analyzed using a database dedicated to microbial siderophores and created by Prof. Samuel Bertrand (http://bertrandsamuel.free.fr/siderophore_base/index.php released on 8 June 2011, accessed on 14 December 2021); compounds were identified through the main adduct encountered using LC–ESI–MS, namely, $[M + H]^+$, $[M - 2H + Fe]^+$, and $[M - H]^-$.

4.6. Statistical Analyses

For statistical analyses, one-way analysis of variance (ANOVA) was used in the Past 4.06b software [61]. Results of plant growth measures and antifungal activity were analysed by Tukey's test; differences were considered significant at $p < 0.05$.

5. Conclusions

Amongst the bacterial strains evaluated, Pvr_9 was found to possess the best characteristics for both promoting the plant growth and acting as biocontrol agent against phytopathogens. The preliminary results achieved not only confirmed the mining of harsh environments as a promising tool for the individuation of potential PGPR, but also provide important clues about the direct antagonistic effect of these strains on *Aspergillus* and *Fusarium* species relevant to crops. Future investigations devoted to deepening and clarifying the mechanism ruling the positive effects on the growth of plants—and in particular of economically important crops—are needed before any possible application in agricultural systems can be proposed. In particular, more research is desirable to elucidate the direct antimicrobial potential of the siderophores identified, which would support the possible use of such bacteria as biocompetitors able to act against phytopathogenic fungal species in different synergistic ways.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11020230/s1, Figure S1: Phosphate solubilization assay; Figure S2: Primary root elongation.

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Debaryomyces hansenii, *Stenotrophomonas rhizophila*, and Ulvan as Biocontrol Agents of Fruit Rot Disease in Muskmelon (*Cucumis melo* L.)

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Abstract: The indiscriminate use of synthetic fungicides has led to negative impact to human health and to the environment. Thus, we investigated the effects of postharvest biocontrol treatment with *Debaryomyces hansenii*, *Stenotrophomonas rhizophila*, and a polysaccharide ulvan on fruit rot disease, storability, and antioxidant enzyme activity in muskmelon (*Cucumis melo* L. var. *reticulatus*). Each fruit was treated with (1) 1×10^6 cells mL^{-1} of *D. hansenii*, (2) 1×10^8 CFU mL^{-1} of *S. rhizophila*, (3) 5 g L^{-1} of ulvan, (4) 1×10^6 cells mL^{-1} of *D. hansenii* + 1×10^8 CFU mL^{-1} of *S. rhizophila*, (5) 1×10^8 CFU mL^{-1} of *S. rhizophila* + 5 g L^{-1} of ulvan, (6) 1×10^6 cells mL^{-1} of *D. hansenii* + 1×10^8 CFU mL^{-1} of *S. rhizophila* + 5 g L^{-1} of ulvan, (7) 1000 ppm of benomyl or sterile water (control). The fruits were air-dried for 2 h, and stored at 27 °C ± 1 °C and 85–90% relative humidity. The fruit rot disease was determined by estimating the disease incidence (%) and lesion diameter (mm), and the adhesion capacity of the biocontrol agents was observed via electron microscopy. Phytopathogen inoculation time before and after adding biocontrol agents were also recorded. Furthermore, the storability quality, weight loss (%), firmness (N), total soluble solids (%), and pH were quantified. The antioxidant enzymes including catalase, peroxidase, superoxide dismutase, and phenylalanine ammonium lyase were determined. In conclusion, the mixed treatment containing *D. hansenii*, *S. rhizophila*, and ulvan delayed fruit rot disease, preserved fruit quality, and increased antioxidant activity. The combined treatment is a promising and effective biological control method to promote the shelf life of harvested muskmelon.

Keywords: antioxidant enzymes; disease incidence; *Fusarium proliferatum*; mixed treatment

1. Introduction

The muskmelon (*Cucumis melo* L.), belonging to the family Cucurbitaceae, is an important horticultural crop cultivated in temperate to arid regions in Asia (74%), America (11.9%), and Europe (7.2%), with a global production of 31,166,896 tons [1]. However, muskmelon is a climacteric ripening fruit, which deteriorates rapidly after harvesting

because of pericarp browning and postharvest disease primarily induced by *Alternaria alternata*, *Rhizopus stolonifer*, *Trichothecium roseum*, and *Fusarium* spp. [2]. Postharvest fruit rot caused by *Fusarium* spp. is considered one of the main diseases that negatively impacts the quality, and influences the commercial acceptability and saleable stock of muskmelon [3]. Thus, muskmelon has a limited shelf life, which further limits their storage, transportation, and marketing [4]. Therefore, handling postharvest muskmelon, which is a key production concern, necessitates further research.

Many synthetic fungicides, such as acibenzolar-S-methyl, azoxystrobin, copper sulfate, imazalil, iprodione, and thiabendazole, are the most common commercial methods employed in muskmelon postharvest handling to retard fruit decay and prolong storage life [5,6]. Nonetheless, their indiscriminate use has led to residue accumulation in fruit, environmental pollution, carcinogenic risk to consumers, and pathogen resistance [7]. In addition, there is a trend to consume residue-free fruits, with stricter government regulations regarding agrochemical products [8]. Consequently, there is an essential need to find alternative methods such as biological control to inhibit decay in harvested fruit. Previous studies have shown that biological control by applying microbial antagonists, such as *Bacillus subtilis* [9], *Burkholderia* sp. [10], and *Pseudomonas graminis* [11], or by applying secondary metabolites such as phenylethyl alcohol from *Trichoderma asperellum* [12] and lactic acid from *Lactobacillus plantarum* [13], is a promising method for managing decay in harvested muskmelon.

Most microbial antagonists have been sourced from the fruit surface (epiphytic), but they can also be isolated from other nearby related areas, i.e., soil, roots, and the phyllosphere [14], or distant sources such as extremophile environments [15]. The marine yeast *Debaryomyces hansenii* has shown significant results as a biocontrol agent by diverse mechanisms of action, such as competition for space (i.e., inhibition of spore germination) and nutrients, and secondary metabolite excretion (i.e., volatile organic compounds and lytic enzymes) [16,17]. The marine bacteria *Stenotrophomonas rhizophila* have shown significant results as biocontrol agents by direct inhibition, excretion of volatile organic compounds, nutrient competition, and lytic enzymes [18,19]. Moreover, previous studies have demonstrated that *D. hansenii* and *S. rhizophila* are safe to humans [17,20].

However, microbial antagonists applied as a single treatment considerably vary in their efficiency and are inconsistent at high levels (>95%) of disease control than that of chemical fungicides [14]. Thus, the integrated approaches could be the key in the successful development of safe and sustainable alternatives for effective postharvest disease management in fruits [21]. Ulvan, a polysaccharide isolated from the green algae *Ulva* spp., has been demonstrated to induce resistance with no direct activity against other microorganisms such as *D. hansenii*, *S. rhizophila*, and *Fusarium proliferatum* [19]. However, the effects of individual or mixed postharvest treatment with *D. hansenii*, *S. rhizophila*, and ulvan on the quality and storability of harvested muskmelon have not been studied before.

In this study, the effects of *D. hansenii*, *S. rhizophila*, and ulvan as individual or mixed treatments on fruit rot disease, storage quality, and antioxidant enzyme activity in muskmelon (*Cucumis melo* L. var. *reticulatus*) was investigated. The aim of this study was to develop an effective and safe biological control strategy for inhibiting fruit decay and prolonging the shelf life of muskmelon.

2. Results

2.1. In Vivo Control Assay and Microscopic Visualization

The mixed treatment of *D. hansenii*, *S. rhizophila*, and ulvan significantly reduced the lesion diameter (3.5 mm) and significantly improved DC (73.5%) of fruit rot induced by *F. proliferatum* in muskmelon compared to that of the individual treatments, and observed better results than those treated with benomyl (Figure 1). Nevertheless, muskmelon fruit inoculated with only ulvan presented the highest lesion diameter (16.3 mm) and the lowest DC (14.3%). By applying Abbott's formula, it was inferred that in comparison with single treatments, all the mixed treatments exhibited a synergistic effect on DC (Table 1).

The mixed treatment with *D. hansenii*, *S. rhizophila*, and ulvan demonstrated the highest predicted synergistic effect.

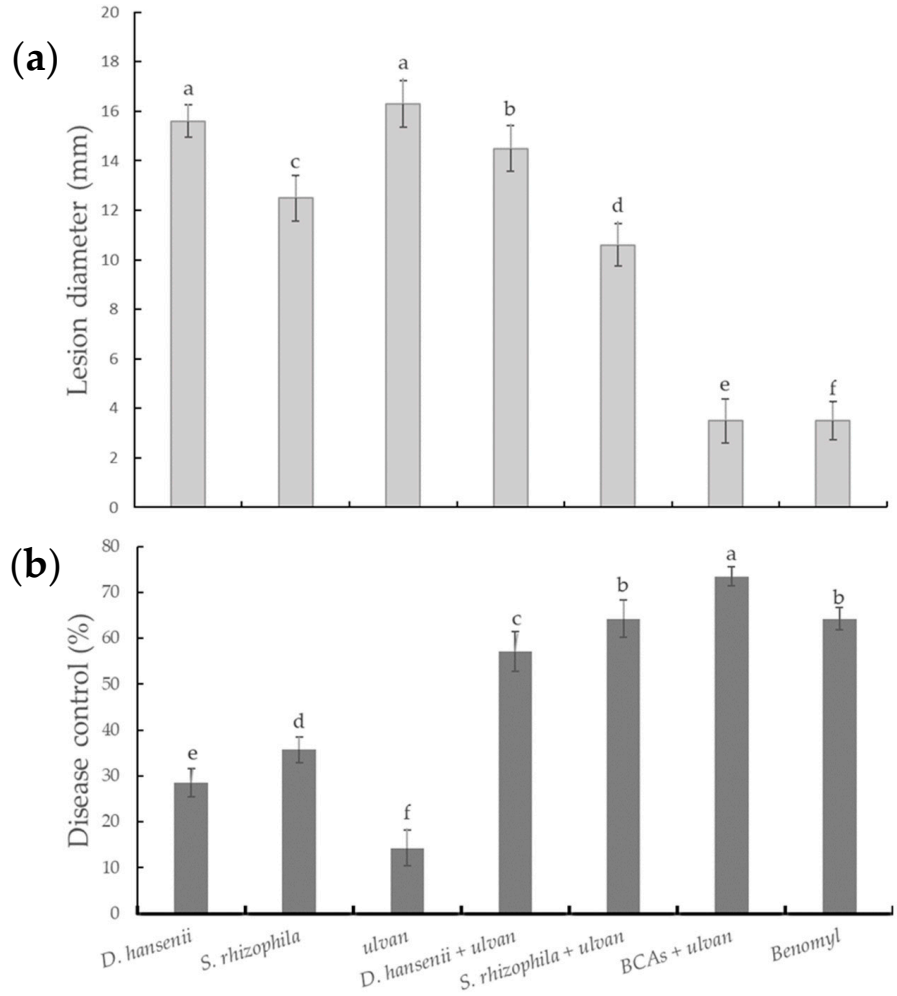

Figure 1. Effect of *D. hansenii*, *S. rhizophila,* and ulvan as individual or mixed treatments on the lesion diameter (**a**) and disease control (**b**) of fruit rot induced by *F. proliferatum* on muskmelon. Bars are the mean of five replicates with three fruits per replicate ± Standard deviation. Different letters in each column indicate significant difference ($p \leq 0.05$). BCAs: Biological control agents (*D. hansenii* + *S. rhizophila*).

Table 1. Synergistic biocontrol activity of *D. hansenii*, *S. rhizophila,* and ulvan against *F. proliferatum* on muskmelon fruit.

Treatment	DCE *	SF
D. hansenii	-	-
S. rhizophila	-	-
Ulvan	-	-
D. hansenii + *S. rhizophila*	49.2	1.7
D. hansenii + ulvan	38.4	1.5
S. rhizophila + ulvan	44.4	1.4
BCAs + ulvan	55.1	1.8
Benomyl	-	-

The disease control was calculated by comparing the treatments with the control (*F. proliferatum* + sterile distilled water) and was presented as percentage for each treatment. * DCE: Expected effect or expected control percentage, SF: Synergy factor, more information on materials and methods. BCAs: Biological control agents (*D. hansenii*+ *S. rhizophila*).

Scanning electron micrograph imaging demonstrated that the spores and mycelia of *F. proliferatum* appeared and grew normally on muskmelon fruit in the control treatment (Figure 2a). When treated with biological control agents (BCAs) as a single treatment, *F. proliferatum* cells developed adhesion capacity (Figure 2b) with limited (Figure 2c) mycelial growth. In the mixed treatment with *D. hansenii* and *S. rhizophila*, the mycelial surface appeared abnormally shaped and notably damaged (Figure 2c).

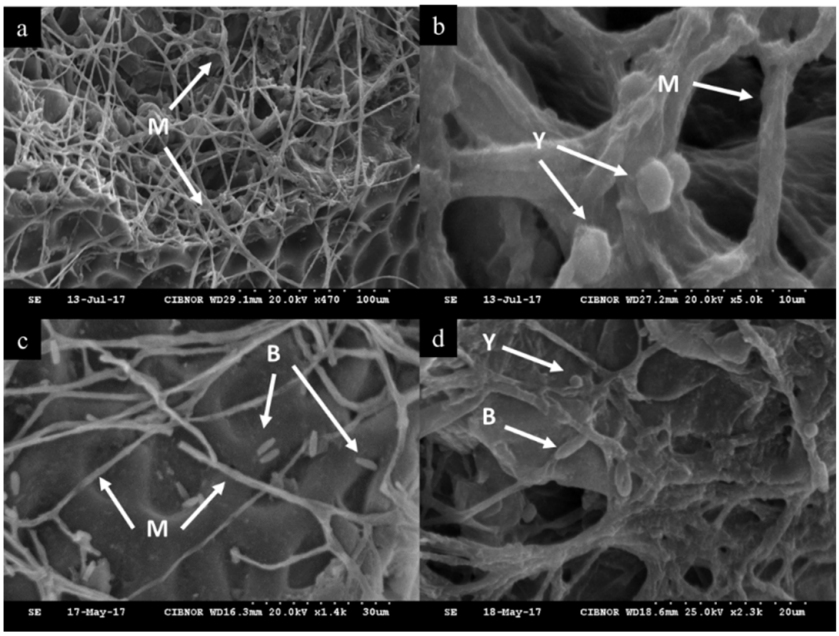

Figure 2. Scanning electron micrograph imaging of biocontrol activity of microbial antagonists against *F. proliferatum* in muskmelon fruit. (**a**) Mycelium of *F. proliferatum*; (**b**) Mycelium of *F. proliferatum* and cells of *D. hansenii*; (**c**) Mycelium of *F. proliferatum* and cells of *S. rhizophila*; (**d**) Mycelium of *F. proliferatum* and cells of *D. hansenii* and *S. rhizophila*. M = mycelium of *F. proliferatum*, Y = *D. hansenii*, and B = *S. rhizophila*.

2.2. Effect of Biocontrol Treatment Time on Their Biocontrol Efficacy

The effect of *D. hansenii*, *S. rhizophila*, and ulvan treatment time after or before the inoculation of *F. proliferatum* significantly affected DC (Table 2) and lesion diameter (Table 3). All muskmelon fruits treated before inoculating *F. proliferatum* had the highest DC and smaller lesion diameter than those treated after inoculating the phytopathogen. The longer the treatment time of the BCAs and ulvan before *F. proliferatum* inoculation, the higher the DC and the smaller the lesion diameter. The fruit inoculated with mixed treatment of *D. hansenii*, *S. rhizophila*, and ulvan 24 h before the inoculation of *F. proliferatum* presented the best results in DC (87.6%) and reduction in lesion diameter (1.7 mm). The fruits treated with ulvan 24 h after inoculating *F. proliferatum* had the lowest DC and the largest lesion diameter (27.7 mm). The DC and lesion diameter of muskmelon fruit treated with benomyl before or after *F. proliferatum* did not differ significantly. The results showed that *D. hansenii*, *S. rhizophila*, and ulvan are effective as preventive treatments rather than curative treatments.

Table 2. Effect of inoculation timing of *D. hansenii*, *S. rhizophila*, and ulvan on disease control (%DC) by *F. proliferatum* on muskmelon fruit.

Treatment	Before (h)			After (h)	
	24	12	2	12	24
D. hansenii	39.7 ± 2.3 ^{g,*}	35.2 ± 2.3 ^{g}	28.6 ± 3.1 ^{f}	17.2 ± 1.4 ^{g}	10.7 ± 1.8 ^{g}
S. rhizophila	44.3 ± 3.1 ^{f}	40.2 ± 3.3 ^{f}	35.7 ± 2.8 ^{e}	20.6 ± 2.3 ^{f}	13.6 ± 3.3 ^{e}
Ulvan	48.9 ± 2.1 ^{e}	27.4 ± 1.4 ^{h}	14.3 ± 3.9 ^{g}	7.2 ± 2.1 ^{h}	5.3 ± 1.7 ^{h}
BCAs	75.8 ± 1.1 ^{b}	72.5 ± 1.8 ^{b}	68.2 ± 3.5 ^{b}	39.2 ± 3.8 ^{c}	18.3 ± 3.4 ^{b}
D. hansenii + ulvan	70.2 ± 1.3 ^{c}	60.3 ± 2.2 ^{e}	57.1 ± 4.3 ^{d}	34.3 ± 1.6 ^{d}	11.1 ± 2.1 ^{f}
S. rhizophila + ulvan	75.3 ± 2.4 ^{b}	68.8 ± 4.1 ^{c}	64.3 ± 4.1 ^{c}	25.7 ± 2.0 ^{e}	15.7 ± 1.1 ^{d}
BCAs + ulvan	87.6 ± 2.3 ^{a}	80.7 ± 3.4 ^{a}	73.5 ± 2.1 ^{a}	40.1 ± 1.2 ^{b}	17.4 ± 1.3 ^{c}
Benomyl	66.5 ± 2.2 ^{d}	65.7 ± 3.2 ^{d}	64.3 ± 2.4 ^{c}	60.5 ± 2.4 ^{a}	59.5 ± 1.2 ^{a}

The disease control was calculated by comparing the treatments with the control (*F. proliferatum* + sterile distilled water) and was expressed as percentage for each treatment. BCAs: Biological control agents (*D. hansenii* + *S. rhizophila*). * Each value is the mean of five replicates with three fruits per replicate ± Standard deviation. Different letters in each column indicate significant difference ($p \leq 0.05$).

Table 3. Effect of inoculation timing of *D. hansenii*, *S. rhizophila*, and ulvan on lesion diameter (mm) by *F. proliferatum* on muskmelon fruit.

Treatment	Before (h)			After (h)	
	24	12	2	12	24
D. hansenii	11.5 ± 0.5 ^{b,*}	12.5 ± 0.7 ^{b}	15.6 ± 0.7 ^{c}	20.8 ± 0.9 ^{c}	23.4 ± 0.7 ^{d}
S. rhizophila	9.8 ± 0.6 ^{d}	10.5 ± 0.8 ^{c}	12.5 ± 0.9 ^{e}	18.3 ± 0.5 ^{e}	24.5 ± 1.3 ^{c}
Ulvan	8.5 ± 0.3 ^{e}	10.6 ± 0.6 ^{c}	16.3 ± 0.9 ^{b}	24.7 ± 1.1 ^{b}	27.7 ± 0.8 ^{b}
BCAs	7.8 ± 0.2 ^{f}	8.7 ± 0.3 ^{d}	10.6 ± 0.2 ^{f}	15.3 ± 0.9 ^{g}	17.1 ± 1.3 ^{g}
D. hansenii + ulvan	10.2 ± 0.9 ^{c}	12.4 ± 1.2 ^{b}	14.5 ± 0.9 ^{d}	19.4 ± 0.8 ^{d}	20.6 ± 0.9 ^{e}
S. rhizophila + ulvan	5.4 ± 0.2 ^{h}	6.0 ± 0.3 ^{e}	6.5 ± 0.9 ^{g}	17.7 ± 1.1 ^{f}	18.1 ± 1.2 ^{f}
BCAs + ulvan	1.7 ± 0.2 ^{i}	2.3 ± 0.1 ^{f}	3.5 ± 0.2 ^{h}	14.5 ± 0.3 ^{h}	18.5 ± 0.1 ^{f}
Benomyl	6.2 ± 0.3 ^{g}	6.4 ± 0.2 ^{e}	6.5 ± 0.7 ^{g}	6.9 ± 0.5 ^{i}	6.7 ± 0.3 ^{h}
Control	27.2 ± 0.7 ^{a}	27.8 ± 1.3 ^{a}	28.4 ± 1.2 ^{a}	28.8 ± 0.9 ^{a}	29.1 ± 1.2 ^{a}

BCAs: Biological control agents (*D. hansenii* + *S. rhizophila*). * Each value is the mean of five replicates with three fruits per replicate ± Standard deviation. Different letters in each column indicate significant difference ($p \leq 0.05$).

2.3. Efficacy of Biocontrol Treatments on Natural Fruit Rot Development and Fruit Quality Parameters

Muskmelon fruits were dipped in either single or mixed treatments containing *D. hansenii*, *S. rhizophila*, and ulvan to assess natural fruit rot development and quality parameters. After 7 d of storage, DI significantly reduced with all treatments in comparison with the control treatment (70%) (Table 4). Muskmelon fruit immersed in the mixed treatment of BCAs and ulvan had the lowest DI (8.3%), which was even lower than that of benomyl (10%). All the mixed treatments had lower DI values than that of the single treatments. Regarding quality parameters, muskmelon fruit immersed in benomyl lost a significant amount of weight (0.68 g) and firmness (4.1 N) in comparison with those immersed in BCAs and ulvan as mixed or single treatments. Furthermore, TSS observed no significant difference between muskmelon treatments. Muskmelon fruit immersed in solutions of mixed treatments and a single treatment containing ulvan had the lowest pH values.

Table 4. Efficacy of *D. hansenii*, *S. rhizophila*, and ulvan on natural fruit rot development and fruit quality parameters.

Treatment	DI (%)	Weight Loss (g)	Firmness (N)	TSS (%)	pH
D. hansenii	$33.3 \pm 1.2^{b,*}$	0.30 ± 0.02^{c}	4.2 ± 0.5^{c}	9.2 ± 0.08^{a}	6.5 ± 0.1^{a}
S. rhizophila	26.7 ± 1.6^{c}	0.30 ± 0.01^{c}	4.2 ± 0.4^{c}	9.2 ± 0.09^{a}	6.5 ± 0.1^{a}
Ulvan	23.3 ± 0.8^{d}	0.24 ± 0.03^{e}	4.2 ± 0.4^{c}	9.3 ± 0.06^{a}	6.1 ± 0.1^{b}
BCAs	17.2 ± 1.1^{f}	0.26 ± 0.02^{d}	4.3 ± 0.3^{a}	9.3 ± 0.04^{a}	6.2 ± 0.2^{b}
D. hansenii + ulvan	20.0 ± 1.2^{e}	0.21 ± 0.03^{f}	4.3 ± 0.3^{a}	9.3 ± 0.08^{a}	6.2 ± 0.1^{b}
S. rhizophila + ulvan	13.3 ± 0.7^{g}	0.22 ± 0.02^{f}	4.3 ± 0.3^{a}	9.3 ± 0.06^{a}	6.1 ± 0.1^{b}
BCAs + ulvan	8.3 ± 0.8^{i}	0.21 ± 0.02^{f}	4.3 ± 0.2^{a}	9.3 ± 0.05^{a}	6.0 ± 0.3^{c}
Benomyl	10.0 ± 0.4^{h}	0.68 ± 0.05^{b}	4.1 ± 0.8^{b}	9.2 ± 0.08^{a}	6.6 ± 0.1^{a}
Control	70.0 ± 1.4^{a}	1.06 ± 0.08^{a}	4.0 ± 0.6^{d}	9.2 ± 0.07^{a}	6.6 ± 0.1^{a}

BCAs: Biological control agents (*D. hansenii* + *S. rhizophila*). * Each value is the mean of five replicates with three fruits per replicate ± Standard deviation. Different letters in each column indicate significant difference ($p \leq 0.05$). DI means disease incidence, and TSS means total soluble solids.

2.4. Antioxidant Enzymatic Activity on Muskmelon Fruit after Biocontrol Treatments

The antioxidant enzymatic activity in muskmelon was measured after treating with single or mixed solutions containing *D. hansenii*, *S. rhizophila*, and ulvan (Figure 3). SOD activity increased significantly in muskmelon fruit after 4 and 6 d of inoculation with the mixed treatment of BCAs and ulvan (Figure 3a), respectively. In all muskmelon fruits, SOD activity decreased considerably during the first 2 d of incubation and increased to the maximum activity level after 6 d of incubation. CAT activity in muskmelon fruits significantly decreased in all the treatments during the first 6 d of incubation and slightly increased after 8 d (Figure 3b). However, single and mixed treatments with *D. hansenii*, *S. rhizophila*, and ulvan maintained a higher CAT activity than that of the control treatment. The POX activity in muskmelon significantly increased with the single BCAs treatment after 6 d of inoculation in comparison with the control treatment (Figure 3c). The highest POX activity was quantified 4 d after inoculating muskmelon fruit with mixed treatment of *D. hansenii*, *S. rhizophila*, and ulvan. In all treatments, POX decreased gradually after incubating for 4 d. PAL activity significantly increased in all muskmelon fruits compared with that of the control treatment (Figure 3d). The highest PAL activity was quantified 2 d after inoculating the mixed treatment containing *D. hansenii*, *S. rhizophila*, and ulvan, which was maintained throughout the incubation period.

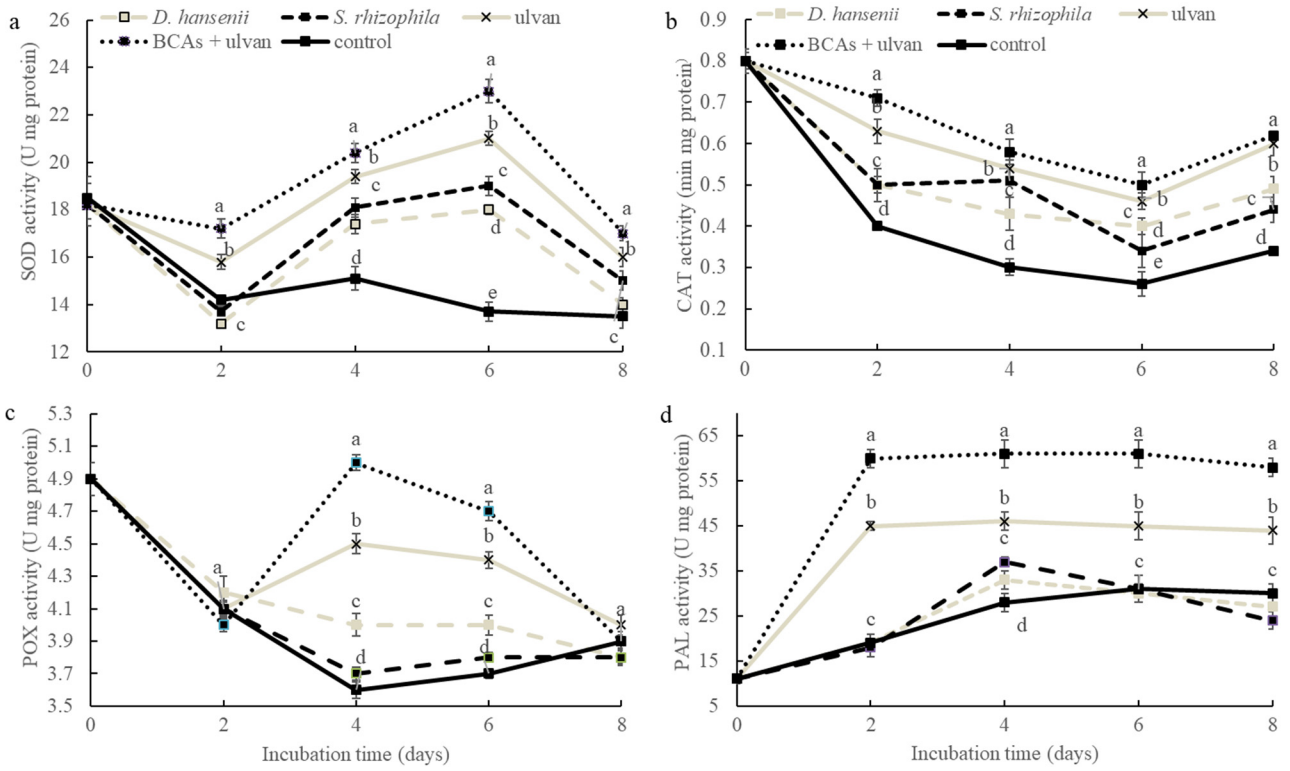

Figure 3. Effect of *D. hansenii*, *S. rhizophila* and ulvan on the antioxidant enzymatic activity on muskmelon fruit. BCAs = Biological control agents (*D. hansenii* + *S. rhizophila*). Each value is the mean of five replicates with three fruits per replicate ± Standard deviation. Different letters indicate significant difference ($p \leq 0.05$). (**a**) superoxide dismutase (SOD) estimation; (**b**) catalase (CAT) estimation; (**c**) peroxidase (POX) estimation; (**d**) superoxide dismutase (SOD) estimation.

3. Discussion

Since publishing the first report on using *Bacillus subtilis* to treat brown rot caused by *Monilinia fructicola* on peaches in 1985, the use of microbial antagonists (i.e., yeast, bacteria, and fungi) as BCAs have been promoted as an alternative to chemical products [22]. Nonetheless, BCAs exhibit certain limitations because they are usually effective against specific hosts and well-defined phytopathogens, and are also affected by adverse environmental conditions [23]. Moreover, BCAs individually cannot eradicate established infections and cannot provide a broad-spectrum DC compared with that of chemical fungicides [24]. Additionally, BCAs must demonstrate a control efficiency comparable to that of conventional fungicides to be considered as a promising alternative [25]. Thus, combining BCAs with its compatible physical or chemical treatments is being investigated in recent years to enhance their individual performance through a synergistic effect [8]. Previous studies have developed several alternatives and compatible treatments, including physical treatments [26], resistance inducers [27], food additives [28], essential oils [29], low fungicidal doses [30], and mixed antagonist cultures [31].

In this study, the results demonstrated that mixed treatments containing BCAs and ulvan significantly enhanced the biocontrol effect of fruit rot disease in muskmelon compared with that of the single treatments. Mixed treatments with resistance inducers have been evaluated previously to enhance the activity of BCAs [27,32–35]. In a previous study, methyl jasmonate was inoculated as a mixed treatment to enhance the biocontrol effect of *Meyerozyma guilliermondii*, which reduced the disease incidence using the combined treatment (21%) in comparison with that of the individual yeast treatment (42%), which further affected the fungal morphology and upregulated resistance-related enzymes. The mixed treatments that include BCAs and resistance inducers are better than individual BCAs treatments because of their wide spectrum of action, and better efficiency for an expanded

disease control under wide environmental conditions [36]. In this study, the compatible activity of the mixed treatment could be attributed to different ecological requirements of both BCAs [37], with ulvan not directly affecting these microorganisms [38].

Furthermore, the inoculation time of the BCAs in this study indicated that the reduced lesion size and DC are related to their high reproduction rate compared to that of the phytopathogen, which rapidly colonize the tissue during pre-treatment [37,39]. Ulvan inoculation time indicates that the reduction in lesion size and the decrease in disease are related to its ability to induce resistance and priming in fruits [40]. Therefore, the BCAs proposed in this study should be used in pretreatment to counteract melon fruit rot caused by *F. proliferatum*, Zhao et al. ref. [41] obtained similar results, wherein the efficiency of *Pichia guilliermondii* against *Rhizopus nigricans* was better when tomato fruits were treated 24 h before inoculating the phytopathogen. Besides, Lima et al. [42] reported that the combination of *Wickerhamomyces anomalus* and *Meyerozyma guilliermondii* inoculated 12 and 24 h before *Colletotrichum gloeosporioides* inoculation, reduced the disease incidence by 13.8% and 30%, respectively.

BCAs colonize more effectively the fruit host and limit the space and nutrients availability when they are inoculated before the phytopathogen (Figure 1). Thus, studying the effect of timing inoculation on the effectiveness of BCAs is essential to develop postharvest control strategies [14]. The time-related experiments in this study demonstrate the importance of applying BCAs immediately after fruit harvesting to control postharvest diseases and to preserve their quality parameters. The ability of BCAs as a preventive treatment rather than a corrective one is closely related to nutrient competition mechanisms [22,43–45].

The innate resistance to postharvest fungal decay is closely related to certain physiological parameters, such as senescence, which is remarkably decreased [46]. In a previous study, the effectiveness of *Pichia membranifaciens* as antagonist against *Penicillium expansum* in peach fruit could be enhanced by adding 0.2 g L^{-1} of benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester without reducing its quality parameters [47]. In this study, the mixed treatments of BCAs and ulvan significantly decreased the natural disease incidence, and preserved the firmness and weight of muskmelon. Initially, the TSS content in the fruit increased, probably due to the degradation of the non soluble polysaccharides to simple sugars, which later decreased with increase in storage time, and related to the increased respiration rate [48]. Furthermore, the respiration rate in muskmelon was delayed by the mixed treatment of BCAs and ulvan because of the increase in TSS post-treatment. The pH of muskmelon fruit decreased from an initial pH of 5.3 to 6.8 during fruit ripening [46]. Moreover, the enzyme polygalacturonase is associated with *F. proliferatum* pathogenicity and virulence, which acts more efficiently after an increase in pH during muskmelon fruit ripening [49,50]. In the results obtained, the mixed treatment with BCAs and ulvan had the lowest pH values, which could be associated with the lowest DI and lesion diameter according to the data presented previously in Section 2.3.

The efficiency of *D. hansenii*, *S. rhizophila*, and ulvan as single or mixed treatment(s) to control muskmelon fruit rot caused by *F. proliferatum* could be related to the increase in the defense response mechanism in fruit (i.e., priming, PR protein synthesis, and oxidative burst) [51]. *Debaryomyces hansenii* reportedly induces resistance in citrus fruits by increasing the synthesis of phytoalexins [52], which produce molecules that confer resistance in fruits against fungal phytopathogens [53]. The results obtained in this study are the first to report the induction of antioxidant enzymes in muskmelon by *S. rhizophila* to reduce the rot caused by *F. proliferatum*. Dumas et al. [54] determined that the defense induction in *Medicago truncatula* by ulvan is mediated by the jasmonate signaling pathway. In rice and wheat, ulvan induces priming and increases the first oxidative burst, increasing resistance against mildew [55]. Cluzet et al. [56] concluded that using microarrays helps ulvan increase the expression of genes coding for phytoalexins, PR proteins, and structural proteins. In this study, ulvan moderately affected the control of disease incidence; however, its effect is attributed to the induction of systemic acquired response (SAR) and priming mechanism, which operate after induced systemic response (ISR) [54–56]. However, elucidating the

mechanisms involved in resistance induction in melon fruits by *D. hansenii*, *S. rhizophila*, and ulvan, requires further exhaustive investigation.

In previous reports, resistance induction was evidently promoted in melon fruits [57–59]. One of the initial defense responses against pathogens is the oxidative burst, which increased the reactive oxygen species (O^{2-} and H_2O_2) [60]. Although reactive oxygen species can contribute to defense in fruits, they can be degraded by antioxidant enzymes such as CAT, SOD, and POX [61]. CAT converts H_2O_2 to O_2 and H_2O, and POD degrades H_2O_2 by oxidizing phenolic compounds [62]. High levels of these enzymes are associated with reduced oxidative damage and delayed senescence [63]. PAL activity can be increased as part of the response mechanisms to numerous stress factors in the fruit [64]. According to Jetiyanon [65], the increase in PAL activity obtained by using the control can sufficiently inhibit pathogen invasion, and reduce disease incidence and lesion diameter [65].

4. Materials and Methods

4.1. Marine Microbial Antagonists Source and Concentration

Debaryomyces hansenii and *S. rhizophila* were obtained from the Phytopathology laboratory of Centro de Investigaciones Biologicas del Noroeste (CIBNOR), La Paz, Baja California Sur, Mexico. *Debaryomyces hansenii* and *S. rhizophila* were maintained and stored in potato dextrose agar (PDA; 39 g L^{-1}) and trypticase soy agar (TSA, 40 g L^{-1}) plates, respectively, at 4 °C. Liquid cultures of *D. hansenii* and *S. rhizophila* were prepared in 250 mL Erlenmeyer flasks containing 50 mL of potato dextrose broth (PDB, 39 g L^{-1}) and trypticase soy broth (TSB, 40 g L^{-1}), respectively, and were incubated at 27 °C for 24 h on a rotary shaker set at 180 rpm. *Debaryomyces hansenii* concentration was adjusted to 1×10^6 cells mL^{-1} using a hemocytometer, and *S. rhizophila* concentration was adjusted to 1×10^8 CFU mL^{-1} using a UV/Vis spectrophotometer (HACH, Dusseldorf, Germany) at 660 nm and absorbance of 1. *Debaryomyces hansenii* and *S. rhizophila* were adjusted to these concentrations prior to use in each of the following experiments.

4.2. Chemical Treatments Source and Concentration

Ulvan (OligoTech®, Elicityl Ltd., Crolles, France) solution was prepared by dissolving 5 g L^{-1} ulvan in sterile deionized water. The chemical fungicide benomyl was used at 1000 ppm. Ulvan and benomyl were adjusted to these concentrations prior to use in each of the following experiments.

4.3. Fusarium proliferatum Source and Concentration

Fusarium proliferatum was isolated from infected muskmelon fruit (*Cucumis melo* L. var. *reticulatus*) [38], and provided by CIBNOR. The fungus was stored on PDA at 4 °C. Prior to use, the culture was reactivated, and its pathogenicity was assessed by re-inoculating into wounded melon fruits, which was subsequently re-isolated onto PDA after establishing infection. Spore suspensions were obtained from 10-d old cultures maintained on PDA at 25 °C, and spore concentration was determined using a hemocytometer and adjusted to 1×10^4 spores mL^{-1} with sterile distilled water (SDW) containing 0.05% (v/v) Tween 80. *Fusarium proliferatum* was adjusted to this concentration prior to use in each of the following experiments.

4.4. Muskmelon Fruit Source and Pre-Treatment

Muskmelon (*Cucumis melo* L. var. *reticulatus*) fruit were sampled at El Pescadero, Baja California Sur, Mexico from a commercial orchard. Fruits without mechanical injury, disease symptoms, physiological maturity, and of uniform size were chosen for the experiments. The fruit surface was disinfected with 1% sodium hypochlorite, washed with SDW, and air-dried at 27 °C.

4.5. In Vivo Biocontrol Assay and Microscopic Visualization

The biocontrol activity of *D. hansenii*, *S. rhizophila*, and ulvan was tested according to the method described by Zhang et al. [66]. Six equidistant wounds of 3-mm diameter were created on each fruit and inoculated with 20 µL of the following treatments: (1) *D. hansenii*, (2) *S. rhizophila*, (3) ulvan, (4) *D. hansenii* + *S. rhizophila*, (5) *D. hansenii* + ulvan, (6) *S. rhizophila* + ulvan, (7) *D. hansenii* + *S. rhizophila* + ulvan, and (8) benomyl. The fruits were dried for 2 h and then each wound was inoculated with 20 µL of a suspension adjusted of *F. proliferatum*. The treatments concentration was adjusted as described in Section 4.1, Section 4.2 and Section 4.3. Fruit were incubated at 27 °C and 90% relative humidity (RH) for 7 d. Disease control (DC) and lesion diameter (mm) were measured. The DC was calculated using the following formula:

$$DC = 100 - \left[\left(\frac{Fi}{Tf}\right) \times 100\right] \quad (1)$$

where Fi = is the number of infected fruits in each treatment, and Tf = is the total number of infected fruits in the control treatment.

The advantage of in vivo mixed biocontrol treatments were assessed with respect to the individual treatments (*D. hansenii*, *S. rhizophila*, and ulvan) and the type of interactions (additive, synergistic, or antagonistic). The synergy factor (SF) was calculated according to de Abbott's formula [67]:

$$SF = \frac{DC}{DCE} \quad (2)$$

where DC and DCE are the observed and expected disease control (%) of the mixed treatments, respectively. DCE was calculated using the following formula:

$$DCE = (DCa + DCb + DCc) - \left(\frac{DCa \times DCb \times DCc}{100}\right) \quad (3)$$

where DCa, DCb, and DCc are the DCs of postharvest *D. hansenii*, *S. rhizophila*, and ulvan as single treatments, respectively.

For microscopic visualization, tissue samples of approximately 0.5 cm^2 were collected from in vivo biocontrol assay and fixed as described by Rivas-Garcia et al. [37]. Samples were examined by scanning electron microscopy (SEM) (Hitachi®, S-3000 N, Tokyo, Japan). Each treatment was represented by five replicates with three fruits per replicate.

4.6. Effect of Biocontrol Treatment Time on the Control of Fruit Rot Disease

The in vivo effect of treatment time of *D. hansenii*, *S. rhizophila*, and ulvan on the suppression of *F. proliferatum* on muskmelon was assessed following the method described by Zhimo et al. [68] with some modifications. Muskmelon fruits were collected and prepared as described in Section 2.4 and inoculated with 20 µL suspensions of the following treatments: (1) *D. hansenii*, (2) *S. rhizophila*, (3) ulvan, (4) *D. hansenii* + *S. rhizophila*, (5) *D. hansenii* + ulvan, (6) *S. rhizophila* + ulvan, (7) *D. hansenii* + *S. rhizophila* + ulvan, and (8) benomyl either prior to (2, 12, and 24 h) or after (12 and 24 h) inoculating 20 µL of *F. proliferatum*. The treatments concentration was adjusted as described in Sections 4.1–4.3. The experiments were performed as previously described in Section 4.5. The fruits were dried for 2 h and then incubated at 27 °C and 90% RH for 7 d. The DC and lesion diameters (mm) were measured. Each treatment was represented by five replicates with three fruits per replicate.

4.7. Efficacy of Biocontrol Treatments on Natural Fruit Rot Development and Fruit Quality Parameters

Muskmelon fruits were collected, and immersed without a pre-treatment (Section 2.4) into 2 L plastic containers with the following treatments: (1) *D. hansenii*, (2) *S. rhizophila*, (3) ulvan, (4) *D. hansenii* + *S. rhizophila*, (5) *D. hansenii* + ulvan, (6) *S. rhizophila* + ulvan,

(7) *D. hansenii* + *S. rhizophila* + ulvan, and (8) benomyl, for 2 min. The fruits were dried for 2 h and then each wound was inoculated with 20 µL of a suspension adjusted of *F. proliferatum*. The treatments concentration was adjusted as described in Sections 4.1–4.3. Fruits were incubated at 27 °C and 90% relative humidity (RH) for 7 d. The percentage of disease incidence (DI) was calculated using the formula:

$$\mathrm{DI} = \frac{\mathrm{Fi}}{\mathrm{Tf}} \times 100 \quad (4)$$

The quality parameters measured in muskmelon included weight loss (%), fruit firmness (N), total soluble solids (%), and pH. For weight loss estimation, muskmelon fruit was weighed before and after storage. Firmness was measured by compressing the muskmelon fruit on two opposite sides along the equatorial region, after applying a load of 9.8 N with a texture analyzer. For total soluble solids (TSS) and pH, 10 g of muskmelon fruit was macerated to obtain fruit juice. TSS was determined using a digital Abbe refractometer (PR-32, Atago Co., Tokyo, Japan) at room temperature. The pH was measured using a digital pH meter (PHS-550; Lohand Co., Hnagzhou, China). Each treatment was represented by five replicates with three fruits per replicate.

4.8. Antioxidant Enzymatic Activity on Muskmelon Fruit

Muskmelon fruits were collected and prepared as described above (Section 2.4). Six equidistant wounds with 3-mm diameter in each fruit were inoculated with 20 µL of the following treatments: (1) *D. hansenii*, (2) *S. rhizophila*, (3) ulvan, (4) *D. hansenii* + *S. rhizophila*, (5) *D. hansenii* + ulvan, (6) *S. rhizophila* + ulvan, and (7) *D. hansenii* + *S. rhizophila* + ulvan. SDW was used as the control. The treatments concentration was adjusted as described in Sections 4.1 and 4.2. The fruits were dried for 2 h, and incubated at 27 °C and 90% relative humidity (RH) for 8 d. Tissues adjacent to the inoculated area were sampled with a scalpel every 2 d (1 × 1 cm, length and depth), and were stored at −80 °C until enzymatic quantification. The collected samples were disrupted using liquid nitrogen and suspended in chilled phosphate buffer (0.1 M, pH 7.4) for catalase (CAT), peroxidase (POX), and superoxide dismutase (SOD) estimation, and suspended in chilled sodium borate buffer (0.1 M, pH 8) for phenylalanine ammonium lyase (PAL) quantification. The homogenate samples were centrifuged at 10,000× *g* for 20 min at 4 °C, and the supernatant was subjected to the enzymatic assay. CAT, POX, SOD, and PAL activities were measured using a commercial assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Protein content was determined using the Bradford assay, with standard curve plotted using bovine serum albumin [69]. One unit of CAT activity is defined as the amount of enzyme that reacts with 1 nmol of formaldehyde per min and is expressed in min mg^{-1} of protein [70]. One unit of POX activity is defined as the amount of enzyme that causes the formation of tetra guaiacol in the presence of H_2O_2 per min and is expressed in U mg^{-1} of protein [71]. One unit of SOD activity is defined as the amount of enzyme necessary to inhibit 50% of the O_2 reaction in the presence of nitro-blue tetrazolium reagent (NBT) and is expressed as U mg^{-1} of protein [72]. One unit of PAL activity is defined as µmol of cinnamic acid formed per minute per milligram of protein (min mg^{-1} of protein) [73]. Each treatment was represented by five replicates with three fruits per replicate.

4.9. Data Analysis

One-way analysis of variance (ANOVA) were performed to analyze the obtained data by using STATISTICA software (version 10.0; StatSoft, Tulsa, OK, USA). Post hoc least significant difference Fisher test ($p \leq 0.05$) was used to compare means.

5. Conclusions

In this study, the mixed pre-treatment of *D. hansenii*, *S. rhizophila*, and ulvan enhanced the biocontrol effect on fruit rot disease in muskmelon, delayed natural fruit rot, lowered percentages of decay and weight loss, maintained higher antioxidant and defense-related

enzymes (CAT, POX, SOD, and PAL), and preserved fruit quality (firmness, TSS, and pH). These results provide convincing evidence that postharvest treatment using 1×10^6 cells of *D. hansenii*, 1×10^8 cells of *S. rhizophila*, and ulvan displays higher disease resistance, better storability of harvested muskmelon fruit, and retains higher fruit quality, which suggests that postharvest mixed treatment containing BCAs and ulvan is a promising, safe, and effective biological control method in preserving the storage time of harvested muskmelon fruit. Omic technologies like metatranscriptomics and metagenomics analysis will be the future for the study of this complex tri-trophic interactions between microbial antagonists–fruit host–Pathogen.

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Review

A Sustainable Alternative for Postharvest Disease Management and Phytopathogens Biocontrol in Fruit: Antagonistic Yeasts

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Abstract: Postharvest diseases of fruits caused by phytopathogens cause losses up to 50% of global production. Phytopathogens control is performed with synthetic fungicides, but the application causes environmental contamination problems and human and animal health in addition to generating resistance. Yeasts are antagonist microorganisms that have been used in the last years as biocontrol agents and in sustainable postharvest disease management in fruits. Yeast application for biocontrol of phytopathogens has been an effective action worldwide. This review explores the sustainable use of yeasts in each continent, the main antagonistic mechanisms towards phytopathogens, their relationship with OMIC sciences, and patents at the world level that involve yeast-based-products for their biocontrol.

Keywords: biocontrol; antagonistic mechanisms; OMIC sciences; patents

1. Introduction

Fruit is an important resource in human diet because of its contribution in vitamins, minerals, organic acids, fiber, among others [1]. Moreover, obesity, cardiovascular, cognitive, skin, eye, lung, and bone diseases could be prevented through regular fruit intake [2,3]. Nowadays, the consumer demands fruit with a high-quality standard, both in appearance and in nutritional content [4,5]. However, postharvest fruit quality is affected by various factors, especially fungal diseases [6], which decrease its organoleptic properties and cause significant losses during storage, affecting up to 25% of total production in industrialized countries and more than 50% in developing countries [7,8].

The main strategy to control fungal infections at the postharvest level in fruit is the application of synthetic fungicides [9]. Nevertheless, these products have negative effects on human, animal, and environmental health [10,11] and induce resistance in phytopathogens [12,13].

The rise of biotechnology in the last decade has made biocontrol one of the most studied sustainable alternatives in reducing postharvest diseases by using antagonistic microorganisms against phytopathogens [14,15], which is considered a viable alternative to synthetic fungicides [16]. Among the microorganisms, yeasts stand out for their antagonistic capacity, for example, they have certain characteristics, such as genetic stability,

efficacy at low concentrations; control towards different phytopathogens [17]; simple nutritional requirements; survival under adverse environmental conditions; compatibility with other chemical and physical treatments; resistance to synthetic fungicides; and absence of pathogenicity towards the host [18,19]. Additionally, the yeasts do not produce metabolites potentially toxic to humans or animals and do not contaminate the environment [20–22].

In this review, we describe the use and applications of yeasts as biocontrol agents and its role in global sustainable postharvest disease management of fruits, including the characteristics of antagonist yeasts, their mechanisms of action, interaction with OMIC sciences, and future trends in their application.

2. Global Overview of the Use of Yeasts for Fruit Disease Biocontrol

Around the world, different yeast species have been evaluated for in vitro and in vivo control of postharvest fruit pathogens (Table 1). Although biocontrol commercial products for postharvest disease control have been developed, the search for new antagonists continues to allow the development of more effective biocontrol products that can be incorporated into crop sustainable management including fruits [23].

Table 1. Yeast antagonists evaluated for the biocontrol of postharvest diseases in five continents of the earth.

Continent	Yeast	Pathogen	Disease	Fruit	Inhibition Range (%)		Reference
					In Vitro	In Vivo	
ASIA							
China	*Candida oleophila*	*Botrytis cinerea*	Gray mold	Kiwifruit	-	17–45	[24]
Thailand	*Papiliotrema aspenensis*	*Colletotrichum gloeosporioides*	Anthracnose	Mango	66	94	[25]
India	*Candida tropicalis*	*Colletotrichum musae*	Anthracnose	Banana	70–85	84–96	[26]
Malaysia	*Trichosporon asahii*	*Colletotrichum gloeosporioides*	Anthracnose	Papaya	55–70	51	[27]
Israel	*Candida oleophila*	*Penicillium digitatum*	Green mold	Grapefruit	-	50–85	[28]
Taiwan	*Aureobasidium* sp.	*Botrytis cinerea*	Gray mold	Strawberry	18–36	-	[29]
Indonesia	*Aureobasidium pullulans*	*Colletotrichum acutatum*	Anthracnose	Chili	32–45	-	[30]
Saudi Arabia	*Pichia anomala*	*Botryodiplodia theobromae*	Fruit rot	Guava	-	39–50	[31]
EUROPE							
Italy	*Wickerhamomyces anomalus*	*Botrytis cinerea*	Gray mold	Strawberry	87	89	[32]
France	*Metschnikowia pulcherrima*	*Penicillium expansum*	Blue mold rot	Apple	52–91	15–18	[33]
Spain	*Hanseniaspora uvarum*	*Botrytis cinerea*	Gray mold	Strawberry	60–67	54–72	[22]
Poland	*Debaryomyces hansenii*	*Monilinia fructicola*	Brown rot	Apple	69	70–85	[34]
Germany	*Rhodosporidium paludigenum*	*Penicillium expansum*	Blue mold rot	Apple	-	67–86	[35]
Portugal	*Metschnikowia andauensis*	*Penicillium italicum*	Blue mold rot	Orange	62–70	90	[36]
AMERICA							
Uruguay	*Candida sake*	*Penicillium expansum*	Blue mold rot	Apple	25–74	25	[37]
Argentina	*Vishniacozyma victoriae*	*Botrytis cinerea*	Gray mold	Pear	-	70–100	[21]
Mexico	*Debaryomyces hansenii*	*Colletotrichum gloeosporioides*	Anthracnose	Papaya	15–36	66–83	[20]

Table 1. *Cont.*

Continent	Yeast	Pathogen	Disease	Fruit	Inhibition Range (%) In Vitro	Inhibition Range (%) In Vivo	Reference
Ecuador	*Candida inconspicua* and *Pichia kluyveri*	*Alternaria alternata*	Black rot	Pitahaya	-	7–20	[38]
Brazil	*Candida oleophila*	*Botrytis cinerea*	Gray mold	Apple			[39]
	Aureobasidium pullulans	*Penicillium digitatum*	Green mold	Citrus	30–41	-	[40]
Chile	*Crptococcus antarcticus*	*Botrytis cinerea*, *Penicillium expansum* and *Geotrichum candidum*	-	-	65–70	-	[41]
AFRICA							
Stellenbosch	*Pichia kluyveri*	*Botrytis cinerea* and *Monilinia laxa*	-	Apple	-	95–100	[42]
Tunisia	*Wickerhamomyces anomalus*	*Penicillium digitatum*	Green mold	Orange	100	100	[43]
South Africa	Various yeasts isolated from surface citrus fruits	*Penicillium digitatum*	Green mold	Citrus	-	95	[44]
Morocco	*Pichia anomala*, *Debaryomyces hansenii* and *Hanseniaspora guilliermondii*	*Penicillium digitatum*	Green mold	Citrus	-	65–80	[45]
AUSTRALIA							
Sidney	*Pichia guilliermondii*	*Botrytis cinerea*, *Alternaria alternata* and *Rhizopus nigricans*	Gray mold, black spot and *Rhizopus* rot	Cherry tomato fruit	-	25–90	[46]
Sidney	*Pichia guilliermondii*	*Colletotrichum acutatum*	Anthracnose	Loquat fruit	-	100	[47]
Sidney	*Cryptococcus laurentii*	*Botrytis cinerea*	Gray mold	Tomato fruit	-	55–90	[48]
Brisbane	*Rhodotorula glutinis*	*Penicillium expansum*	Blue mold	Pear	-	90–95	[49]

3. Mechanisms of Action of Antagonistic Yeast towards Fruit Fungal Phytopathogens

3.1. Competition for Space and Nutrients

Competition for nutrients and space has been suggested to be the major mechanism of action by which yeasts exert their antagonistic action in inhibiting pathogenic fungi. Yeasts consume the necessary nutrients for their colonization and growth faster than the pathogens resulting in inhibiting spore germination, reducing its growth and infection level and, thus, decreasing infection and diseases development [50,51]. In addition, the synthesis of inhibitory compounds in yeasts is increased by the absorption of nutrients in situ or ex situ, improving their ability to biocontrol plant diseases [52].

The carbon sources that yeast consume include glucose, maltose, fructose, melezitose, and lactose, among others [53]. The determination of the nutritional needs and adaptation to the host of each yeast are important for their capacity as an antagonist [54].

3.2. Killer Toxin

Killer toxins are often glycosylated proteins produced by yeast of different species and can disrupt specific cell wall components (β-1,3-D-glucans, β-1,6-D-glucans, mannoproteins, and chitin), which result in fungal cell death (Table 2) [55,56]. Killer toxins attach to the cell membrane where they interact with a secondary receptor that result in changes in cell membrane permeabilization, DNA synthesis inhibition, cell cycle disruption, and RNA fragmentation [57,58].

Table 2. Inhibition of phytopathogens cause of postharvest disease of fruits by yeasts producing killer toxins.

Killer Yeast	Phytopathogen	Fruit	Control (%)	Reference
Debaryomyces hansenii	*Alternaria brassicicola*, *Alternaria citri*, *Aspergillus niger* and *Rhizopus stolonifer*	Apple, tomato, and lemon	80–100	[59]
Wickerhamomyces anomalus	*Colletotrichum gloeosporioides*	Papaya	100	[60]
Debaryomyces hansenii	*Monilinia fructigena* and *M. fructicola*	Peach and plum	33–86	[61]
Debaryomyces hansenii	*Aspergillus niger*	-	80	[62]
Pichia fermentans	*Penicillium digitatum* and *P. italicum*	Lemon	40	[63]
Wickerhamomyces anomalus and *Meyerozyma guilliermondii*	*Colletotrichum gloeosporioides*	Papaya	20–24	[64]
Saccharomyces cerevisiae and Wickerhamomyces anomalus	*Penicillium digitatum*	Orange	87	[65]

Genetic studies in *Saccharomyces cerevisiae* have shown that the ability to produce killer toxins is cytoplasmically inherited and related to the presence of double-stranded linear RNA (dsRNA) plasmids, which are then encapsulated, forming non-infectious virus-like particles (VLP) within the cell cytoplasm [66]. All killer toxins are produced under acidic conditions, and their activity decrease with the increase in pH and temperature of the medium in which they are found—an increase in these variables is sufficient for the yeasts to stop producing them [67,68].

3.3. Lytic Enzymes

One of the antagonistic mechanisms of yeasts against phytopathogens is the production of lytic enzymes, such as glucanases, chitinases, and proteases, which act on different sites of the fungal cell wall, causing cell lysis and death (Figure 1) [69,70].

β-glucanases are enzymes that hydrolyze the β-glucosidic bond of β-glucans. There are two types of glucanases: those that randomly hydrolyze intra-chain bonds giving rise to oligosaccharides (endoglucanases) and those that release glucose molecules by hydrolyzing bonds at the non-reducing end of the chain (exoglucanase). There are also yeasts that can produce both types of enzymes [71, 72]. Different mechanisms for glucanase synthesis and secretion have been suggested, but the most important one involves a synthesis regulated by repression in glucose when it is not found in sufficient quantities in the medium [73]. In relation to chitinases, these enzymes hydrolyze β-1,4 bonds of chitin *N*-acetyl-β-D-glycosamide, which is one of its main cellular fungus components, breaking it into oligomers and monomers of *N*-acetyl-β-D-glucosaminidase and causing cell death [74,75].

Five types of chitinases have been identified, of which the most common is type I with a molecular weight of around 30 kDa. In its sequence, it has an N-terminal domain similar to hevein and type II, which possess a lower molecular weight of 25 kDa and lack the N-terminal hevein domain [76,77]. Finally, proteases have a molecular weight of approximately 35 kDa, stability at a pH from 2 to 5, a low isoelectric point, are insensitive to metal and heavy metal chelators, and have a high capacity to hydrolyze a wide range of peptide bonds of the mannoproteins that make up the fungus cell wall [51,78].

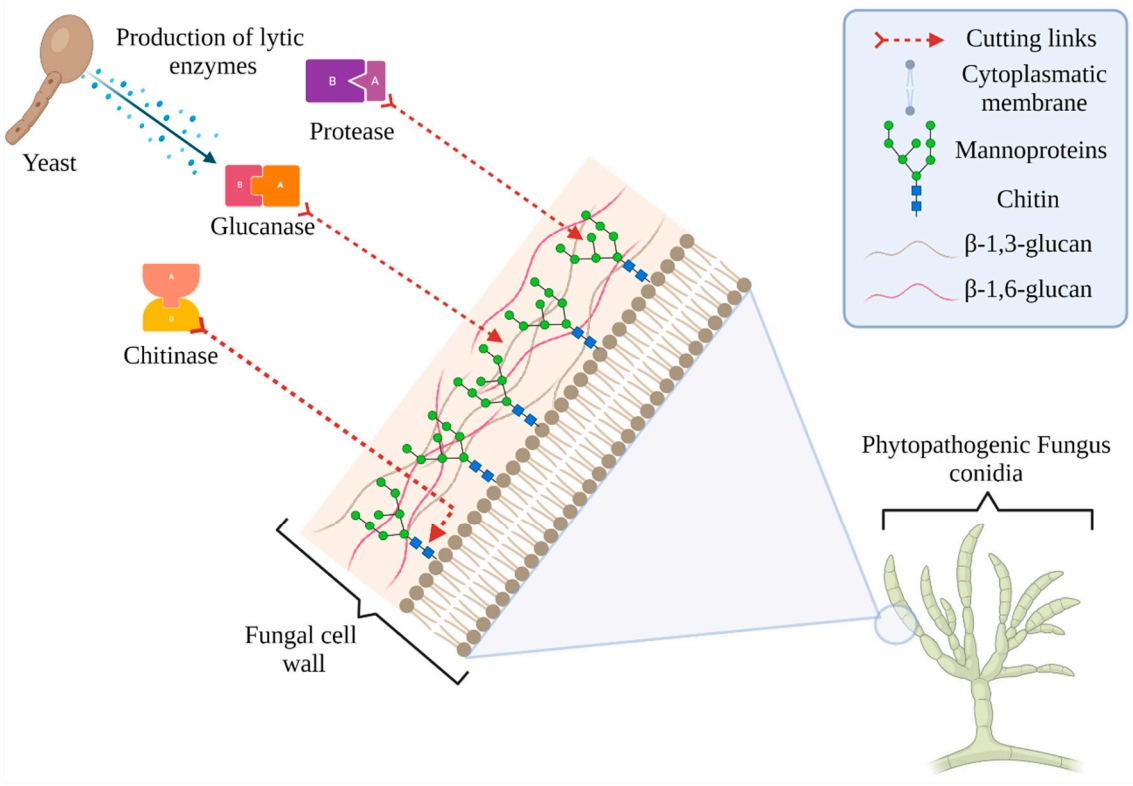

Figure 1. Enzyme production by antagonistic yeasts and their lytic effect on phytopathogenic fungus cell wall.

3.4. Induction of Host Resistance

Yeasts can induce resistance in the host as an indirect mechanism to prevent infections caused by fungi [79]. At the initial stages of fungus invasion into the tissue, the fruit or plant cells begin with a hypersensitivity reaction (HR), which necrotizes the tissue invaded by the fungus to isolate the infection, to prevent, or to slow the advance towards healthy cells [80]. HR can be activated by many agents called inducers, such as synthetic products, phytopathogens, non-pathogenic microorganisms (such as yeasts, fungi, and bacteria), ultraviolet light, and insects, among others [81,82]. This reaction in the host can be systemic; due to this characteristic, induction is defined as systemic acquired resistance (SAR) [83].

In response to any inducer, the plant overexpresses genes and enzymes related to plant defense by increasing the production of substances. For example, these substances include proteins related to pathogenesis (PR-proteins, classified in 14 families) [84] and phytoalexins (characterized around 300, including coumarins, flavonoids, diterpenes, and benzofuran, among others) [85] and/or lytic enzymes (proteases, glucanases, and chitinases) [86] and reactive oxygen species (ROS) [87], among others, which have resulted in inhibition effects and/or cell lysis or disruption of the phytopathogenic fungus.

PR proteins are defined as proteins that are absent or detected at a low basal level in healthy tissues but significantly accumulate during pathological conditions in both compatible and incompatible host–pathogen interactions [88]. Research studies involved PR-proteins following yeast treatment of fruit, i.e., *Pichia membranaefaciens* induced PR-9 and PR-10 in peach fruit [89]. However, PR-protein responses are too variable in relation to specific host tissue as well as microbial stimuli. The gene expression of PR-5 and PR-8 was characterized in apple fruit after treatment with *Candida oleophila* as a biocontrol agent against *Botrytis cinerea*. As a result, PR-8 was significantly overexpressed in response to both microorganisms while neither *B. cinerea* nor *C. oleophila* treatment significantly overexpressed the PR-5 gene [39].

Phytoalexin and lytic enzyme production by yeast resistance induction was demonstrated by Nantawanit et al. [90], who concluded that resistance induction in chili fruit treated with *Pichia guilliermondii* significantly enhanced the activities of phenylalanine ammonia-lyase (PAL), chitinase, and

β-1,3-glucanase, and capsidiol phytoalexin accumulation in chili tissue. PAL is a fundamental enzyme during the first steps of the phenylpropanoid pathway to synthetize lignin, phenols, phytoalexins, and other compounds related to the plant resistance process [91].

Moreover, biocontrol yeast agents can enhance antioxidant enzyme activity to alleviate the oxidative damage caused by ROS produced in response to pathogen infection [65]. After cherry treatment with *P. membranaefaciens* at 5×10^7 cells mL^{-1}, peroxidase (POD) activity was enhanced, but catalase (CAT) and superoxide dismutase (SOD) decreased [92]. Many mechanisms related to resistance induction are simultaneously promoted by yeast antagonists. For example, *Rodosporidium* spp., *Pichia* spp., and *Cryptococcus laurentii* enhanced the activity of antioxidant enzymes and enzymes related to defense [93,94].

4. Antagonistic Yeasts and OMIC Sciences

Conventionally, the study of the mechanisms of action is related to the evaluation of the production of antibiotics, lytic enzymes, or other metabolites in vitro or in co-culture against the phytopathogen [95]. Information of the antagonistic mechanisms of antagonist yeasts is crucial for improving their efficiency against phytopathogens. Therefore, OMIC approaches, such as genomic, transcriptomic, and proteomic, are modern molecular technologies that help in their characterization [96]. Information on efficacy and consistency of an antagonist yeast helps to select the best antagonist against a specific phytopathogen [39]. The study of the microbial antagonist genome helps to understand the potential genes involved in biocontrol activity, characterizes groups of genes with unknown functions, compares the genome with other biological control agents, and, finally, helps in study gene transcription [97].

Proteomic approaches provide information on changes in metabolic/physiological functions within the cell. Additionally, any biotic or abiotic factors that induce changes during microbial growth can be studied by this molecular tool [98]. Proteomic analysis plays a key role in host–phytopathogen interactions, and this technique can help identify key proteins involved in antagonist–phytopathogen–host interaction [71].

Metabolomics analyses allow an understanding of cell physiology in real time. The production of secondary metabolites, antibiotics, and lytic enzymes is one of the main mechanisms of action for the control of phytopathogens [99]. The interaction of microbial antagonists with phytopathogens can change the proteome and transcriptome of plants or fruit, as well as their response to biotic stress through the induction of defense-related metabolic pathways [100].

Transcriptomic studies of biological control agents provide useful information on the genes involved in the production of secondary metabolites mostly studied in bacteria and yeasts [101]. In the case of fungal biocontrol agents, studies have focused on the genes involved in the influence of lytic enzymes, such as glucanases, proteases, and chitinases on fungal cell wall [102]. Transcriptomic analysis is not limited to the study of biological control agents; the study of phytopathogens also provides useful knowledge associated with its virulence [103].

Another important aspect is microbial interaction on the fruit's surface with antagonistic microorganisms since they are an integral part of the host's composition. The study of the microbiome is important to understand the key role of the microorganisms present and their role in fruit health and physiology, as well as their possible positive or negative interactions with artificially applied antagonists [104,105].

5. Patents on Yeast-Based Products for Plant and Fruit Disease Biocontrol

A patent, understood as the title that the state grants for the exclusive exploitation of an invention for a specified period [106,107], is a method used to protect intellectual property and, in many cases, is required to advance on the development of a biological product for the control of plant diseases. The first yeast-related patents date from 1842 in Finland [108] and 1873 in the United States by Luis Pasteur (US141972) [109].

Globally, from 2009 to 2021, 163 patents were reported in the Derwent Innovation database related to yeasts as biological control agents for plants or parts of them (Figure 2). Germany, USA, Australia, and China account for 53% of all patents with yeasts worldwide.

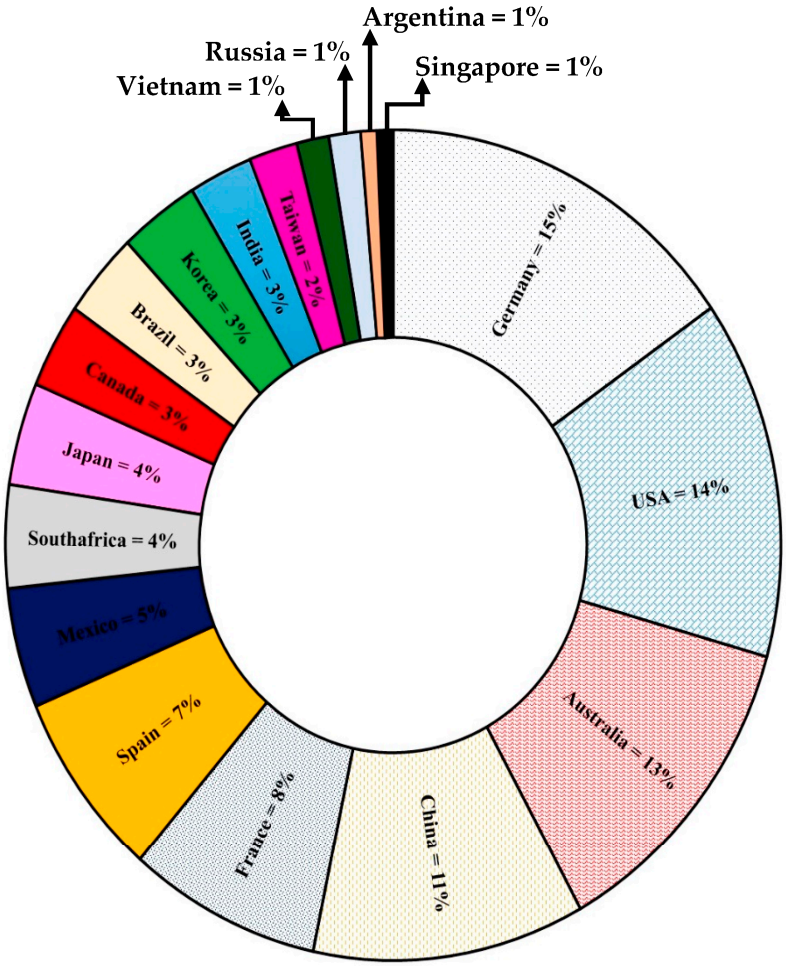

Figure 2. Percentage of patents by country of biocontrol products containing yeasts used in plants or plant parts.

Of the 163 patents, 73.68% of the records have the name of the genus or genus and species of the yeast contained in the patented product, and 26.31% only indicated the word "yeast" among its components. Generally, the products contain yeasts and other microorganisms. Related to these products, 32.89% contained *Metschnikowia fructicola*; 11.18% contained *Candida* sp.; 11.18% contained a mixture of *Candida oleophila*, *Metschnikovia fructicola*, and *Pichia anomala*; 9.86% contained *Pichia* sp.; 7.89% contained *Rhodotorula* sp.; 5.92% contained *Cryptococcus* alone or mixed with *Rhodotorula* sp.; and 1.97% contained *Debaryomyces* sp.

Moreover, 84.21% of the patents belong to companies, where Bayer® is the predominant one. A low percentage is occupied by academic institutions (15.78%). This analysis reflects little participation of academics belonging to higher education institutions such as universities or public research centers in intellectual property registries. Much of the valuable information generated in universities has not been recorded, probably because the main objective is teaching and in addition to the lack of equipment to carry out mass formulations of the new product or an entity dedicated to marketing within these institutions. The development of biocontrol products containing yeasts for use in the post-harvest period is in high demand by entrepreneurs related to post-harvest and end consumer because it is a harmless product.

6. Conclusions

The use of yeasts as a post-harvest treatment to reduce decay caused by various phytopathogenic fungi in fruit of commercial interest is a sustainable and efficient alternative to the utilization of synthetic fungicides. The application of yeasts will be able to reduce the levels of fruit losses caused by phytopathogens, which will increase economic gains because of a greater volume of production for commercialization. Its implementation in postharvest will improve shelf life of the fruit and may

lower crop costs by reducing the use of synthetic products. The acceptance of the consumer for product acquisition—not treated with any chemical—allowed opening new markets since it is a fruit not treated with synthetic fungicides.

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Nutrient Status of Cucumber Plants Affects Powdery Mildew (*Podosphaera xanthii*)

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Abstract: We examined the effects of applications of N, P, K, Mg, and Ca through an irrigation solution and spraying K, Ca, and Mg salts on cucumber powdery mildew (CPM, *Podosphaera xanthii*) in potted plants and under commercial-like conditions. Spraying $CaCl_2$ and $MgCl_2$, or KCl and K_2SO_4, decreased CPM. There were significant negative correlations between the anion-related molar concentrations of the salts and disease severity. Among the sprayed treatments, NaCl provided significantly less CPM control when applied at a low (0.05 M) concentration, as compared with $CaCl_2$ and $MgCl_2$. When sprayed applications of Mg and K salts were analyzed separately from the untreated control, the Cl^- salts were found to be more effective than the $SO_4{}^{-2}$ salts. High N and Mg concentrations in the irrigation water delivered to young, fruit-less cucumber plants reduced CPM, whereas more CPM was observed when the irrigation solution contained a medium amount of P and a high amount of K. In contrast, mature, fruit-bearing plants had less severe CPM at higher N, lower P, and higher K levels. Spraying mature plants with monopotassium phosphate, polyhalite ($K_2Ca_2Mg(SO_4)_4{\cdot}2H_2O$), and the salts mentioned above over an entire growing season suppressed CPM. CPM severity was also reduced by spray applications of Ca, Mg, and $KSO_4{}^{-2}$ and Cl^- salts. Spray applications provided better CPM control than fertigation treatments. Induced resistance is probably involved in the effects of nutrients on CPM.

Keywords: agrotechnical control; calcium; *Cucumis sativus*; cultural control; integrated management; powdery mildew; magnesium; plant disease; potassium

1. Introduction

Podosphaera xanthii (syn.: *Podosphaera fuliginea*, *Sphaerotheca fuliginea*, *Sphaerotheca fusca*) is one of the causal agents of powdery mildew of cucurbits [1]. It is a biotrophic pathogen that is distributed by asexual conidia that germinate on green plant organs (mainly leaves), producing germ tubes that form appressoria. The appressoria form penetration pegs that penetrate the epidermal cells and then form haustoria in close contact with the host cells. The pathogen grows over the leaf surface and superficially inside the epidermis and forms chains of conidia attached to conidiophores, which are visible as white marks on the leaves [2]. The white marks spread from older to younger leaves and the cucumber powdery mildew (CPM) severity is measured according to the percentage of leaf surface covered in white [3]. CPM is managed using fungicide sprays and, to a lesser extent, by biocontrol; partial resistance is also available in some cultivars [3–5].

Mineral nutrients are needed for plant development and play important roles in all plant-life processes. Nitrogen, phosphorus, and potassium are essential for biomass

production and plant growth. Calcium and magnesium are essential for many cellular processes and are less important for plant growth [6]. Microelements are important for plant life but are beyond the scope of the present publication. Therefore, we will review only their roles in plant diseases. Minerals are important in plant–pathogen interactions [7–9]. All essential mineral nutrients affect the progress of plant diseases [8,9]. However, those effects vary by microelement and between different plant–pathogen systems [10]. Different nutrients' concentrations influence the resistance of plants to pathogens through metabolic changes, changes in the surrounding conditions, effects on the activity of enzymes, the synthesis of cell walls, the cell membrane's permeability, the synthesis of amino acids, polyphenols, and sugars [11], and plant resistance gene upregulation, as was demonstrated for foliar applications of N, K, Mn, and Zn [7].

The effects of N on plant disease depend on the form of N, the species and organ of the affected plant, and the pathogen's identity [12]. It is generally understood that obligate plant parasites thrive in the presence of excess N fertilization. This has been attributed to the associated increased proliferation of young plant tissue and to the increase in the amino acid apoplast concentration on the leaf surface that encourages conidia germination and penetration [13]. A high N concentration in tomato plants increased *Fusarium oxysporum* f. sp. *lycopersici* severity and decreased the expression of phenylpropanoid pathway enzymes and the levels of lignin, callose, tylose, and rishitin, which all play important roles in plant defense [12]. Grape downy mildew increased and the synthesis of the phytoalexin resveratrol decreased at higher concentrations of N [14]. Rust and downy mildew of cereals increased when high levels of NO_3^- were applied as fertilizer [15]. In sweet basil, the necrotrophs *Botrytis cinerea* and *Sclerotinia sclerotiorum* are more prevalent when there are higher concentrations of N in the plant shoots [16,17]. Increasing the total N concentration in the irrigation solution was shown to increase the level of sweet basil downy mildew in potted sweet basil plants as well as sweet basil grown under field conditions. Moreover, when NH_4^+ accounted for a large proportion of the total N in the irrigation solution, less severe downy mildew was observed [18].

Phosphorus has been reported to decrease soilborne diseases such as take-all in wheat (*Gaeumannomyces graminis* in *Triticum aestivum*), charcoal rot (*Macrophomina phaseolina*) in maize (*Zea mays*) [9] and soybean (*Glycine max*) [19], and *Verticillium dahlia* wilt in potato (*Solanum tuberosum*) [20], as well as the foliar disease powdery mildew in wheat [21]. P fertigation has been found to increase the severity of onion downy mildew (*Peronospora destructor*) [22]. It has also been argued that P plays a role in induced resistance in some pathosystems [23–25].

Potassium at optimal tissue concentrations decreases the susceptibility of plants to disease [9]. K reduced the incidence of disease caused by the ascomycete pathogens *B. cinerea* and *S. sclerotiorum* [16,17]. K also suppressed downy mildew (*Peronospora plantaginis*) severity in *Plantago ovata*, the plant used to produce isabgol [26], and suppresses sweet basil downy mildew when it is sprayed on the plant [27].

Calcium plays a role in plant defense systems [28]. It is an important constituent of pectin and other cell-wall components. Ca binds pectin oligomers and by that it prevents pathogen penetration. Ca acts against cell-wall-degrading enzymes of pathogens [28,29]. Ca also plays a role in the activity of pathogenesis-related proteins, the expression of defense-related genes, and hypersensitive reactions [30–32]. Ca increases the resistance of plants to pathogens such as species of *Pythium*, *Sclerotinia*, *Botrytis*, and *Fusarium* [9]. Ca reduced in sweet basil the incidence of *B. cinerea* and *S. sclerotiorum* [16,17]. Sprayed applications of Ca reduced the severity of downy mildew (*Sclerospora graminicola*) in pearl millet (*Pennisetum glaucum*) [33]. Irrigation with increased levels of $CaCl_2$ decreased sweet basil downy mildew under field conditions [27].

Magnesium affects plant diseases, directly and indirectly, through its antagonistic interactions with other nutritional minerals (e.g., K, Ca, and Mn) [34]. *Fusarium oxysporum* f. sp. *conglutinans* disease in cotton (*Gossypium arboreum*) was reduced when Mg availability was at optimal levels. A high Mg concentration that interfered with Ca absorption

increased the severity of bacterial speck (*Xanthomonas campestris* pv. *vesicatoria*) in tomato (*Solanum lycopersicum*) [34]. Mg decreased downy mildew (*Peronospora arborescens*) severity in poppy (*Papaver somniferum*) [35] and decreased tobacco downy mildew (*Peronospora tabacina*) severity [36]. Irrigation with increased $MgCl_2$ also decreased sweet basil downy mildew under field conditions [27].

There have been previous studies on nutritional elements' effects on diseases of cucumber. Fertigation of cucumber with P in a hydroponic system and spray applications of P-containing salts were found to decrease powdery mildew [24,37,38]. Spray applications of Ca and K reduced gray mold (*B. cinerea*) on fruits and stems of cucumber plants and K fertigation reduced downy mildew (*Pseudoperonospora cubensis*) on cucumber leaves [39]. Nitrate was found to protect cucumber plants against *Fusarium oxysporum* f. sp. *cucumerinum* [40]. Monopotassium phosphate (MKP) is effective against cucumber powdery mildew under commercial conditions [41,42].

We examined the effects on CPM of salts containing the cations Ca, Mg, and K and the anions Cl and SO_4 applied as foliar sprays (alone or in combination) to young plants and mature, fruit-bearing plants. We also examined the application of N, P, and K as well as Ca and Mg, which were applied via irrigation water. In this study, we first tested in potted plants the effects of spray treatments and determined the optimal concentrations for the application of each of the mineral cations. Later, we tested the effects of a limited number of concentrations and combinations of nutrients on CPM severity under commercial-like conditions.

2. Results

2.1. Effect of Sprayed Applications of Salt Solutions on CPM in Potted Cucumber Plants (Expt. A-s1)

Sprayed applications of Ca and Mg Cl^- salts and K salts with Cl^- and ${SO_4}^{-2}$ at concentrations of 0.5 to 1.0% (Table 1) were made to mature cucumber plants. CPM developed on the leaves and was significantly suppressed by most of the salts (Figure 1a). The concentrations of $CaCl_2$ and KCl did not affect their suppressive effects. We evaluated the relationships between CPM severity and the molar concentrations of the applied salts according to the cations and the anions of the salts. A significant negative correlation was observed between the anion-related molar concentrations of the salts and disease severity (Figure 1b), whereas the relationship between the cation molar weight of the salts and CPM severity was insignificant (data not presented). Thus, in our subsequent experiments, we considered the anion-related molar concentrations of the salts.

2.2. Effect of Sprayed Applications of Cl^- Salt Solutions on CPM in Potted Plants (Expts. B-s1 and B-s2)

To evaluate the effects of different concentrations of spray-applied Cl^- on CPM, chloride salts of Ca, Mg, and Na were applied to cucumber plants (Table 1, Figure 2). In one experiment, only NaCl was applied (Expt. B-s1, Figure 2a,b). In a second experiment (Expt. B-s2, Figure 2c), Cl salts of Na, Ca, and Mg and a mixture of Ca and Mg salts were applied. All of the salts significantly reduced CPM severity (Figure 2a–c). The relationship between the concentration of Cl^- in the spray solution and the Cl^- concentration in the leaves was positive and significant (Figure 2a) and the Cl^- concentration in the leaves was strongly negatively correlated with CPM severity (Figure 2b). NaCl was significantly less effective at the lower 0.05 M concentration (Figure 2c). Interestingly, the relationship between the degree of suppression and the salt concentration was similar across the different salts, with the greatest control observed at salt concentrations of less than or equal to 0.1 M and no further disease reduction observed at the higher concentrations. Furthermore, the combination $MgCl_2$+$CaCl_2$ did not result in an additive effect, as compared with each salt alone (Figure 2c).

Table 1. Experimental setup, factors tested, application methods, and growing seasons.

Site	Code	Growing Setting	Materials Tested	Additional Treatment	Application	Season
A	A-s1	Pots	K_2SO_4, KCl, $MgCl_2$, $CaCl_2$		Foliar (spray, "s")	All year
B	B-s1	Pots	NaCl		Foliar	All year
B	B-s2	Pots	$MgCl_2$, $CaCl_2$, NaCl, $MgCl_2$+$CaCl_2$		Foliar	All year
B	B-s3	Pots	$MgSO_4$, $MgCl_2$, K_2SO_4, KCl		Foliar	All year
B	B-s4	Pots	$MgSO_4$, $MgCl_2$, K_2SO_4, KCl	penconazole	Foliar	All year
B	B-N-f	Pots	N		Fertigation (f)	All year
B	B-P-f	Pots	P		Fertigation	All year
B	B-K-f	Pots	K		Fertigation	All year
B	B-Ca-f	Pots	Ca (Cl)		Fertigation	All year
B	B-Mg-f	Pots	Mg (Cl)		Fertigation	All year
A	A-SCs-a	Large pots (semi-commercial)	Monopotassium phosphate (MKP), $K_2Ca_2Mg(SO_4)_4{\cdot}2(H_2O)$ (polyhalite as polysulfate)		Foliar	Autumn–Winter
A	A-SCs-b	Large pots (semi-commercial)	$MgCl_2$, $CaCl_2$, K_2SO_4, $MgCl_2$+$CaCl_2$+K_2SO_4		Foliar	Winter–Spring
C	CL1	Boxes (commercial-like)	N, P, K in irrigation (Table 2) X $MgCl_2$+K_2SO_4	triadimenol	Fertigation and Foliar	Spring
C	CL2	Boxes (commercial-like)	$MgCl_2$, K_2SO_4, $MgCl_2$+K_2SO_4, MKP	-"-	Foliar	Spring

2.3. Effects of Spray Applications of Cl^- and $SO_4{}^{-2}$ Salt-Solutions and a Fungicide on CPM in Potted Plants (Expts. B-s3 and B-s4)

In Expt. B-s3, Mg and K salts containing either Cl^- or $SO_4{}^{-2}$ anions were applied at two concentrations (Table 1, Figure 3a). The salts effectively suppressed CPM; disease severity in the control treatment reached 86%, while all of the salt treatments suppressed the disease severity down to 0.7 to 19.4%. When the salt-spray treatments were analyzed separately from the untreated control, the Cl^--containing salts were statistically more effective than the $SO_4{}^{-2}$-containing salts and the higher salt concentrations were more effective than the lower salt concentrations ($p < 0.0009$, statistical analysis not presented). In these experiments, there was no difference between K- and Mg-containing salts. In other experiments (Expt. A-s4), all of the tested salts except for KCl provided CPM suppression that was just as good as that provided by the chemical fungicide penconazole (Figure 3b).

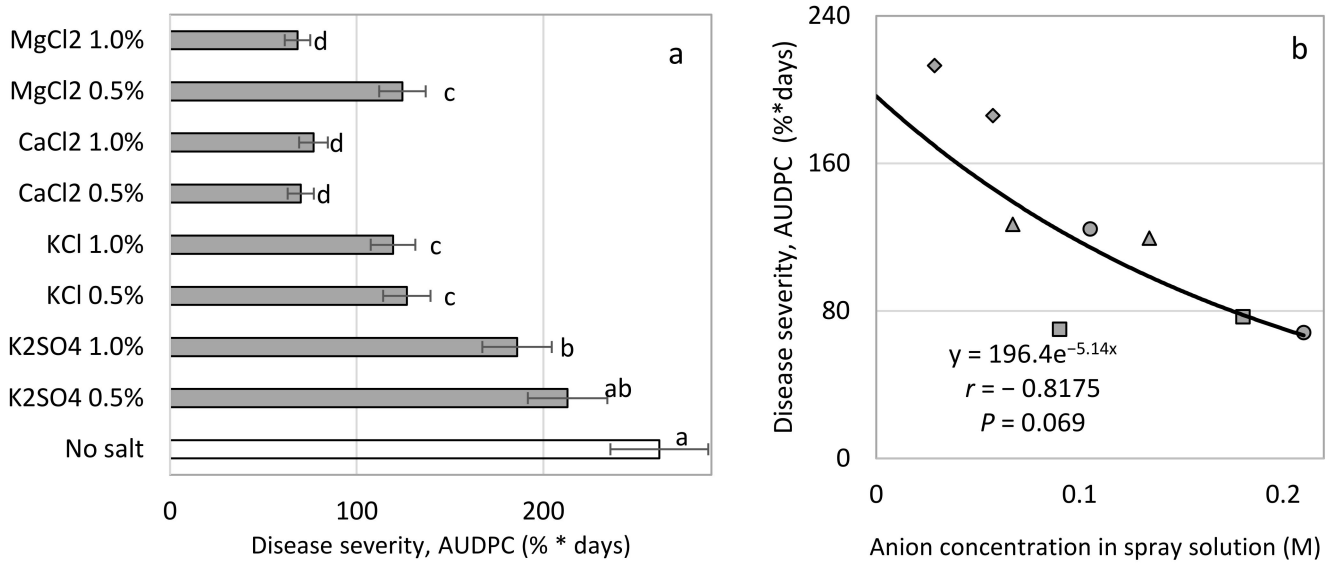

Figure 1. Effects of sprayed applications of salts on the severity of cucumber powdery mildew (CPM, *Podosphaera xanthii*) on leaves (Expt. A-s1). Solutions containing different concentrations of the salts were sprayed on mature plants twice a week. CPM severity was evaluated on a 0 to 100 scale, in which 0 = no disease symptoms and 100 = leaf fully covered by symptoms. (**a**) The area under disease progress curve (AUDPC) was calculated for 18 days from disease onset. Values followed by a common letter are significantly not different from each other according to Tukey-Kramer's HSD test ($P \leq 0.05$). (**b**) The relationships between the anion concentrations of the various spray treatments (presented as molar values) and CPM severity were examined. Markers represent $MgCl_2$ (●), $CaCl_2$ (■), KCl (▲), and KSO_4 (♦). The regression formula is presented and the Pearson regression (*r*) values are presented along with significance levels (*P*). Bars = SE.

2.4. Pot Experiments—Supplemental Nutrients in the Fertigation Solution under Controlled Conditions (Expts. B-#-f)

Effects of N, P, and K applied through the fertigation solution on CPM severity (Expts. B-N/P/K-f).

Different concentrations of N, P, and K in the irrigation solution were achieved without changing the concentrations of the other major ions, as described in the Materials and Methods section and Table 1.

N (Expts B-N-f): Raising the concentration of N in the fertigation solution (15% NH_4^+) from 0.7 to 14.3 mM (Table 1) resulted in a gradual increase in the N concentration in the cucumber leaves, up to 3.91% of the leaf dry weight (Figure 4a). Increasing the N concentration in the irrigation solution resulted in a minor decrease in CPM severity (Figure 4b). Similarly, the major increase in N leaf concentration was associated with a minor decrease in disease severity (Figure 4c).

P (Expts. B-P-f): Raising the concentration of P in the fertigation solution from 0 to 0.65 mM (Table 1) gradually increased the P concentration in the cucumber leaves, up to 0.8% of the leaf dry weight (Figure 5a). Increasing the P concentration in the irrigation solution led to a peak in CPM severity at 0.32 mM P (Figure 5b). Similarly, the change in the leaf concentration of P led to a peak in disease severity at 0.46 mM P (Figure 5c). *K (Expts. B-K-f)*: Raising the concentration of K in the fertigation solution from 0.3 to 2.6 mM (Table 1) gradually increased the K concentration in the cucumber leaves, up to 3.16% of the leaf dry weight (Figure 6a). Increasing the K concentration in the irrigation solution led to a gradual increase in CPM severity (Figure 6b). Similarly, the change in K leaf concentration led to a gradual increase in disease severity (Figure 6c).

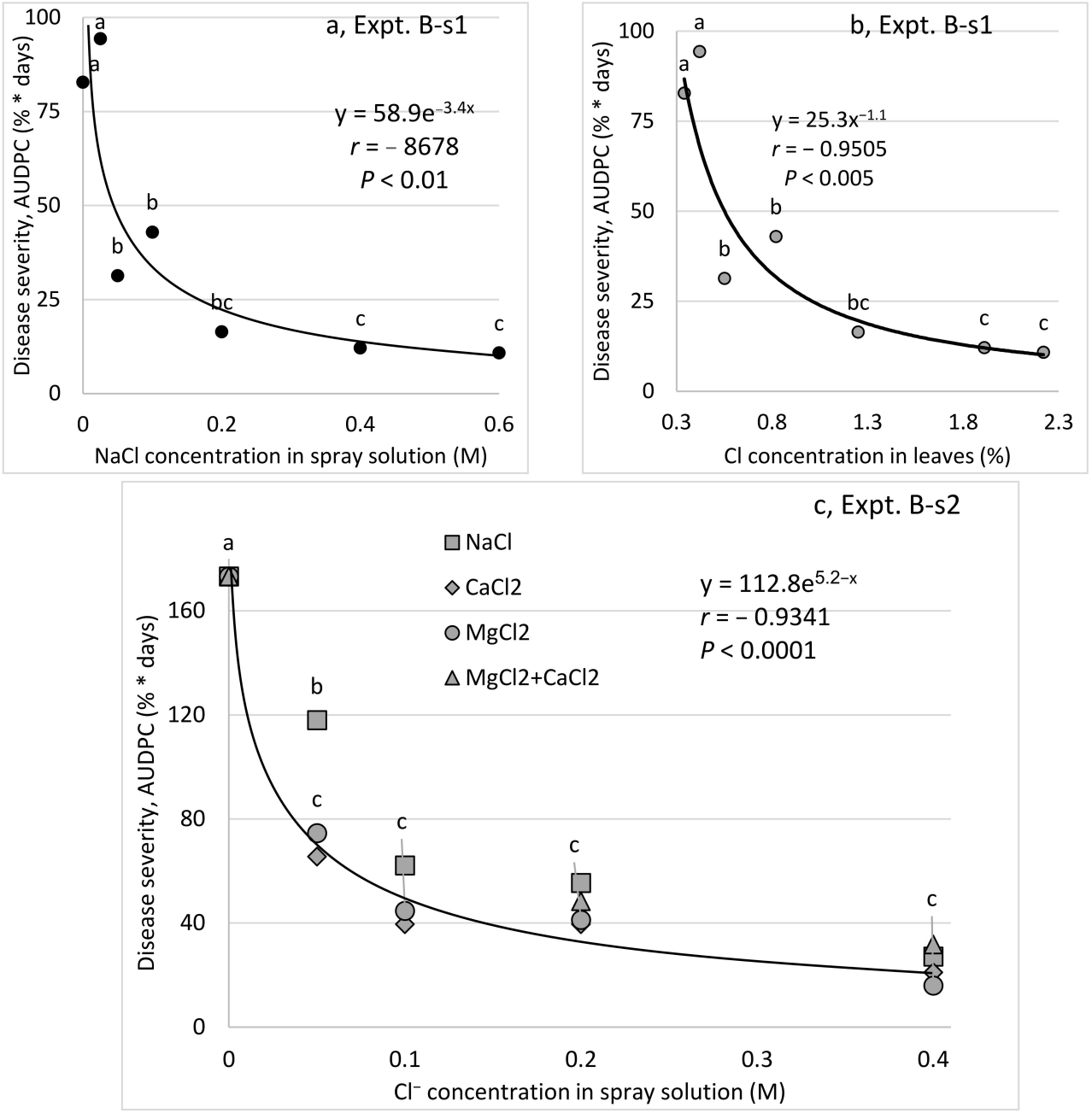

Figure 2. Effects of spray applications of chloride salts of Mg, Ca, and Na on the severity of cucumber powdery mildew (CPM, *Podosphaera xanthii*) on leaves of cucumber plants. Salts were applied to mature plants twice a week. The molar concentrations related to the anion (Cl^-) are presented. (**a**) Effects of NaCl (applied at concentrations of 0 to 0.6 M in Expt. B-s1) on CPM. (**b**) Effects of the subsequent leaf concentrations of Cl^{-1} on the severity of CPM. (**c**) Effects of $MgCl_2$, $CaCl_2$, and NaCl (applied at concentrations up to 0.4 M Cl^- in Expt. B-s2) on CPM severity. CPM severity was evaluated on a 0 to 100 scale, in which 0 = no disease symptoms and 100 = leaf fully covered by symptoms. The area under the disease progress curve (AUDPC) was calculated for 12 days from disease onset. Values in each concentration followed by a common letter are significantly not different from each other according to Tukey-Kramer's HSD test ($p \leq 0.05$).

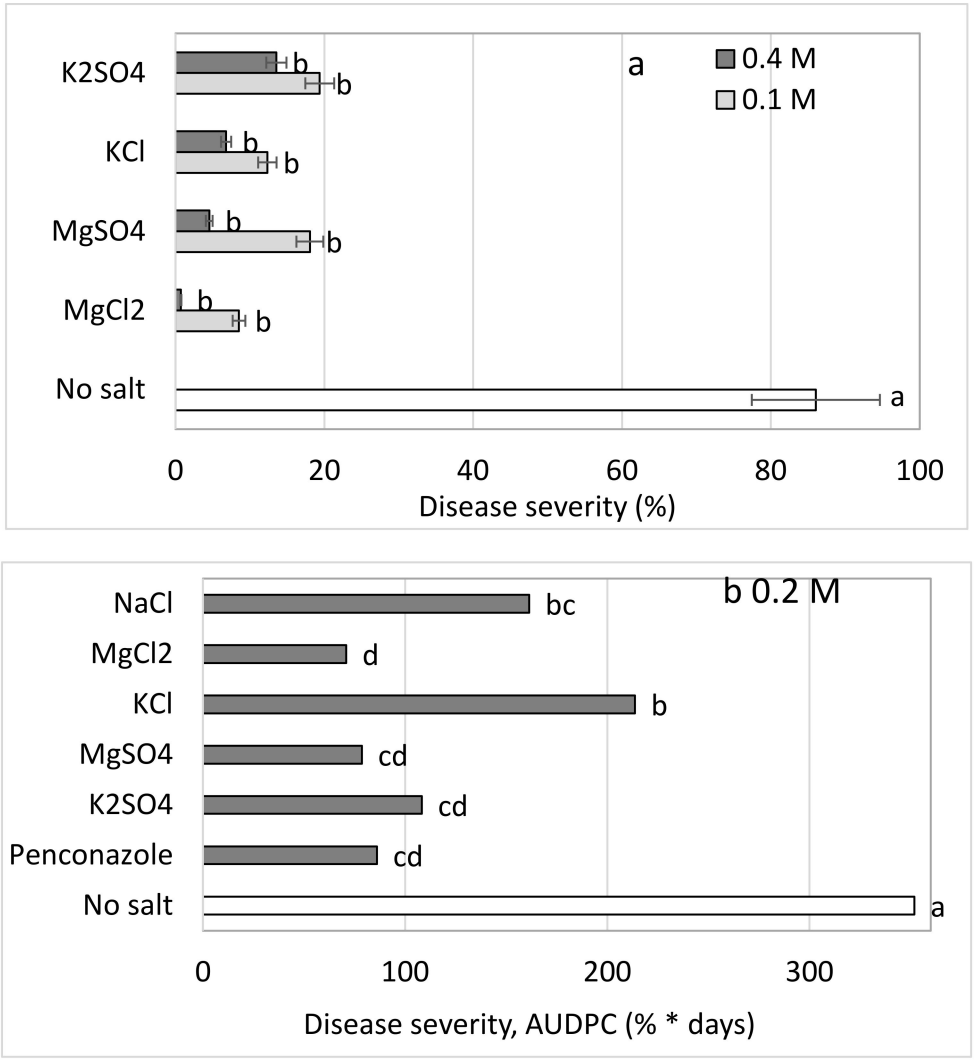

Figure 3. (**a**) Effects on cucumber powdery mildew (CPM, *Podosphaera xanthii*) severity of water (empty, lower column), $MgCl_2$, $MgSO_4$, KCl, and K_2SO_4 sprayed twice a week at concentrations of 0.1 and 0.4 M in Expt. B-s3 and (**b**) a comparison of these salts sprayed at 0.2 M with the chemical fungicide penconazole (0.035% of 200 g/L Ofir, 2000, once a week) in Expt. B-s4. CPM severity was evaluated on a 0 to 100 scale, in which 0 = no disease symptoms and 100 = leaf fully covered by symptoms. The area under the disease progress curve (AUDPC) was calculated. Values for each salt concentration followed by a common letter are significantly not different from each other according to two-way ANOVA with Tukey's HSD ($p < 0.0009$); the analysis was identical for each set at both concentrations (**a**,**b**). The difference between the two salt concentrations (0.1 vs. 0.4 M, **a**,**b**) was significant ($p < 0.0009$). Bars = SE.

2.5. Relationship between CPM Severity and Ca and Mg Supplied through the Fertigation Solution (Expts. B-Ca/Mg-f)

Ca (Expts B-Ca-f): Raising the concentration of Ca in the fertigation solution from 1.0 to 4.0 mM resulted in a gradual increase in the Ca concentration in the cucumber leaves, up to 4.29% of the leaf dry weight (Figure 7). Ca levels in the irrigation solution and leaves did not significantly affect CPM severity (data not shown).

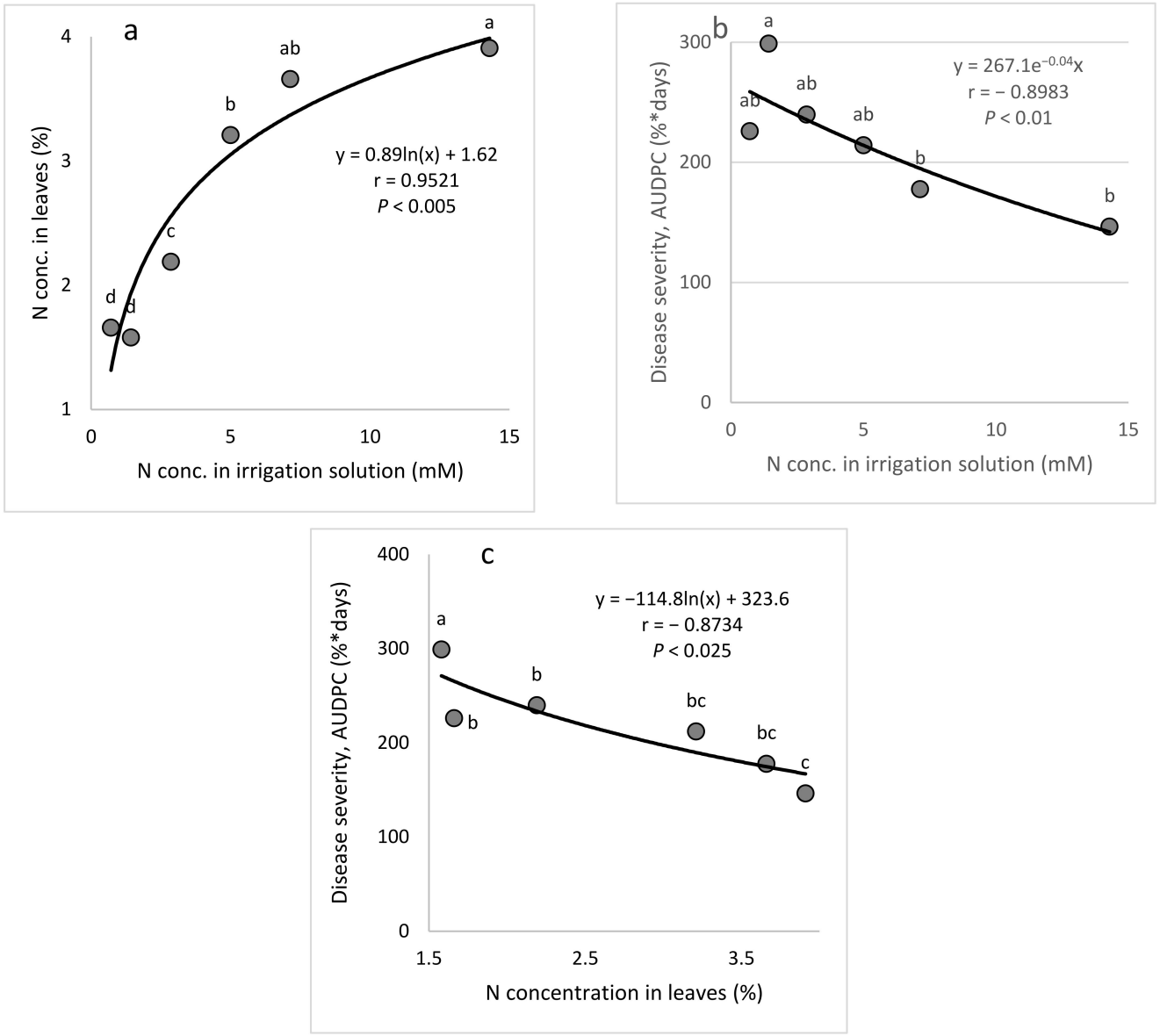

Figure 4. Effect of the N concentration in the irrigation solution on cucumber powdery mildew (CPM, *Podosphaera xanthii*, Expts. B-N-f). (**a**) The N concentration in the leaves relative to the N concentration in the irrigation solution. (**b**) CPM severity relative to the N concentration in the irrigation solution. (**c**) CPM severity relative to the N concentration in the leaves. Disease severity was evaluated on a 0 to 100 scale, in which 0 = healthy leaves and 100 = leaves completely covered by disease symptoms. The area under the disease progress curve (AUDPC) was calculated for 18 days from disease onset. In each graph, values for each N treatment followed by a common letter are significantly not different from each other according to Tukey-Kramer's HSD test ($p \leq 0.05$). The regression formulas are presented and the Pearson regression (r) values are presented along with significance levels (p).

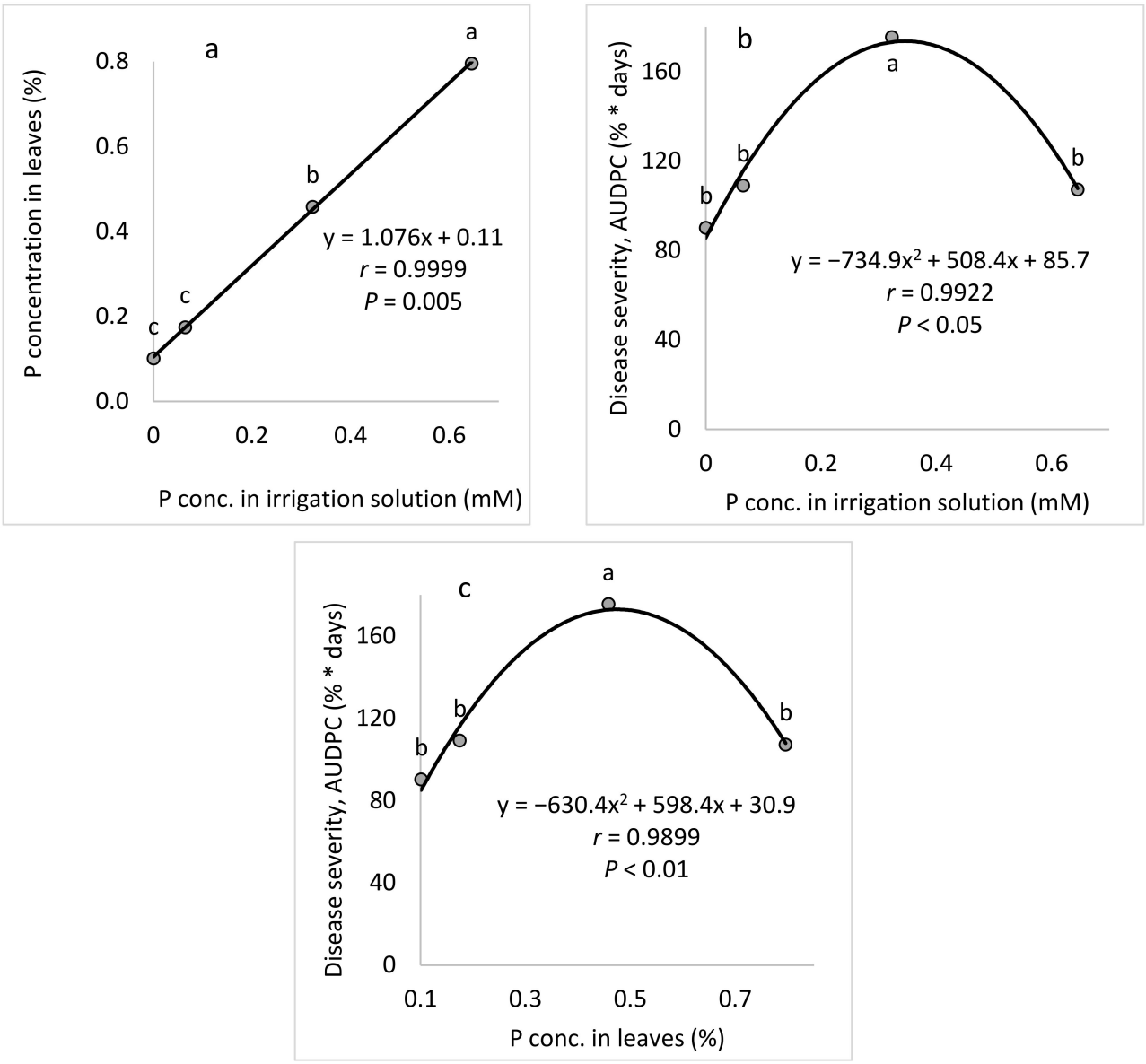

Figure 5. Effects of different concentrations of P in the irrigation solution on cucumber powdery mildew (CPM, *Podosphaera xanthii*, Expts. B-P-f). (**a**) P concentration in the leaves relative to the P concentration in the irrigation solution. (**b**) CPM severity relative to the P concentration in the irrigation solution and (**c**) CPM severity relative to the P concentration in the leaves. Disease severity was evaluated on a 0 to 100 scale, in which 0 = healthy leaves and 100 = leaves completely covered by disease symptoms. The area under disease progress curve (AUDPC) was calculated for 18 days from disease onset. In each graph, values for each P treatment followed by a common letter are significantly not different from each other according to Tukey-Kramer's HSD test ($p \leq 0.05$). The regression formulas are presented and the Pearson regression (r) values are presented along with significance levels (p).

Mg *(Expts B-Mg-f)*: Raising the concentration of Mg in the fertigation solution from 0.82 to 4.94 mM resulted in a gradual increase in the Mg concentration in the cucumber leaves, up to 1.94% of the leaf dry weight (Figure 8a). Increasing the Mg concentration in the irrigation solution led to a gradual decrease in CPM severity (Figure 8b). Similarly, the change in Mg leaf concentration resulted in a gradual decrease in disease severity (Figure 8c).

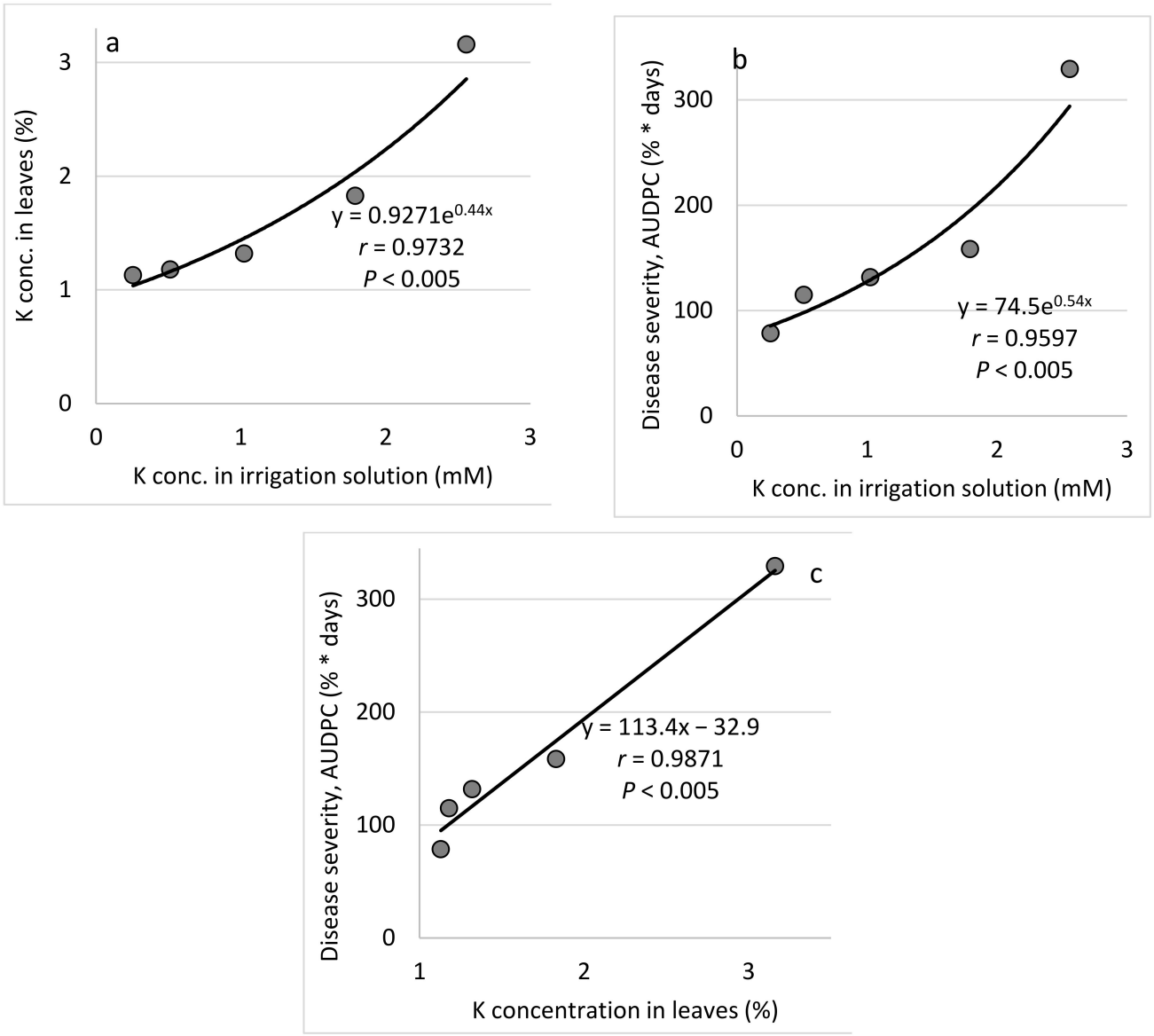

Figure 6. Effect of the concentration of K in the irrigation solution on cucumber powdery mildew (CPM, *Podosphaera xanthii*, Expts. B-K-f). (**a**) K concentration in the leaves relative to the K concentration in the irrigation solution. (**b**) CPM severity relative to the K concentration in the irrigation solution and (**c**) CPM severity relative to the K concentration in the leaves. Disease severity was evaluated using a 0 to 100 scale, in which 0 = healthy leaves and 100 = leaves completely covered by disease symptoms. The area under disease progress curve (AUDPC) was calculated for 18 days from disease onset. In each graph, values for each K treatment followed by a common letter are significantly not different from each other according to Tukey-Kramer's HSD test ($p \leq 0.05$).

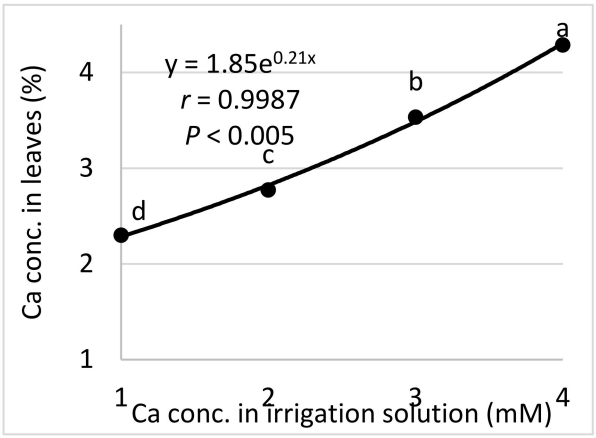

Figure 7. The Ca concentration in the leaves relative to the Ca concentration in the irrigation water (Expts. B-Ca-f). The regression formula is presented and the Pearson regression (r) value is presented along with the significance level (p); values for each Ca treatment followed by a common letter are significantly not different from each other according to Tukey-Kramer's HSD test ($p \leq 0.05$).

2.6. Effects of Spray Applications of Various Salts on CPM under Semi-Commercial Conditions (Experiments B-SCs-a/b)

The semi-commercial experiments involved mature, fruit-bearing plants and included: (a) sprays of MKP and polyhalite (Expt. B-SCs-a, Table 1), which are commercially available as complex fertilizers and contain K or Mg, Ca, and S, respectively; and (b) $MgCl_2$, $CaCl_2$, and K_2SO_4 at 0.05 and 0.1 M (Cl^- or $SO_4{}^{-2}$-related) and a combination of 0.05 M of these salts and 0.1 M $K_2(SO_4)$ (Expt. B-SCs-b, Table 1). CPM severity was significantly reduced by MKP and polyhalite at the three evaluation times (Figure 9a) and in terms of the AUDPC (Figure 9b). The salts generally reduced CPM severity at the three evaluation times and the calculated AUDPC. The calculated AUDPC revealed better disease suppression by the combination of the salts as compared with applications of the same concentrations of the individual salts (Figure 10a,b).

2.7. Commercial-Like (CL) Net-House Experiments to Test the Effects of Fertigation and Spray Applications

N, P, and K fertigation was tested under commercial-like conditions (Expt. CL1) with mature plants over a whole growing season (Table 1). A somewhat lower CPM severity was observed among the plants that were fertigated with low N (2.9 vs. 7.1 and 14.3 M N, Figure 11a1) and high P (0.65 vs. 1.29 M P, Figure 11a2). A lower CPM severity was observed among plants treated with the low concentration of K (1.0 vs. 2.6 and 5.1 M K, Figure 11a3). In the same experiment, the fungicide and the mixture of $MgCl_2$ and K_2SO_4 provided significant CPM control (57.9 and 75.6%, respectively; Figure 11).

Except for the low-N treatment, the respective cumulative number of fruits and the weight of the yield per plot in the various fertigation treatments were similar and ranged between 10.8 and 13.2 fruits/plant and between 1.226 and 1.356 kg/plant, respectively. In contrast, the low-N treatment yielded significantly less than the rest of the fertigation treatments, with a cumulative 8.8 fruits/plot and 0.908 kg/plant. The three spray treatments in Expt. CL1 had cumulative yields of 11.0–11.8 fruits/plant and 1.115–1.235 kg/plant, with no significant differences between them.

Spray treatments were also applied in the second commercial-like experiment (Expt. CL2). CPM severity was significantly suppressed by MKP and the fungicide triadimenol: 75.6 and 61.8%, respectively. $MgCl_2$ provided better CPM control (97.6% disease reduction). The control provided by the mixed-salts treatment (73.2% disease reduction) was no better than that provided by each salt alone (Figure 12). The various treatments yielded 12.1–14.3 fruits/plant and 1.2–1.6 kg fruits/plant, with no significant differences between the treatments.

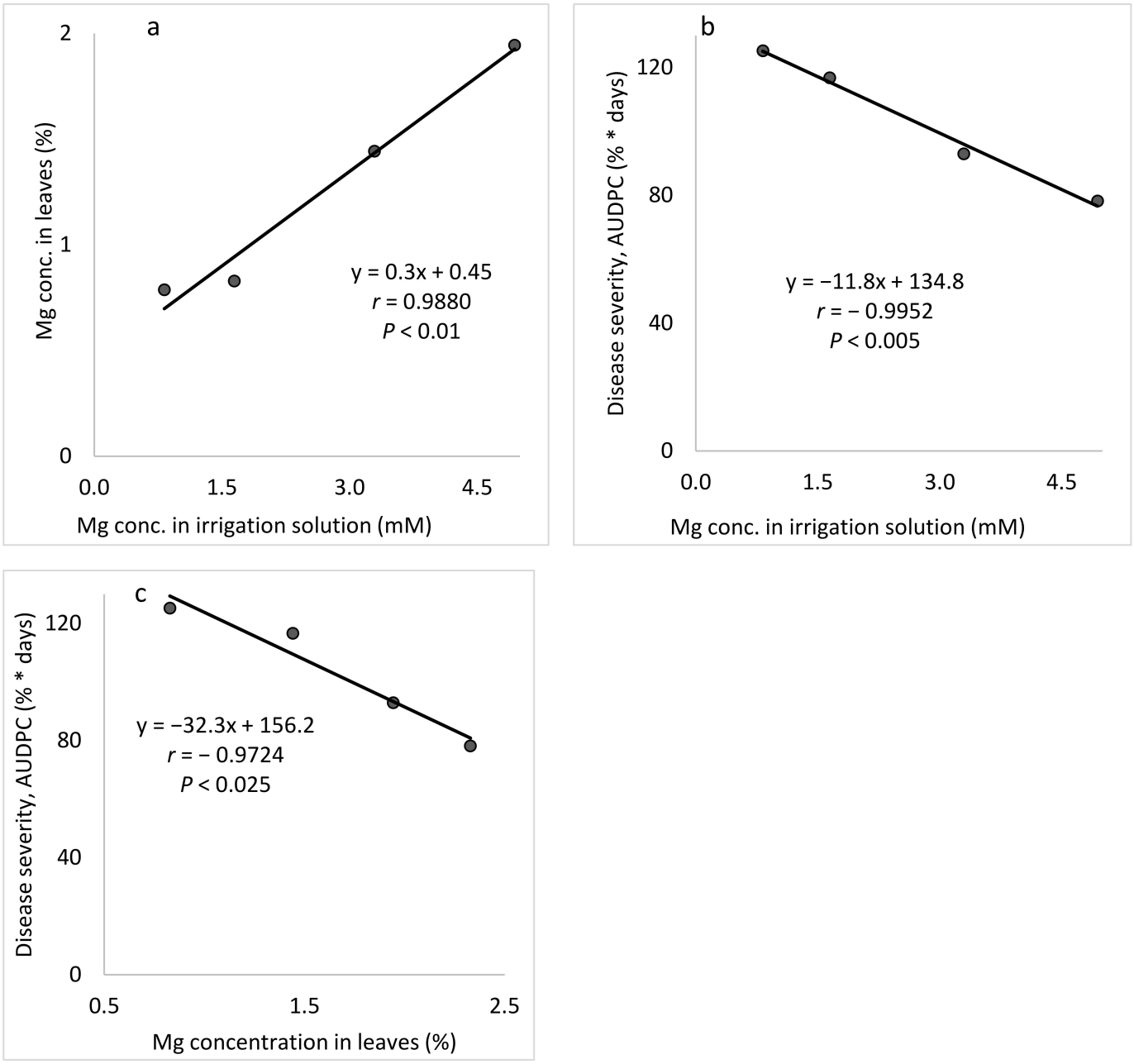

Figure 8. Effect of the concentration of Mg in the irrigation solution on cucumber powdery mildew (CPM, *Podosphaera xanthii*, Expts. B-Mg-f). (**a**) Mg concentration in the leaves relative to the Mg concentration in the irrigation solution. (**b**) CPM severity relative to the Mg concentration in the irrigation solution and (**c**) CPM severity relative to the Mg concentration in the leaves. Disease severity was evaluated on a 0 to 100 scale, in which 0 = healthy leaves and 100 = leaves completely covered by disease symptoms. The area under the disease progress curve (AUDPC) was calculated for 18 days from disease onset. In each graph, values for each Mg treatment followed by a common letter are significantly not different from each other according to Tukey-Kramer's HSD test ($p \leq 0.05$). The regression formulas are presented and the Pearson regression (r) values are presented along with significance levels (p).

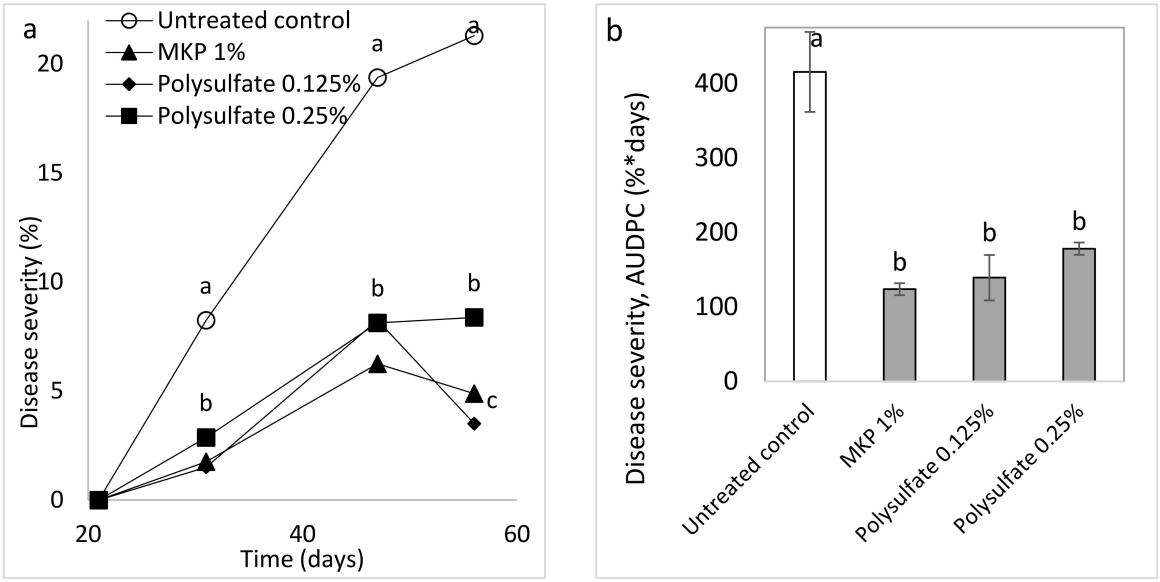

Figure 9. Effect of weekly sprays with polyhalite (polysulfate) and monopotassium phosphate (MKP) on cucumber powdery mildew (CPM, *Podosphaera xanthii*) (Expt. A-SCs-a). (**a**) CPM severity was evaluated on a 0 to 100 scale, in which 0 = no disease symptoms and 100 = leaf fully covered by symptoms. (**b**) The area under disease progress curve (AUDPC) through Day 57. Values for each date and in each graph followed by a common letter are significantly not different from each other according to one-way ANOVA with Tukey's HSD ($p \leq 0.05$). Bars = SE.

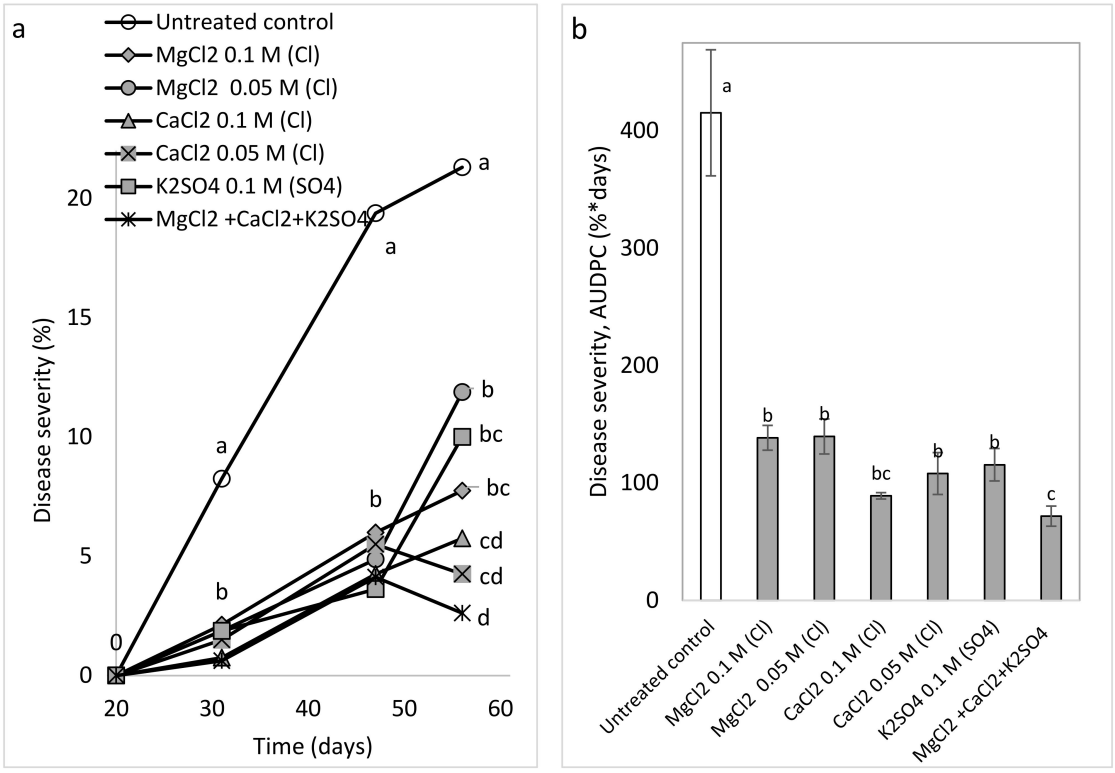

Figure 10. Effect of weekly sprays of various salts at two concentrations, individually and in combination ($MgCl_2$ 0.05 M (Cl), $CaCl_2$ 0.05 M (Cl), and K_2SO_4 0.1 M (SO_4)), on cucumber powdery mildew (CPM, *Podosphaera xanthii*) (Expt. A-SCs-b). (**a**) CPM severity was evaluated on a 0 to 100 scale, in which 0 = no disease symptoms and 100 = leaf fully covered by symptoms. (**b**) The area under disease progress curve (AUDPC) was calculated through Day 57. Values for each date and in each graph followed by a common letter are significantly not different from each other according to one-way ANOVA with Tukey's HSD ($p \leq 0.05$). Bars = SE.

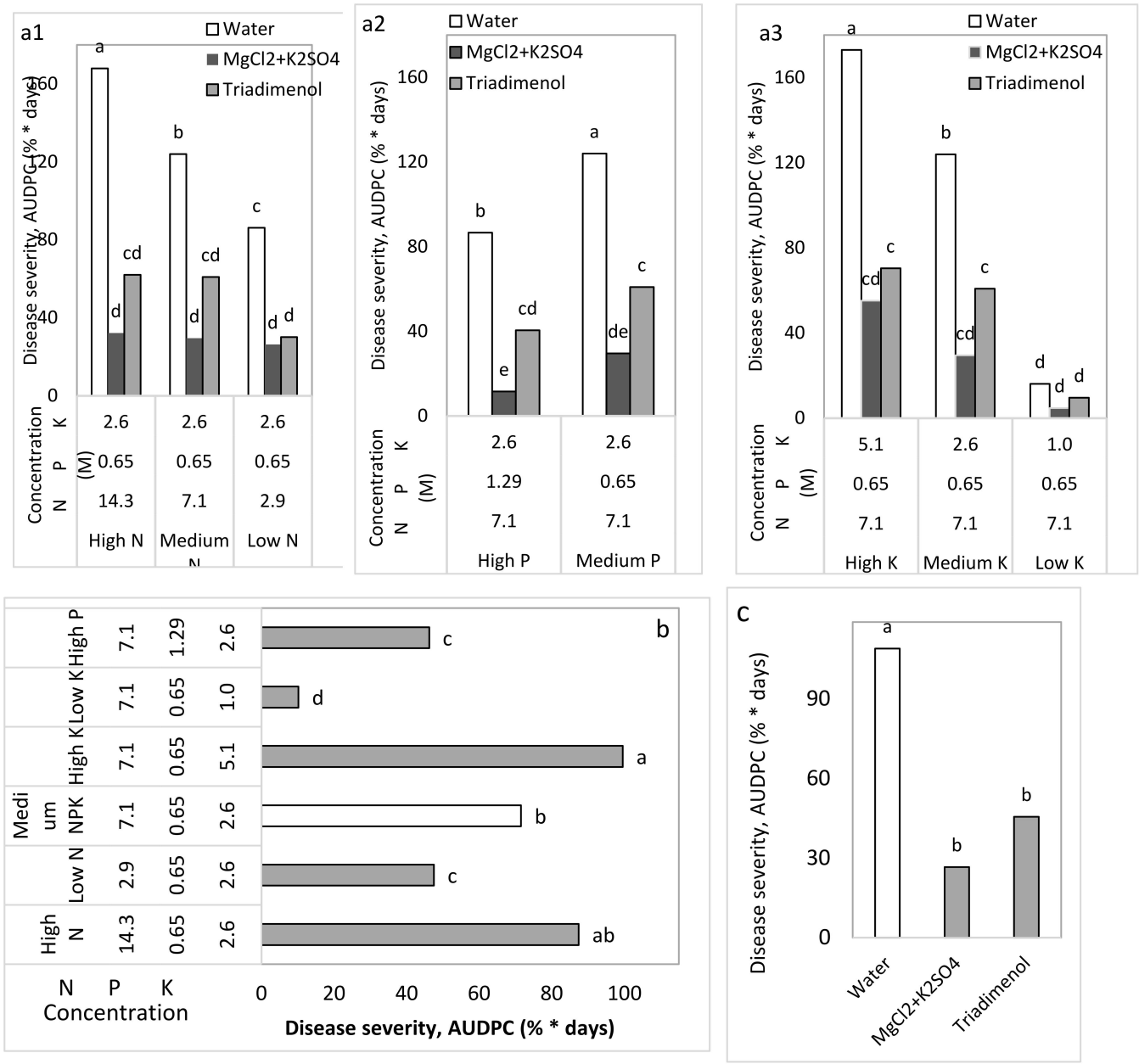

Figure 11. Effects of N, P, and K levels in the irrigation solution and spray applications, every 3 to 5 days, with $MgCl_2$+K_2SO_4 0.1 M (Cl^-) + 0.1 M ($SO_4{}^-$) and the fungicide triadimenol on cucumber powdery mildew (CPM, *Podosphaera xanthii*) under commercial-like conditions (Expt. CL1). The effects of three N concentrations (**a1**), two P concentrations (**a2**), and three K concentrations (**a3**) in the irrigation solution are presented alongside the three spray regimes (**a1**–**a3**); the medium N, P, and K concentrations are the same in all of the irrigation treatments. CPM severity was evaluated on a 0–100 scale, in which 0 = no disease symptoms and 100 = leaf fully covered by symptoms. The area under the disease progress curve (AUDPC) was calculated through Day 57. Disease severity is described for (**a**) all single combination treatments (irrigation × spray treatments) and for the major treatments either for the (**b**) NPK fertilization levels or (**c**) the spray treatments across irrigation treatments. Values in each graph followed by a common letter are significantly not different from each other according to two-way ANOVA with Tukey's HSD ($p \leq 0.05$).

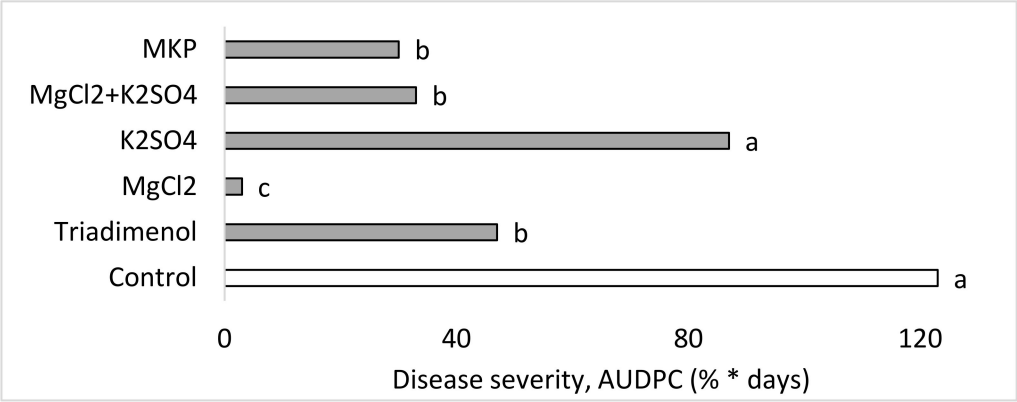

Figure 12. Effects of spray applications of $MgCl_2$ 0.1 M (Cl^-), K_2SO_4 0.1 M (SO_4^-),$MgCl_2$+K_2SO_4, monopotassium phosphate (MKP), and the fungicide triadimenol on cucumber powdery mildew (CPM, *Podosphaera xanthii*) under commercial-like conditions (Ept. CL2). CPM severity was evaluated on a 0 to 100% scale, in which 0 = no disease symptoms and 100 = leaf fully covered by symptoms. The area under the disease progress curve (AUDPC) was calculated through Day 57. Values in each graph followed by a common letter are significantly not different from each other according to one way ANOVA with Tukey's HSD.

3. Discussion

A great deal of information is available in the literature on the effects of mineral nutrition on the development of plant diseases (e.g., [43]). It is widely recognized that nutrition can influence disease, but much of the available information is contradictory, with both disease suppression and disease promotion described [43,44]. Effects of individual ions and combinations of ions on plant diseases have been demonstrated. In soybean (*Glycine max*), K, Ca, Mg, S, and Fe all induce resistance to *Fusarium oxysporum* infection [45]. As mentioned above, we observed an effect on disease by Ca, Mg, and K treatments [18], by Mn and Zn treatments [46], and by N in the irrigation solution [27]. In the present study, we tested the effects of N, P, K, Ca, and Mg ions applied as part of the irrigation solution and Ca, Mg, and K salts applied in spray solutions.

3.1. N Involvement

There is a general notion that high levels of N will increase the severity of diseases that are caused by obligate parasites [7]. In this study, increasing the N concentration in the irrigation water somewhat decreased the severity of CPM in younger plants that had no fruit and increased the disease severity in mature, fruit-bearing plants that were grown under commercial-like conditions. Similar to our results for young cucumber, a previous study found that N (300 mg/L) applied with an irrigation solution to potted plants under greenhouse conditions reduced the severity of downy mildew of cucumber (*Pseudoperonospora cubensis*) by 24% [47]. Similar to our results with mature plants, higher N concentrations in the irrigation water were previously shown to lead to more severe downy mildew in sweet basil under commercial-like conditions [27]. Wang et al. [40] reported that nitrate significantly suppressed Fusarium wilt caused by *F. oxysporum* f. sp. *cucumerinum* (FOC), as compared with ammonium, in both pot and hydroponic experiments. The researchers found that nitrate protects cucumber plants against *F. oxysporum* by decreasing root citrate exudation and FOC infection, suggesting a role of N in disease suppression in cucumber.

Previous studies have found that P affects disease levels differently in different pathosystems. Mustafa et al. [21] found that P inhibits wheat powdery mildew (*Blumeria graminis*), whereas Develash and Sugha [22] found that P increases the severity of onion downy mildew (*Peronospora destructor*). Although we have researched the effects of plant nutrients in various pathosystems, the present study is the first to study the effect of P on a crop disease. We found that P increased the severity of CPM among both young plants and mature, fruit-bearing plants.

3.2. K Involvement

In the present study, K was applied as part of the irrigation solution and also as salts in a spray solution. Increasing the K concentration in the irrigation water increased CPM in young plants, but decreased disease severity in older plants and when it was applied as sprayed salts. The different effects of K observed in the present study suggest that K might also have different effects in the pathosystems that have been examined, but further investigation into the age effect on the response to K is needed. Sweet basil downy mildew, which is caused by an obligate parasite (*P. belbahrii*), was reduced by spray-applied K, but not by K that was applied as part of the irrigation solution [18]. In earlier studies, the necrotrophs *Botrytis cinerea* and *Sclerotinia sclerotiorum* were suppressed by K applied as part of the irrigation water or as part of a spray solution [16,17].

The application of K_2SO_4 reduced the downy mildew (*Plasmopara viticola*) incidence on grapevine (*Vitis vinifera*) leaves. An increased K concentration in grapevine petioles increases the constitutive and post-inflectional accumulation of phenolic acids, such as o-coumaric acid and p-coumaric acid, and of total phenols [48]. K was also associated with increased leaf activity of the phenylalanine ammonia-lyase and increased disease resistance [48]. The addition of K to a low-nitrate fertilizer reduced cucumber fruit gray mold (*B. cinerea*) and stem infections [49]. In addition, foliar-applied KNO_3 reduced the incidence and severity of cotton Alternaria leaf blight (*Alternaria macrospora* and *A. alternata*) [49].

Application of 4–30 mM KNO_3 before inoculation greatly reduced the incidence of stem rot (*Phytophthora sojae*) in soybean. The extent of that disease reduction was related to the increased K levels in the plants, particularly in plants' cortex layer [50]. Dipotassium hydrogen phosphate reduced downy mildew (*S. graminicola*) of pearl millet (*Cenchrus americanus* syn. *Pennisetum glaucum*) under experimental and commercial-greenhouse conditions. However, unlike our findings, the disease suppression in that system was related to the phosphate component [51]. Contrasting results have been reported for KCl fertilization, which reduced the severity of wheat leaf rust (*Puccinia triticina*); however, that response may have been partially related to the chloride in the KCl fertilizer [23].

Potassium enrichment in cucumber reduced the natural incidence of downy mildew (*Pseudoperonospora cubensis*) [39]. A reduction in cucumber powdery mildew was achieved through the application of potassium dihydrogen phosphate, magnesium sulfate, ferrous sulfate, and potassium monohydrogen phosphate [41]. Effective control of cucumber powdery mildew, as expressed by a 99% reduction in symptoms, was achieved one or two days post application of a single spray of phosphate and K salt solutions and those treatments were also effective at a later stage [37,38]. The authors of those works concluded that these properties of potassium and phosphate salts make them appropriate for use as foliar fertilizers with potential beneficial effects on disease control.

3.3. P Involvement

MKP has been reported to be beneficial for the control of powdery mildews. MKP sprayed alone, mixed with, or in alternation with fungicides suppressed powdery mildews of apple, peach, and nectarine (*Podosphaera leucotricha* in *Prunus persica* var. *nucipersica*), grapevine (*Uncinula necator*), rose (*Sphaerotheca pannosa* in *Rosa* × *hybrida*), cucumber and melon (the fungus named in that research was *S. fuliginea*; the plants were *Cucumis sativus* and *C. melo*, respectively), and mango (*Oidium mangifera* in *Mangifera indica*) [42]. In addition, nutrient solutions containing P at concentrations of 5 to 40 ppm in a hydroponic system induced systemic resistance against *S. fuliginea* in young cucumber plants. Once the pathogen had been established, root-applied P did not affect well-developed colonies. However, foliar application of a 1% solution of MKP effectively protected the foliage against powdery mildew, regardless of the P concentration in the water [24]. In the present work, we describe the suppression of CPM by MKP under semi-commercial and commercial-like conditions.

3.4. Ca and Mg Involvement

Adding Ca to the irrigation solution reduced gray mold on fruits and stems of cucumber plants. Ca also reduces gray mold in pepper (*Capsicum annum*) and eggplant (*Solanum melongena*) [49]. Seed treatment with 90 mM $CaCl_2$ suppressed pearl millet downy mildew (*Sclerospora graminicola*) and reduced the biomass of the pathogen in the treated plants [33]. Chardonnet and Donèche [52] suggested that Ca treatment of cucumber fruit prior to infection can increase the cell-wall-bound Ca and thereby

decrease pectin digestion by fungal pectinolytic enzymes and fruit infection by the necrotrophic fungus *Botrytis cinerea*. Protection against cucumber powdery mildew in a greenhouse was obtained with the application of calcium nitrate and potassium phosphate in doses of 20 g/L and a 6 mL/L spray [53]. In the present work, we were able to increase the Ca concentration in the cucumber plants, but this did not affect the level of CPM.

Tomato (*Solanum lycopersicum*) root and crown rot (F. *oxysporum* f. sp. *radicis-lycopersici*) was reduced by $Ca(NO_3)_2$ but not by $MgSO_4$; the disease reduction was related to the level of nitrate [54]. Mg and Ca suppressed sweet basil downy mildew in sweet basil [18]. In the present study, both Mg in the irrigation solution and Mg applied as a spray suppressed CPM.

Since the combination of salts gave only a slight improvement in CPM control over the control that was observed with the single treatments, we cannot at all generalize that a combination of ions or salts can provide any major improvement in disease control. In a recent study involving sweet basil downy mildew, Ca and Mg did not have an additive suppression effect on sweet basil downy mildew [18]. It has been suggested that the deleterious effect of Ca on Mg load in the canopy was the reason that the combination of supplemental Mg and supplemental Ca did not improve disease control when both elements were applied together. There is no evidence in the literature for any such effect of this cation combination on plant disease. Furthermore, the observed suppression of the downy mildew by Ca and Mg treatments suggests a general mode of action that is triggered by either of those cations (i.e., induced resistance).

Nevertheless, the mixture of ions in a spray solution was also tested using polyhalite ($K_2Ca_2Mg(SO_4)_4{\cdot}2(H_2O)$). Polyhalite effectively suppressed CPM severity. This seems to be the first report of any disease control provided by this fertilizer.

3.5. Role of Anions

Interestingly, the suppressive effects of spray-applied K, Ca, and Mg salts on CPM of young cucumber plants were correlated with the anion concentration and not with the cation concentration. The choice of anion to be used with the cations that were used was a question that was raised during the present research. Cl^- was more effective than $SO_4{}^{-2}$ in two sets of experiments (Expts. A-s1 and A-s3), and repeated experiments revealed a relationship between the Cl^- concentration and the efficacy of disease control (i.e., at concentrations greater than 0.05 and 0.1 M, no improvement in CPM control was observed). Nevertheless, at the 0.05 M concentration, NaCl was significantly less effective than a 0.05 M chloride concentration of Mg and Ca salts. Under commercial-like conditions, fertigation-applied $MgCl_2$ suppressed sweet basil downy mildew better than fertigation-applied $MgSO_4$. Cl^- was also the anion of choice in other greenhouse experiments [18].

There have been a few other reports on the effects of these anions on plant diseases. The effects of Cl fertilizer (e.g., KCl) on winter wheat were investigated in field trials, which revealed that the applied Cl suppressed the foliar diseases powdery mildew (*Erysiphe graminis*) and leaf rust (*Puccinia recondite*) [55]. Not much information is available about the effects of the anion on plant diseases. The application of KCl at 150 to 400 μg of K per gram of dry soil increased the incidence of Phytophthora root and stem rot (*Phytophthora sojae*) on susceptible soybean plants. That increased disease appears to be due to the presence of chloride [56].

4. Materials and Methods

4.1. Plants and Experiments

Cucumber (*Cucumis sativus*) cv. Bet Alpha seeds were planted in seedling trays containing 4 × 4 × 10 cm invert-pyramidal cells filled with perlite. The perlite (medium size, 1.2 mm) was washed in water prior to use (Agrifusia, Fertilizers & Chemicals Ltd., Haifa, Israel) for potted-plant experiments. The developed seedlings were transplanted to pots at 3 weeks of age. Seeds were also germinated directly in pots, as described below. To prevent damping-off in the pots harboring germinating seeds, we treated the growth medium once with a fungicide (0.25% Dynon in water drench, containing 722 g/L Propamocarb HCl, Bayer, Germany). Cucumber Cv. 501 was used in the commercial-like (CL) experiments, as described below.

The experiments involving plants grown in pots were performed at two sites in Israel: the Volcani Institute in Rishon LeZion (31°58′09″ N 34°48′02″ E) (Site A) and the Gilat Research Center in the

northern Negev region (31°23′12″ N 34°43′15″ E) (Site B), 68 km south of Site A. Experiments were also carried out using plants grown in containers under commercial greenhouse conditions in a net-house at the Gilat Research Center (Site C). At Site A, experiments were carried out at an experimental greenhouse, in 2-L pots and 10-L pots (potted and semi-commercial conditions, respectively). At Site B, experiments were carried out in 2-L pots. The commercial-like experiments (Site C) were carried out in 136-L containers. For the fertigation experiments, cucumber plants were planted in pots or containers filled with perlite.

A potting mixture consisting of coconut fiber:tuff (unsorted to 8 mm; 7:3 vol.:vol.) was used in the experiments that involved foliar salt treatments. The plants were irrigated to excess via a drip system two to four times a day, depending on the season, at a volume calibrated to lead to >30% water leaching. The daily irrigation volume was determined after analyzing the irrigation and drainage solutions once every 2 weeks to prevent over-salinization or acidification of the root-zone solution. Plants in pots and containers were maintained according to the local extension service's recommendations. All pot experiments were irrigated with fresh water (electrical conductivity (EC): 0.4 dS/m = desalinated water). The tested elements were applied with the irrigation solution (fertigation) or as a foliar spray with no added surfactants, as described below and as summarized in Table 1.

4.2. Pathogen and Disease

Cucurbit powdery mildew (*Podosphaera xanthii*, CPM) occurred naturally and the cucumber cultivar used is known to be susceptible to CPM. Nevertheless, to ensure even distribution of the pathogen across the plants, every potted-plant experiment was artificially inoculated with a conidial suspension (10^4 conidia/mL) using a hand sprayer that produced a fine mist that dried within 10 min. The pathogen formed the typical white symptoms of CPM on leaves and disease severity was evaluated according to the symptoms coverage area of the leaves, on a 0 to 100 scale, in which 0 = healthy leaves and 100 = leaves completely covered by disease signs/symptoms. The evaluated leaves were as follows: Leaf 5 from the plant base as a mature leaf; Leaves 6–9 as medium leaves; and Leaves 9–12 as younger leaves. For whole-plant CPM severity, the average ratings for the evaluated leaves on each plant were calculated. The area under the disease progress curve (AUDPC) was calculated throughout the period of epidemic development.

4.3. Foliar Application of Salt Solutions to Potted Cucumber Plants (Experiments A-s 1 and B-s 1–4)

Cucumber seedlings were transplanted into pots containing perlite or growth mixture, as described above, unless noted otherwise. Fertigation was carried out with the fertilizer 4-2-6 (N-P_2O_5-K_2O) + 3% microelements (Fertilizers and Chemical Compounds Ltd., Haifa, Israel) throughout the experiments. Spray with water with no salt served as an untreated control. In young cucumber plants, following the formation of two or three leaves, when the plants had reached a height of 20 to 30 cm (Expt. B-s) and in mature plants bearing at least 18 leaves (Expt. A-s), the foliar treatments were initiated and plants were artificially inoculated with conidia of *Podosphaera xanthii*. Experiments A-s 1 and Experiments B-s 1 through 4 were each repeated twice, with five replicates each time, and arranged in randomized blocks.

Sprays were conducted twice a week with a hand sprayer that formed a mist of small drops and contained up to 1 L of water. Solutions contained 0.1 and 1.0% of K_2SO_4, KCl, $MgCl_2$, and $CaCl_2$ with various molar concentrations of the applied ions (Expt. A-s1): NaCl at concentrations of 0, 0.05, 0.1, 0.2 0.4, and 0.6 M (Cl^-) (Expt. B-s1); $MgCl_2$, $CaCl_2$, NaCl, and $MgCl_2$ with $CaCl_2$ at concentrations of 0, 0.05, 0.1, 0.2, and 0.4 M (Expt. B-s2); $MgSO_4$, $MgCl_2$, K_2SO_4, and KCl at anion concentrations of 0.1 and 0.4 M (Expt. B-s3); $MgSO_4$, $MgCl_2$, K_2SO_4, and KCl at anion concentrations of 0.2 M; and the fungicide penconazole (200 g/L as Ofir2000, Syngenta, Switzerland) sprayed with a 0.035% formulation once a week (Expt. B-s4).

4.4. Effects of Different Concentrations of N, P, K, Ca, and Mg in the Fertigation Solution on CPM (Experiments B-#-f)

Pot experiments were conducted in an unheated, polyethylene-covered greenhouse located at Site B. The aim of these experiments was to study the effects of different N, P, K, Ca, and Mg concentrations in the fertigation solution ("f" treatments) on the development of CPM in potted cucumber plants. The cucumber plants were planted in 2-L, perlite-filled pots with one plant per pot, in 10 replicates. Each

set of cation concentrations was repeated twice, and pots were arranged randomly. The plants did not bear any fruit.

Nutrient solutions were prepared in 500-L containers containing all of the added nutrients. All of the plants were fertigated with 5-3-8 (N-P_2O_5-K_2O) fertilizer (Fertilizers and Chemical Compounds Ltd., Haifa, Israel) for 2 weeks, until plant establishment. Later on, the effect of cation concentration was tested by tailoring the fertigation solution to each N, P, K, Ca, and Mg concentration of interest, as described below. The concentrations of nutrients that were not part of the experiments and remained the same across all treatments were as follows: 5.7 mM N (90% NO_3^--N and 10% NH_4^+-N, excluding Experiment B1-N below), 0.352 mM P (excluding Experiment B1-P below), 2.6 mM K (excluding Experiment B1-K below), 1.3 mM Ca (excluding Experiment B1-Ca below), 0.54 mM Mg (excluding Experiment B1-Mg below), 1.1 mM SO_4^{-2}, 0.023 mM B, 9.8 μM Fe, 4.9 μM Mn, 2.1 μM Zn, 0.31 μM Cu, and 0.16 μM Mo. Solutions were prepared by dissolving KH_2PO_4, K_2SO_4, KNO_3, $NH_4H_2PO_4$, $NaNO_3$, and NH_4NO_3 in water [17,18,27]. In Experiment B1-Ca-f, Ca was applied as $CaCl_2$ and, in Experiment B1-Mg-f, Mg was applied as $MgCl_2$. The EC in the different B-N/P/K-f treatments was 1.03–1.36 dS/m and the pH of the fertigation solution was 6.99–7.42. The EC in the different B-Ca/Mg-f treatments was 0.99–2.50 dS/m and the pH of the fertigation solution was 6.43–7.12.

4.4.1. N Concentration in the Fertigation Solution (Experiments B-N-f)

The aim of these experiments was to study the effect of the N concentration in the fertigation solution on the development of CPM in potted cucumber plants. To characterize the response of cucumber plants to different concentrations of N in the fertigation solutions, six N concentrations (0.7, 1.4, 2.9, 5.0, 7.1, and 14.3 mM) were used while the concentrations of the other nutritional elements were kept constant.

4.4.2. P Concentration in the Fertigation Solution (Experiments B-P-f)

The aim of these experiments was to study the effect of the P concentration in the fertigation solution on the development of CPM in potted cucumber plants. To characterize the response of cucumber plants to different concentrations of P in the fertigation solutions, four P concentrations (0, 0.065, 0.323, and 0.645 mM) were used while the concentrations of the other nutritional elements were kept constant.

4.4.3. K Concentration in the Fertigation Solution (Experiments B-K-f)

The aim of these experiments was to study the effect of the K concentration in the fertigation solution on the development of CPM in potted cucumber plants. To characterize the response of cucumber plants to different concentrations of K in the fertigation solutions, five K concentrations (0.3, 0.5, 1.0, 1.8, and 2.6 mM) were used while the concentrations of the other nutritional elements were kept constant.

4.4.4. Ca Concentrations in the Fertigation Solution (Experiment B-Ca-f, $CaCl_2$ Supplement)

The aim of these experiments was to study the effect of Ca concentration on the development of CPM in potted cucumber plants. To characterize the response of cucumber plants to different concentrations of Ca (applied as Cl^- salt) in the fertigation solutions, without changing the concentrations of the other major ions (Table 1), four Ca concentrations (1.0, 2.0, 3.0, and 4.0 mM) were used while the concentrations of the other nutritional were kept constant.

4.4.5. Mg Concentrations in the Fertigation Solution (Experiment B-Mg-f, $MgCl_2$ Supplement)

The aim of these experiments was to study the effect of the Mg concentration in the fertigation solution on the development of CPM in potted cucumber plants. To characterize the response of cucumber plants to different concentrations of Mg (applied as Cl^- salt) in the fertigation solutions, without changing the concentrations of the other major ions (Table 1), four Mg concentrations (0.82, 1.65, 3.29, and 4.94 mM) were used while the concentrations of the other nutritional elements were kept the same for all treatments.

4.5. Foliar Application of Salt Solutions to Cucumber Plants Grown under Semi-Commercial Conditions (Experiments A-SC a and b)

At Site A, cucumber seedlings were transplanted into 10-L pots containing growth mixture. Fertigation was carried out with the fertilizer 4-2-6 (N-P_2O_5-K_2O) + 3% microelements (Fertilizers and Chemical Compounds Ltd., Haifa, Israel) throughout the experiments. A spray with water with no salt served as an untreated control. Mature plants bearing at least 20 leaves, as well as flowers and fruits, were used. The foliar treatments were initiated when plants were artificially inoculated with conidia of *Podosphaera xanthii*. Experiments were conducted twice with six replicates each time and arranged in randomized blocks.

Spray treatments were applied twice a week with a hand sprayer containing up to 1 L of water and forming a mist of small droplets. The experiments included the treatments of MKP (Fertilizers and Chemical Compounds Ltd., Haifa, Israel) at 1% as recommended and polyhalite ($K_2Ca_2Mg(SO_4)_4{\cdot}2(H_2O)$ (polysulfate, Fertilizers and Chemical Compounds Ltd., Haifa, Israel) [57] at concentrations of 0.125 and 0.25% (Expt. A-SC a). The salts $MgCl_2$, $CaCl_2$, and K_2SO_4 were sprayed at concentrations of 0.05 and 0.1 M (anion) and a combination of $MgCl_2$ 0.05 M (Cl), $CaCl_2$ 0.05 M, and K_2SO_4 0.1 M ($SO_4{}^{-2}$) resulting in a total of 0.1 M Cl^- and $SO_4{}^{-2}$ (Expt. A-CS b).

4.6. Commercial-Like (CL) Net-House Experiments to Test the Effects of Minerals Applied as Part of the Fertigation Solution and/or as Spray Treatments

At Site C, experiments were carried out in a net-covered greenhouse (net = 50 mesh) having natural conditions with fluctuating RH and temperature. One experiment was performed to test the effects of fertigation treatments coupled with spray treatments (Expt. CL1). The second experiment was conducted to evaluate the effects of different spray treatments (Expt. CL2). Cucumber plants (Line 501, Hazera Genesis, Berorim, Israel) were maintained in a commercial nursery (Shetil Neto, Gevaram, Israel), transplanted on 1 March 2019 to the perlite boxes mentioned below, and grown until the end of May 2019, at which point they were all bearing fruit.

Cucumber plants were planted in perlite (medium size, 1.2 mm, Agrifusia) growth medium in polystyrene containers (1.0 × 0.8 × 0.17 m), with six plants per container and 12 plants in an experimental plot. Plants were irrigated daily according to local extension service recommendations. During the initial 5 days, plants were sprinkler-fertigated with 4.3 mM N (10% $NH_4{}^+$), 1.6 mM K, and 0.65 mM P in the fertigation solution to aid their establishment. After that initial period, the plants were irrigated through drippers and fertilized with 8.57 mM N, 0.65 mM P, and 3.2 mM K in water for 2 weeks until the fertigation treatments were initiated as described below (Table 2). Fertigation was performed from 1000-L tanks dedicated to each treatment, with a 17-mm drip-irrigation pipe that had a 2 L/h dripper embedded every 20 cm along its length. Plants were irrigated three times a day. There were six fertigation treatments with three N concentrations (2.9, 7.1, and 14.3 M), two P concentrations (0.65 and 1.29 M), and three K concentrations (1.0, 2.6, and 5.1 M). Each time there was any change in the concentration of one nutritional element, the other two elements were kept at the same 'medium' concentrations, so that only the concentration of one element was changed in each fertigation treatment (Table 2).

Table 2. Fertigation regimes in the commercial-like experiment (Expt. CL 1).

Treatment		Fertigation Treatments (M)		
1	High N	Medium P 0.65	Medium K 2.6	High N 14.3
2	Low N	Medium P 0.65	Medium K 2.6	Low N 2.9
3	Medium NPK	Medium P 0.65	Medium K 2.6	Medium N 7.1
4	High K	Medium P 0.65	High K 5.1	Medium N 7.1
5	Low K	Medium P 0.65	Low K 1.0	Medium N 7.1
6	High P	High P 1.29	Medium K 2.6	Medium N 7.1

Each of the fertigation treatments was combined with one of the spray treatments: water, the fungicide triadimenol (250 g/L as Bayfidan, Bayer, Germany) sprayed at a concentration of 0.05% once every 2 weeks, or the combined salt treatment of $MgCl_2$+K_2SO_4 0.1 M (Cl^-) + 0.1 M ($SO_4{}^-$). Experiment

CL2 involved spray applications of $MgCl_2$ 0.1 M (Cl^-), K_2SO_4 0.1 M (SO_4^-), $MgCl_2$+K_2SO_4, 1% MKP, and the fungicide triadimenol, as mentioned above.

In both experiments, spray treatments were applied with a backpack sprayer equipped with a conical nozzle. Sprays were administrated until runoff every 3 to 5 days. The yield was harvested once a week starting from 40 days after planting and until the end of the experiments. The cumulative number of fruits and the weight of the yield were recorded. Experiment CL1 was organized in randomized split plots with four replicates for each combination treatment. Experiment CL2 was organized in randomized blocks with four replicates. Each plot consisted of two perlite boxes, with six plants in each box arranged in two rows, so that each plot had 12 plants.

4.7. Element Analysis

For all experiments, fully expanded mature leaves were sampled randomly at harvest time and used to determine leaf mineral concentrations. The leaves were rinsed with distilled water and dried in an oven at 70 °C for 48 h. The dried plant material was ground and subjected to chemical analysis. N, P, and K concentrations in the leaves were analyzed after digestion with sulfuric acid and peroxide [58]. Ca, Na, and Mg levels were analyzed after digestion with nitric acid and perchlorate [59]. The concentrations of N and P were determined with an autoanalyzer (Lachat Instruments, Milwaukee, WI, USA). Levels of K, Na, Mg, and Ca were analyzed with an atomic absorption spectrophotometer (Perkin-Elmer 460, Norwalk, CT, USA). Cl was extracted from the leaves in water (100:1 water:dry matter) and the Cl^- concentration was determined with a chloride analyzer (Model 926, Sherwood Scientific Ltd., Cambridge, UK).

4.8. Statistics

Data in percentages were arcsine-transformed before further analysis. Area under the disease progress curve (AUDPC) values were calculated. Standard errors (SE) of the means were calculated and are presented alongside the degrees of freedom (DF = $n - 1$ for the controlled-conditions experiments and DF = $n - 2$ for correlations calculated for the field data). The disease-severity data and the AUDPC data were analyzed using ANOVA and the Tukey–Kramer HSD test. Statistical analysis was performed (α = 5%) using JMP 14.0 software (SAS Institute, Cary, NC, USA).

To examine the relationship between the concentration of a nutritional element and disease severity in experimental replicates (plots), correlation coefficients were calculated and best-line formulas were calculated using all individual pairs of data (n). Correlation line types included linear, exponential, logarithmic, and polynomial. In the captions of the relevant figures, the regression formulas and the Pearson correlation coefficient (r) values are presented along with significance levels (P) according to degrees of freedom ($n - 2$).

5. Conclusions

The severity of cucumber powdery mildew was reduced by the foliar application of Ca, Mg, and K salts, among which $SO4^{-2}$ and Cl^- salts both have an effect, but Cl^- is somewhat more effective. MKP and polyhalite also provide effective disease control. Increasing the K concentration in irrigated mature plants was effective as well, but the mentioned foliar application seems to provide more effective CPM control. The combination of salts in a single spray solution provided only a minor additive effect and the combination of spray treatments with irrigation treatments did not yield any additive effect. Coupled with the fact that above a certain concentration (measured as the molar concentration of Cl^-) of the spray-applied salts we saw no additional disease reduction, these findings suggest that induced resistance is a potential mode of action in the CPM pathosystem, as has also been demonstrated in the sweet basil downy mildew pathosystem [18].

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Effects of Microelements on Downy Mildew (*Peronospora belbahrii*) of Sweet Basil

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Abstract: We recently demonstrated that spraying or irrigating with Ca, Mg and K reduces the severity of sweet basil downy mildew (SBDM). Here, the effects of Mn, Zn, Cu and Fe on SBDM were tested in potted plants. The effects of Mn and Zn were also tested under semi-commercial and commercial-like field conditions. Spray applications of a mixture of EDTA-chelated microelements (i.e., Fe-EDTA, Mn-EDTA, Zn-EDTA, Cu-EDTA and Mo) reduces SBDM severity. The application of EDTA chelates of individual microelements (i.e., Fe-EDTA, Mn-EDTA and Zn-EDTA) significantly reduces SBDM in potted plants. Foliar applications of Mn-EDTA and Zn-EDTA are found to be effective under semi-commercial conditions and were, thus, further tested under commercial-like conditions. Under commercial-like conditions, foliar-applied Mn-EDTA and Zn-EDTA decreased SBDM severity by 46–71%. When applied through the irrigation solution, those two microelements reduce SBDM by more than 50%. Combining Mg with Mn-EDTA and Zn-EDTA in the irrigation solution does not provide any additional disease reduction. In the commercial-like field experiment, the microelement-mixture treatment, applied as a spray or via the irrigation solution, was combined with fungicides spray treatments. This combination provides synergistic disease control. The mode of action in this plant–pathogen system may involve features of altered host resistance.

Keywords: agrotechnical control; cultural control; integrated management; downy mildew; magnesium; manganese; microelements; plant disease; *Ocimum basilicum*; zinc

1. Introduction

Microelements are essential for plant development and are needed in lower concentrations than macro-elements. Microelements are active as co-factors of metalo-enzymes, and some of them play roles in the structural stability of membranes in vascular plants. Microelements are involved in metabolic processes, such as the synthesis of primary and secondary metabolites, energy metabolism, cell defense, the control of gene expression, hormone absorption and signal transduction [1]. Microelements affect the concentrations of phenols and lignin in plants [2] and also affect how plants respond to pathogens [3], including the induction of systemic acquired resistance. Substantial information on the effects of specific microelements on plant diseases was published in the second half of the 20th century. In the powdery mildew–cucumber patho-system, Mn and Cu were found to induce the release of Ca ions from membranes. Ca ions play a role in the defense system together with salicylic acid [4]. Interactions between microelements may affect both the pathogen and the host. For instance, Fe is essential for *Fusarium* pathogenicity, while Mn

competes with the pathogen's absorption of Fe [5]. Fe promotes the activity of a toxin of *F. oxysporum* var. *lycopersici*; whereas Cu inhibits the effect of Ca [6].

Mn is important for the development of plant resistance to foliar diseases [2]. It is involved in at least two steps in the biosynthesis of lignin, a process that is important for plant protection [7]. A single spray application of $MnCl_2$ induced systemic-induced resistance to powdery mildew in cucumber plants and increased β-1,3-glucanase content even in non-infected plants [8]. Mn fertilization reduces the severity of foliar diseases such as downy mildews and powdery mildew [9].

Zn is important for the biosynthesis of proteins, energy production, transcription factor function and for maintaining the structure and integrity of membranes. More than 1200 proteins have been reported to contain, bind or translocate Zn [10,11]. There have been several reports on the effects of Zn on plant diseases. As an activator of Cu/Zn-SOD (superoxide dismutase), Zn is involved in protecting membranes against chilling damage by detoxifying superoxide radicals [12]. Protecting membranes against free radicals reduces the leakage of sugars and amino acids across membranes, which may limit parasite activity [2,12]. Zn reduces the susceptibility of cabbage (*Brassica oleracea*) and turnip (*Brassica rapa* subsp. *rapa*) to *Erysiphe polygoni* powdery mildew [13], as well as the severity of take-all disease in wheat, *Gaeumannomyces graminis* var. *tritici* [2].

Fe is involved in photosynthetic processes, mitochondrial respiration, assimilation of N; synthesis of hormones, such as jasmonic acid and gibberellin; the formation and elimination of reactive oxygen species (ROS) and resistance to plant pathogens [1]. Fe is also important for microorganisms, including microbial enzymes, that are important for plant infection [2,14]. Fe has been shown to reduce the severity of wheat rust and anthracnose in banana (*Musa acuminita*) [2]. Spray applications of Fe increased the resistance of apple (*Malus domestica*) and pear (*Pyrus communis*) to *Sphaeropsis malorum*, and the resistance of cabbage to *Olpidium brassicae* [5], but also increased the severity of take-all disease (*Gaeumannomyces graminis* var. *tritici*) in barley (*Hordeum vulgare*) [9].

Cu is essential for photosynthesis and mitochondrial respiration. It is also important for C and N metabolism, protection from oxidation damage, and cell-wall synthesis. Various biochemical reactions are dependent on Cu or Fe, and affected by their relative availability [15]. Enzymes such as polyphenol oxidase and phenolase, which are involved in lignin synthesis, require Cu, and Cu deficiency reduces the lignin content in plants [16]. Cu is a co-factor for the receptor of ethylene; thus, in cases of Cu deficiency, the affinity of the ethylene receptor to ethylene is drastically reduced [17]. Associations between low levels of Cu and increased disease severity have been reported for *Alternaria helianthi* infection of sunflower (*Helianthus annuus*), *Claviceps purpurea* infection (ergot disease) of barley and rye (*Secale cereale*) and *Septoria tritici* infection of wheat [5,18]. Increased Cu fertilization has been shown to reduce *Sclerotinia minor* infection in peanut (*Arachis hypogea*) [19] and *G. graminis* var. *tritici* infection in wheat [20].

We recently demonstrated that spraying or irrigating with N, Ca, Mg and K reduces the severity of sweet basil downy mildew (SBDM; [21,22]). SBDM is caused by *Peronospora belbahrii* [23]. It is a devastating disease in autumn and spring crops of sweet basil that has been reported to be affected by microclimate manipulation [24]. Chemical control options are limited and may fail due to the development of resistance [25]. One finding of that research was that SBDM is suppressed by concentrations of 3.0–4.94 mM Mg in the irrigation water under commercial-like conditions, as compared with a standard Mg concentration of 1.65 mM usually present in the irrigation water [21]. The purpose of the present study was to test the effects of the microelements Mn, Zn, Cu and Fe on SBDM in potted plants and under semi-commercial and field conditions. Selected microelements were also tested under commercial-like conditions, in combination with increased Mg concentration in the irrigation water and foliar applications of chemical fungicides. The research project was carried out in the same locations, in parallel and following the researches on effects of nitrogen and NH_4^+ fertilization [22] and of calcium, magnesium

and potassium on sweet basil downy mildew (*Peronospora belbahrii*) [21]. These projects use similar methods and technical details.

2. Results

2.1. Effects of Foliar Applications of Microelements on Sweet Basil Downy Mildew (Experiments A1 and A2)

Two separate sets of potted-plant experiments were carried out to test the effects of foliar applications of a mixture of microelements and individual microelements on SBDM. A mixture of Fe-EDTA, Mn-EDTA, Zn-EDTA and Cu-EDTA (Koratin) reduced the severity of SBDM, when applied as a spray, at concentrations of 0.1–0.2%, but not at 0.4% (Figure 1a). The higher Koratin concentration was phytotoxic.

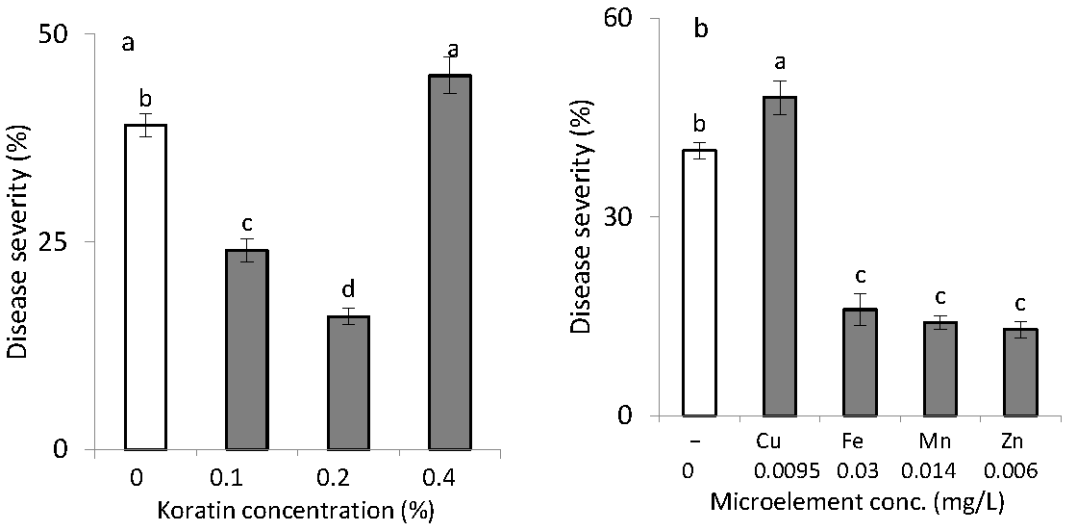

Figure 1. Effects of spray applications of solutions of EDTA chelates of microelements on sweet basil downy mildew (SBDM) severity when applied (**a**) in a mixture or (**b**) as single-microelement solutions. SBDM severity was evaluated on a 0–100% scale, in which 0 = healthy plants and 100% = plants completely covered by disease symptoms. Values followed by a different letter are significantly different according to one-way ANOVA and Tukey's HSD test. Default significance levels were set at $p \leq 0.05$. Bar = SE.

The individual EDTA chelates of Fe, Mn and Zn also provided significant disease control (Figure 1b). The control provided by the combination of microelements was not superior to that provided by the individual microelements (results not presented); thus, further work dealt with applications of individual microelements. The microelements, when applied as a mixture or individually, did not affect shoot yield.

2.2. Effects of Microelement Solutions on Sweet Basil Downy Mildew (Experiment C1, Autumn 2015–2016)

Experiments on the microelements' effect on SBDM in location C were carried out in parallel to experiments in projects that dealt with nitrogen [22] and Ca, Mg and K effects on the disease [21]. In the present project, the severity of SBDM on untreated sweet basil plants reached 60% 151 days after planting. Spray applications of the mixture of EDTA-chelated microelements (Koratin, Fe-EDTA, Mn-EDTA, Zn-EDTA, Cu-EDTA and Mo, as $(NH_4)_2MoO_4$) reduced disease severity until 129 days after planting, and foliar-applied fungicides reduced disease severity through the end of the experiment (Figure 2a). The calculation of AUDPC values (Figure 2b) revealed significant reductions in disease severity of 25.9%, 51.7% and 65.9% in the microelement, fungicides and combined microelement + fungicides treatments, respectively. The application of microelements did not significantly affect shoot yield.

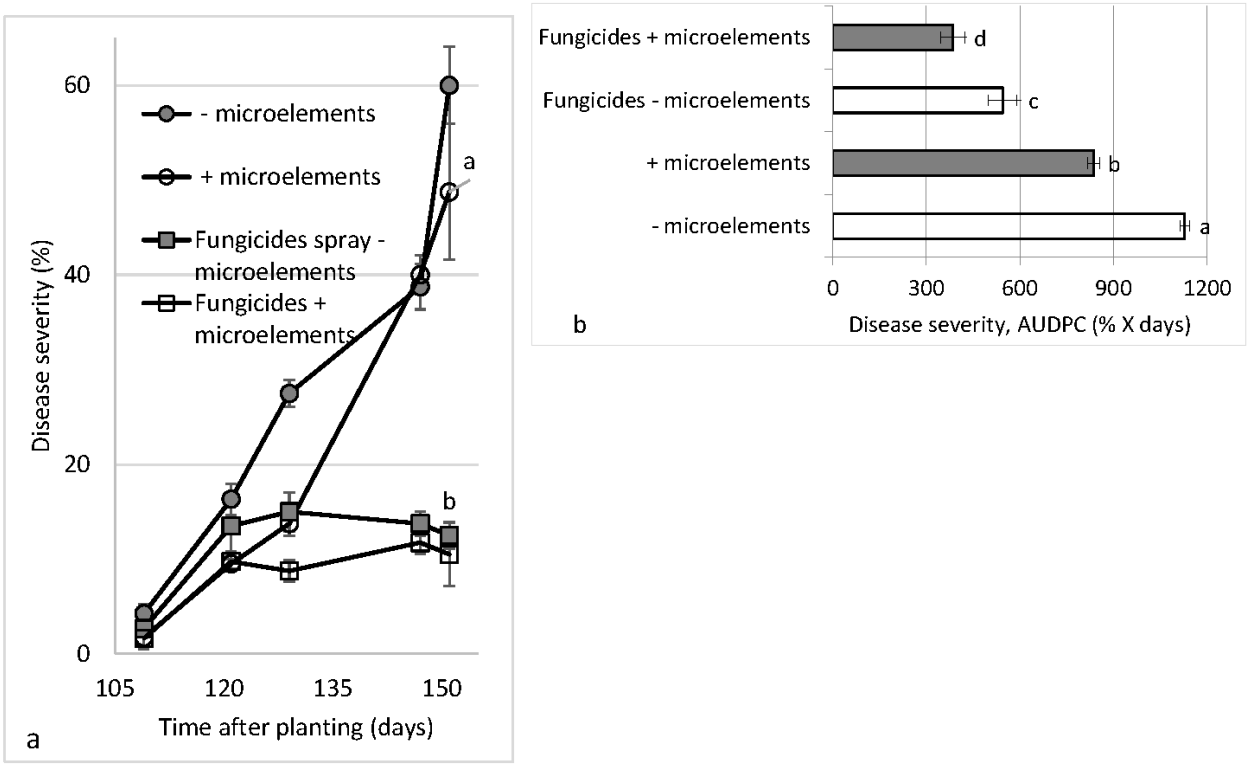

Figure 2. Effects of foliar applications of a microelement solution (Koratin: Fe-EDTA, Mn-EDTA, Zn-EDTA, Cu-EDTA and Mo mixture) and fungicides on sweet basil downy mildew (SBDM) severity in Experiment C1, Autumn 2015–2016. Treatments consisted of microelements (−/+ spray) coupled with fungicides treatments (−/+ spray). SBDM was evaluated (**a**) 109 to 151 days after planting and data are presented separately for (**b**) area under disease progress curve (AUDPC) through 42 days. Disease severity was evaluated on a 0–100% scale, in which 0 = healthy plants and 100% = plants completely covered by disease symptoms. Values in each graph and date followed by a different lower-case letter are significantly different according to one-way ANOVA with Tukey's HSD test. Default significance levels were set at $p \leq 0.05$. Bar = SE.

A two-way statistical analysis of the results of the two main experiment components (i.e., fungicides sprays and microelement sprays) revealed a significant contribution of each of the main components to the reduction in SBDM severity, with the fungicides treatments providing a 52.7% reduction in severity, and the microelement treatments providing a 27.0% reduction in severity ($p < 0.05$). Calculation of the SF of the reduction in severity induced by the microelement and fungicides treatments revealed a significant synergistic effect for disease levels 147 days after planting (SF = 1.095) and for the calculated AUDPC (SF = 1.026).

We also examined the relationship between disease severity and the microelement concentrations in shoots sampled in individual plots in the experiment. At 94 days after planting, concentrations of Zn and Mn in the shoots were negatively correlated with SBDM severity (Figure 3a,b); whereas shoot concentrations of Cu and Fe were positively correlated with disease severity (Figure 3c,d). At 129 days after planting, Zn and Mn concentrations in the shoots were negatively correlated with SBDM severity, and the relationships between the concentrations of Cu and Fe and SBDM severity were insignificant (results not presented).

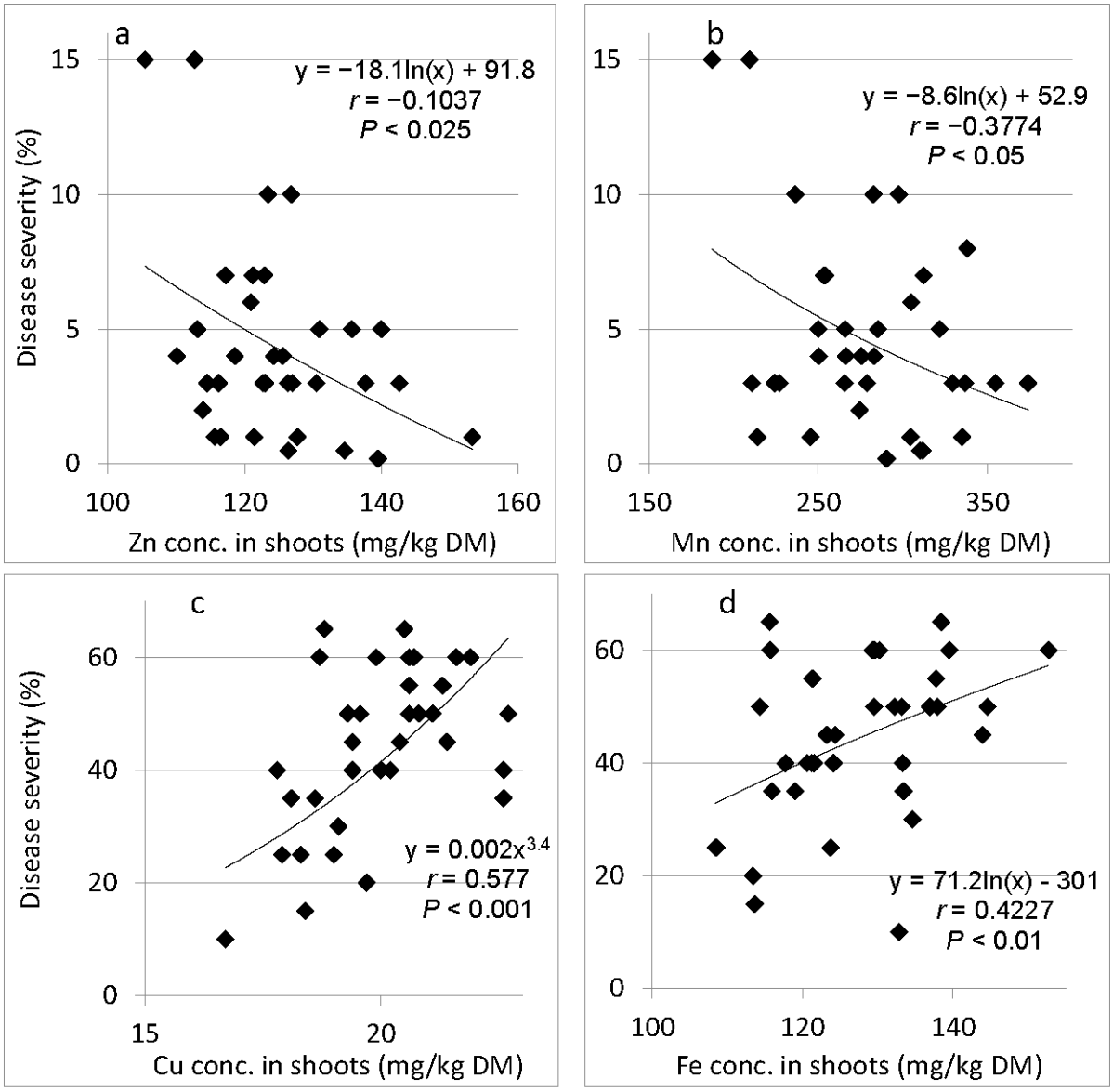

Figure 3. Relationships between the concentrations of microelements (**a**) Zn, (**b**) Mn, (**c**) Cu and (**d**) Fe in shoots of sweet basil sampled in single plots of Experiment C1 at 94 days after planting and the severity of sweet basil downy mildew (SBDM). SBDM severity was evaluated on a 0–100% scale, in which 0 = healthy plants and 100% = plants completely covered by disease symptoms. The best-fit regression formula is presented for each microelement. Pearson regression values (*r*) are presented along with significance levels (*p*).

2.3. Foliar Application of Microelement Chelates to Sweet Basil Plants Grown in Containers (Semi-Commercial Experiments, Experiment B)

Sweet basil plants grown under semi-commercial conditions were treated with 0.1% Koratin. Disease in the untreated control reached an AUDPC of 153.6% × days, and the treatment reduced it by 18%. Further research concentrated on the effects of Mn and Zn on SBDM, because the effects of those microelements were consistent in the earlier potted-plant experiments (Site A) and since, under commercial-like conditions, the concentrations of Mn and Zn in sweet basil shoots were negatively correlated with SBDM severity. Therefore, spray applications of Zn-EDTA and Mn-EDTA were carried out over two months in plants grown in containers under semi-commercial conditions. Both treatments suppressed the severity of downy mildew on the peak disease sampling day, and at the end of the experiment (Figure 4a,b). The spray treatment that included both Mn and Zn did not affect shoot yield. Following the suppression of SBDM

by Mn and Zn under semi-commercial conditions, we applied the two microelements in commercial-like experiments, as described below for experiments C2 through C5.

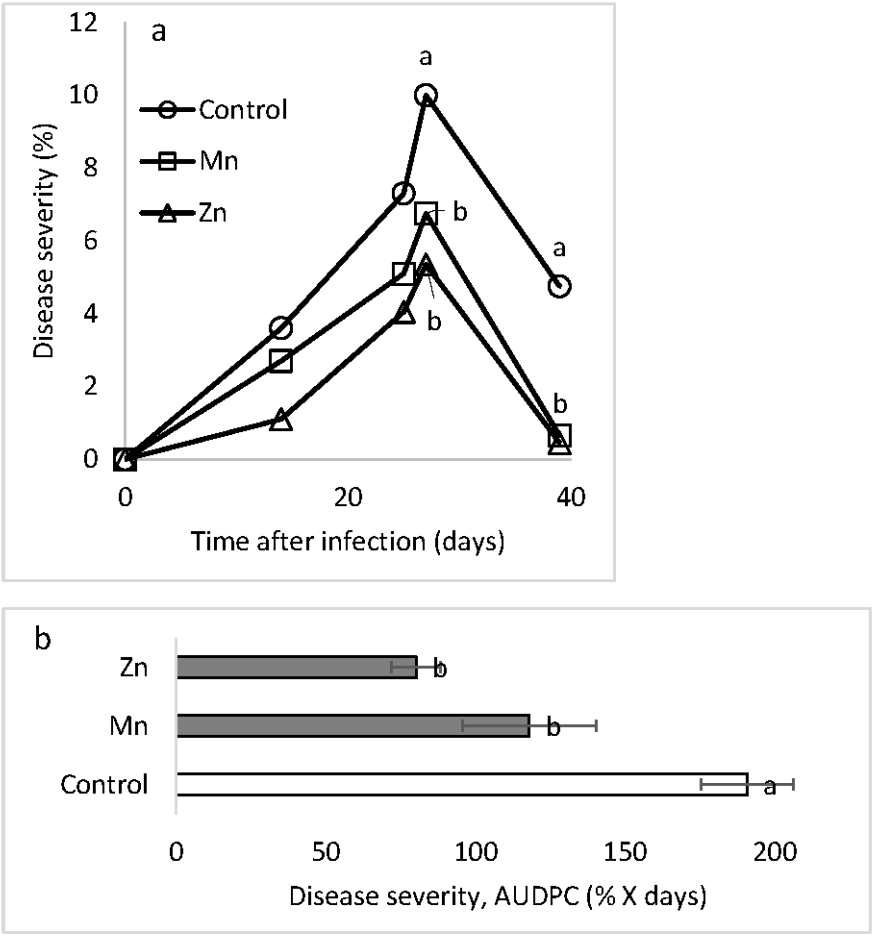

Figure 4. Effects of foliar applications of individual microelement chelates on the severity of sweet basil downy mildew (SBDM) under semi-commercial conditions. Zn-EDTA (0.006%) and Mn-EDTA (0.014%) were sprayed once a week over a two-month period. (**a**) Development of the disease and (**b**) area under disease severity curve (AUDPC) values through 39 days are presented. SBDM severity was evaluated on a 0–100% scale, in which 0 = healthy plants and 100% = plants completely covered by disease symptoms. Values followed by a different letter are significantly different according to one-way ANOVA with Tukey's HSD test. Default significance levels were set at $p \leq 0.05$. Bar = SE.

2.4. Foliar Microelement Treatments under Commercial-like Conditions (Experiment C2, Spring 2016)

In Experiment C2, the effects of foliar-applied Zn, Mn and fungicides on SBDM were compared under commercial-like conditions. The concentrations of Zn and Mn in the shoots were measured in untreated and treated plots at 69 days after planting. The shoot Zn concentrations in the untreated and treated plots were 37.48 ± 1.14 and 42.1 ± 2.1 mg/kg dry weight, respectively. The shoot Mn concentrations in the untreated and treated plots were 50.4 ± 1.84 and 68.5 ± 2.23 mg/kg dry weight, respectively. For each of the microelements, the difference between the treatments was significant ($p \leq 0.05$). The fungicides treatment did not affect the concentrations of Mn and Zn in the shoots.

Disease severity 104 days after planting was 35 ± 4.1% in the untreated control and foliar applications of Zn, Mn and fungicides significantly ($p \leq 0.05$) decreased disease severity by 59–71%. The calculated AUDPC was 773 ± 105.4 % x days in the untreated control and these treatments also significantly ($p \leq 0.05$) reduced the calculated AUDPC by 46–71%.

In the untreated control, the total shoot yield was 6.22 ± 0.04 kg/m^2 and the Grade A shoot yield was 3.88 ± 0.17 kg/m^2. In the foliar-applied Zn plots, the total shoot yield was 6.70 ± 0.39 kg/m^2 and

the Grade A shoot yield was 4.25 ± 0.17 kg/m^2. Finally, in the foliar-applied Mn plots, the total shoot yield was 7.23 ± 0.09 kg/m^2 and the Grade A shoot yield was 4.39 ± 0.06 kg/m^2. The yields of the spray treatments were significantly higher than that of the control ($p \leq 0.05$).

2.5. Effects of Foliar and Fertigation Applications of Zn and Mn in Combination with Mg Supplied through Fertigation under Commercial-like Conditions (Experiment C3, Autumn 2016)

In a previous publication, we reported that SBDM could be suppressed by increasing the concentration of Mg in the irrigation solution, a treatment that was also associated with increased levels of Mg in the sweet basil shoots [21]. In the current experiment, we tested the effects of increased concentrations of Mn and Zn in the irrigation solution and foliar applications of Mn and Zn on SBDM when the irrigation solution contained either 1.65 mM Mg (basic level) or 4.94 mM Mg (supplemented).

Shoot concentrations of Mg in plants grown in the basic Mg irrigation treatments ranged between 0.83% and 1.02%, and for the plants grown in the supplemental Mg irrigation treatments, shoot Mg concentrations ranged between 1.20% and 1.29%. The concentration of Zn in the sweet basil shoots at 55 days after planting significantly increased as a result of the application of Zn through the irrigation solution, as observed in plants irrigated with both Mg concentrations, as well as plants treated with foliar-applied Mg that were irrigated with a solution that contained only the basic Mg concentration (Table 1). Interestingly, the increased concentration of Mg in the irrigation water increased the level of Zn in the shoots (Table 1, analysis not presented). A similar trend was observed for the Mn concentration in the shoots following the application of supplemental Mn through spray or fertigation treatments (Table 1), and as a result of the application of supplemental Mg (analysis not presented).

Table 1. Concentrations of Zn and Mn in shoots of sweet basil plants that were grown under commercial-like conditions and treated with two different concentrations of Mg (applied through the irrigation solution), and Zn and Mn applied as a spray or through the irrigation solution (Experiment C3, Autumn 2016) at 55 days after planting.

Microelements in the Irrigation Solution		**Zn in Shoots (mg/kg DW)**		**Mn in Shoots (mg/kg DW)**	
Microelement	Concentration (mg/L)	Mg in Irrigation Solution (mM)			
		1.65	4.94	1.65	4.94
None	0	71.1 ± 2.78 c	78.8 ± 2.54 b	53.6 ± 4.41 b	49.0 ± 3.41 b
Zn	1	93.8 ± 1.73 b	118.2 ± 9.66 a	45.3 ± 4.05 b	63.8 ± 5.58 b
Mn	2	72.5 ± 2.49 c	77.6 ± 3.09 b	73.0 ± 9.95 a	115.3 ± 15.23 a
Foliar-applied microelements					
Microelement	Concentration (%)				
Zn	0.006	107.4 ± 7.05 a	Nm	49.9 ± 6.33 b	nm
Mn	0.014	69.5 ± 2.06 c	Nm	79.3 ± 5.04 a	nm

Values for each Mg treatment and each microelement treatment that are followed by a different letter are significantly different according to one-way ANOVA with Tukey's HSD test. The default significance level was set at $p \leq 0.05$. nm—not measured. DW—dry weight.

SBDM severity reached 52.5% at 93 days after planting. It was significantly reduced by Zn and Mn when supplied in the irrigation solution. The addition of these microelements to Mg-enriched water did not provide any further disease suppression (Figure 5a). Fungicides treatments drastically and significantly reduced SBDM severity to 7.5%, and only the fertigation-applied Zn supplement provided greater control (Figure 5b). Since the two-way ANOVA revealed a significant interaction between the microelements and the Mg treatment, each of the individual treatments is presented alone. Moreover, the combination of fertigation-applied microelements and fungicides treatments did not have any synergistic disease-control effect at 93 days after planting (results not presented).

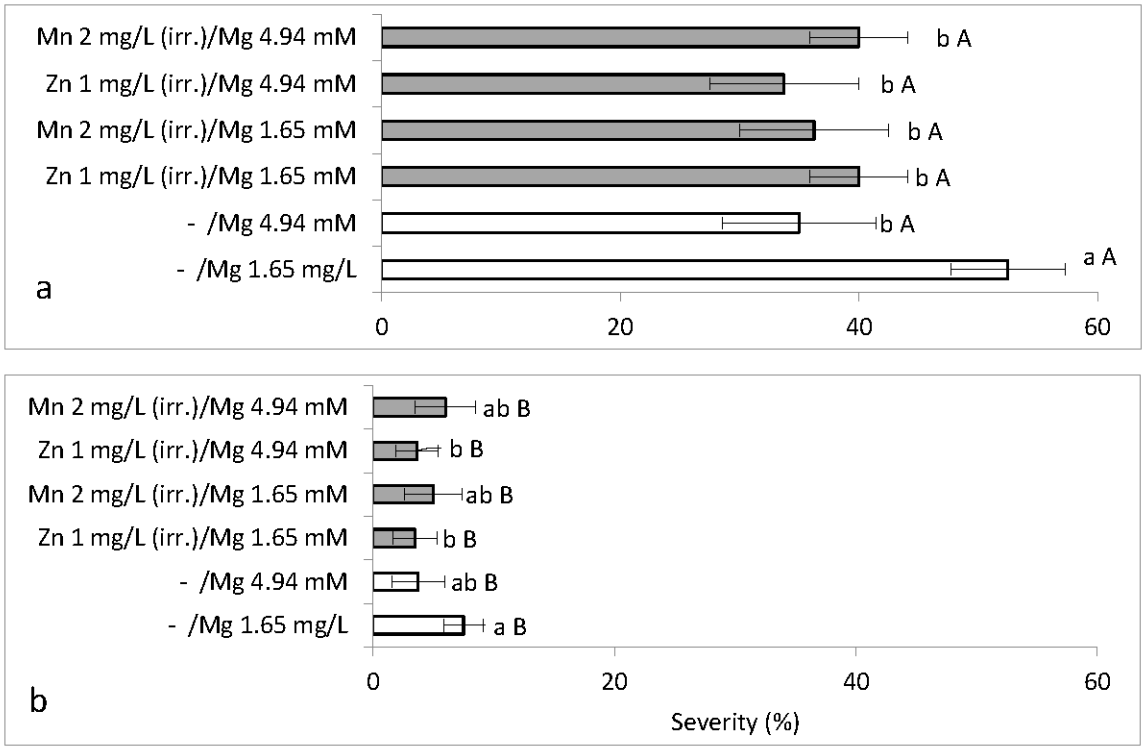

Figure 5. Effects of adding Zn-EDTA (1 mg/L; 1.64 mM) and Mn-EDTA (2 mg/L; 4.94 mM) to the irrigation solution on the severity of sweet basil downy mildew (SBDM) among plants grown under commercial-like conditions (Autumn 2016). Treatments were applied (**a**) alone or (**b**) together with fungicides. Severity of the SBDM at 93 days after planting was evaluated on a 0–100% scale, in which 0 = healthy plants and 100% = plants completely covered by disease symptoms. Values within each graph that are followed by a different lower-case letter and values followed by a capital letter in each pair of treatments without and with fungicides are significantly different according to two-way ANOVA with Tukey's HSD test. The interaction between the main parameters (microelement application × Mg concentration) was significant, so detailed analyses of all of the combination treatments are presented. Default significance levels were set at $p \leq 0.05$. Bar = SE.

The cumulative shoot yield for the control treatment reached 2.41 kg/m^2 at 98 days after planting. None of the microelement-fertigation treatments resulted in any increases in yield. Yield in the fungicides treatment was 2.96 kg/m^2, which was not significantly different from the yield of the plots that were not treated with any fungicide. The yields for the microelement + fungicides treatments were not significantly greater than the yields of plants treated only with the fungicides. The combination of 120 mg/L Mg and either Zn or Mn, provided an additive yield increase in both the treatments without fungicides and those with fungicide: 2.68 to 2.90 and 3.13 to 3.22 kg/m^2, respectively.

Foliar-applied Zn significantly reduced SBDM severity (26.1%; Figure 6a). The fungicides treatments reduced the disease significantly while no additional suppression of the SBDM severity was achieved by the combination of fungicides with the foliar microelement applications (Figure 6b). Yield was not increased by the foliar application of microelements or fungicides (results not presented). Analysis of the microelement concentrations in the shoots of single replicates (experimental plots) did not reveal any relationship between microelement treatments and disease severity (data not shown).

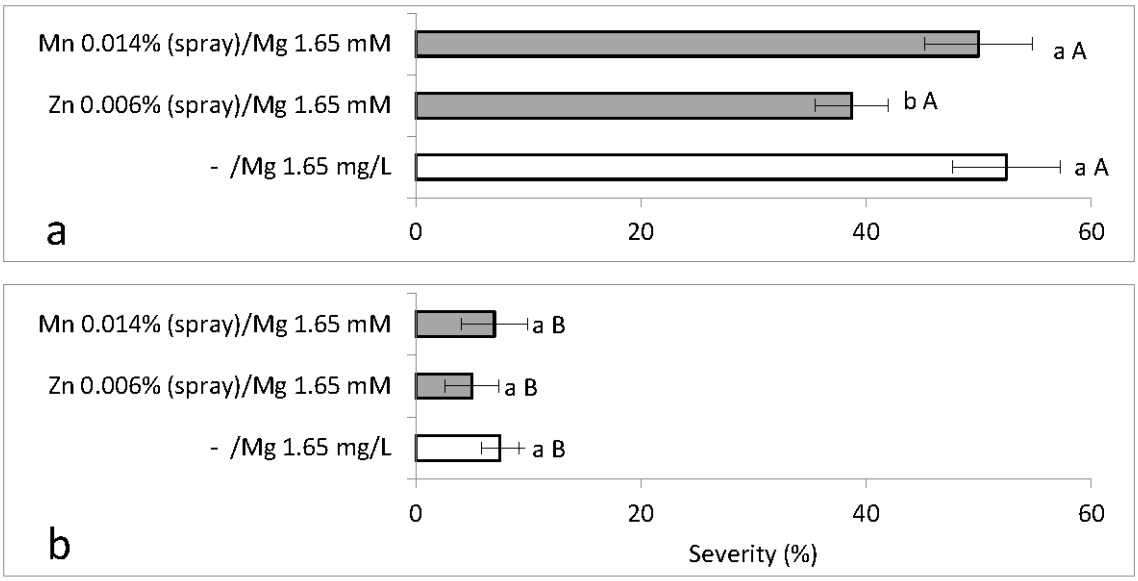

Figure 6. Effects of foliar applications of Zn-EDTA (0.006%), Mn-EDTA (0.014%) and fungicides on the severity of sweet basil downy mildew under commercial-like conditions (Autumn 2016). Plants were irrigated with a solution without added Mg (Mg concentration in water was regarded as 40 mg/L). Severity of SBDM at 93 days after planting (**a**) without fungicides and (**b**) with fungicides. Disease severity was evaluated on a 0–100% scale, in which 0 = healthy plants and 100% = plants completely covered by disease symptoms. Values within each graph that are followed by a different lower-case letter and values followed by a capital letter in each pair of treatments without and with fungicides are significantly different according to two-way ANOVA with Tukey's HSD test. The interaction between the major parameters (fungicides × foliar-applied microelements) was significant, so detailed analyses of all of the combination treatments are presented. Default significance levels were set at $p \leq 0.05$. Bar = SE.

2.6. Effects of Foliar Spray or Fertigation Applications of Zn and Mn Combined with Mg Supplementation under Commercial-like Conditions (Experiment C4, Spring 2017)

In the Spring of 2017 commercial-like experiment, we tested the effects of increased Mn and Zn in the irrigation solution on SBDM, when the irrigation solution contained either 1.65 mM (basic Mg level) or 4.94 mM (Mg supplemented). At 90 days after planting, the shoot Mg concentrations of plants grown in the basic Mg irrigation treatment ranged between 0.70% and 0.93%, and the shoot Mg concentrations for plants grown in the Mg-supplemented irrigation treatments ranged between 0.90% and 1.03%. The concentration of Zn in the sweet basil shoots increased significantly when Zn was applied through the irrigation solution, regardless of the level of Mg in that solution (Table 2). A similar trend was observed for the Mn concentration in the shoots following the application of Mn through the fertigation solution or by spray (Table 2).

Table 2. Concentrations of nutritional elements in the shoots of sweet basil plants grown under commercial conditions and treated with two Mg concentrations (applied through the irrigation solution) and with Zn and Mn (applied through the irrigation solution) at 90 days after planting (Experiment C4, Spring 2017).

Microelements in the Irrigation Solution		**Zn in Shoots (mg/kg DW)**		**Mn in Shoots (mg/kg DW)**	
Microelement	Concentration (mg/L)	Mg in Irrigation Solution (mM)			
		1.65	4.94	1.65	4.94
None	0	62.2 ± 1.11 b	66.0 ± 11.30 b	41.0 ± 1.56 b	44.0 ± 2.93 b
Zn	1	90.9 ± 12.86 a	96.1 ± 3.97 a	43.6 ± 4.09 b	50.0 ± 6.05 b
Mn	2	52.9 ± 2.13 c	55.9 ± 2.05 b	75.6 ± 11.11 a	123.2 ± 13.25 a

Values for each Mg treatment and each microelement treatment that are followed by a different letter are significantly different according to one-way ANOVA with Tukey's HSD test.

The addition of Zn and Mn to the irrigation solution significantly suppressed SBDM severity (by more than 50%) at 104 days after planting, at both examined Mg levels (Figure 7a). The fungicides

treatment significantly reduced disease and the combination of microelements and fungicide reduced disease significantly, as compared with the fungicides treatment alone (Figure 7a,b). There was no synergism between the increased Mg (4.94 mg/L) treatment and either of the microelement treatments in plots that were not treated with any fungicide. In the fungicide-treated plots, the combination of supplemental Mn and supplemental Mg had an additive effect (SF = 1.00). Synergism was obtained between the supplemental Zn treatment and the fungicides treatment (SF = 1.03). Differences in shoot yield were insignificant (results not presented).

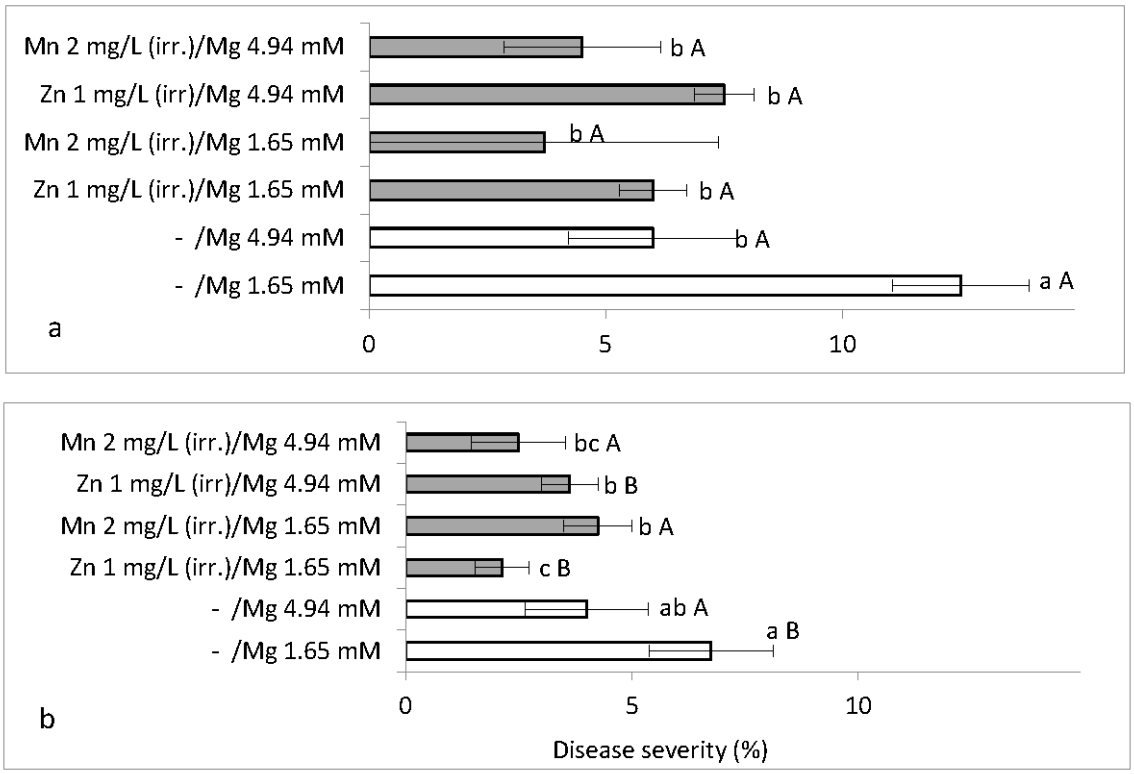

Figure 7. Effects of adding Zn-EDTA (1 mg/L) and Mn-EDTA (2 mg/L) to the irrigation solution so that it contained 1.65 and 4.94 mM/L of those microelements on sweet basil downy mildew (SBDM) severity under field conditions (Spring 2017). Treatments were applied (**a**) alone or (**b**) in combination with fungicides. SBDM severity was evaluated at 104 days after planting on a 0–100% scale, in which 0 = healthy plants and 100% = plants completely covered by disease symptoms. Values within each graph followed by a different lower-case letter and values followed by a capital letter in each pair of treatments without and with fungicides are significantly different according to two-way ANOVA with Tukey's HSD test. Default significance levels were set at $p \leq 0.05$. Bar = SE. DW—dry weight.

Foliar applications of Zn and Mn significantly reduced SBDM severity by 44.0% to 48.0% (Figure 8a) and the fungicide significantly reduced disease severity by 45.6% (Figure 8b). The application of microelement spray treatments to fungicide-treated plants similarly reduced disease severity by 51.5%, as compared with the fungicides alone (Figure 8b). There was no synergism between the different foliar treatments. Cumulative yield in the untreated control was 4.558 kg/m^2 at 110 days after planting. The yield was significantly increased (11.8%) by the application of Zn, and fungicides treatment increased yield by 7.8%.

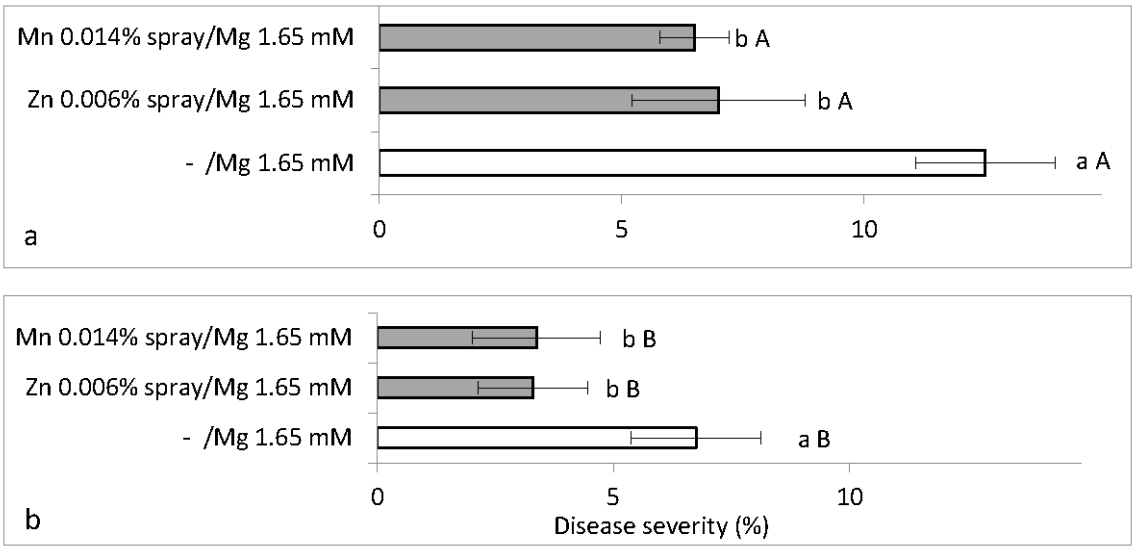

Figure 8. Effects of foliar applications of (**a**,**b**) Zn-EDTA (0.006%) and Mn-EDTA (0.014%) and (**b**) fungicides on the severity of sweet basil downy mildew (SBDM) under field conditions (Spring 2017). The plants were irrigated with no added Mg. (The Mg concentration in the irrigation solution was 1.65 mM.) Severity of SBDM at 104 days after planting (**a**) without fungicides and (**b**) with fungicides. Disease severity was evaluated on a 0–100% scale, in which 0 = healthy plants and 100% = plants completely covered by disease symptoms. Values within each graph that are followed by a different lower-case letter and values followed by a capital letter in each pair of treatments without and with fungicides are significantly different according to two-way ANOVA with Tukey's HSD test. Default significance levels were set at $p \leq 0.05$. Bar = SE.

The concentrations of microelements in the sampled shoots corresponded to the observed levels of disease severity. There were significant negative correlations between SBDM severity and the concentrations of Zn and Mn in the shoots (Figure 9).

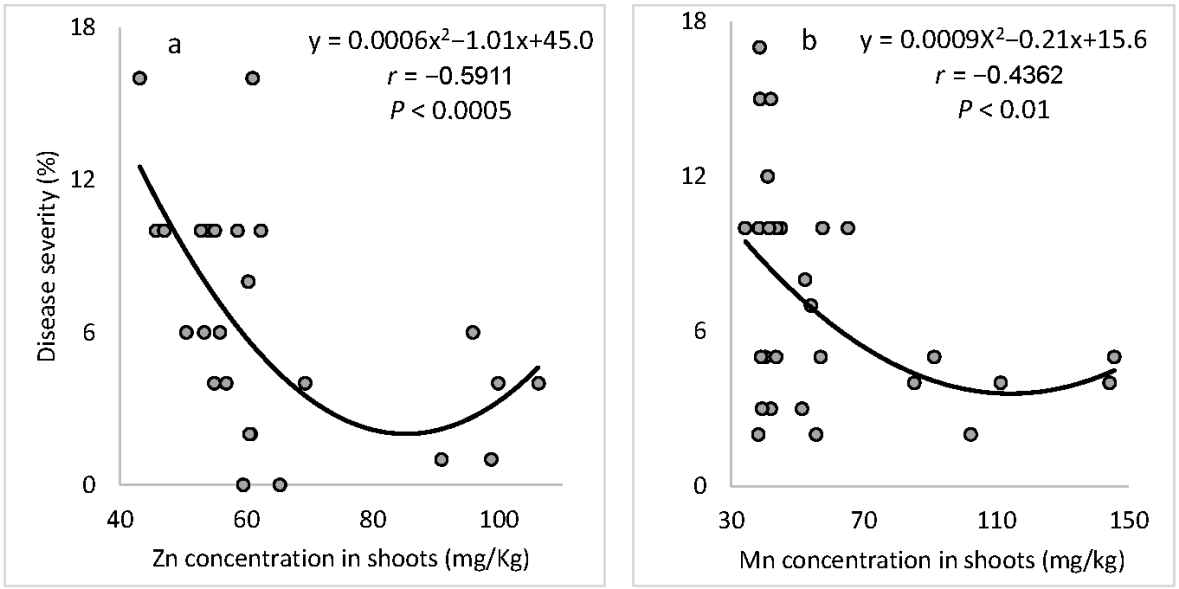

Figure 9. Relationship between the concentrations of microelements (**a**) Zn and (**b**) Mn in the shoots of sweet basil sampled in single plots of Experiment C4 and severity of sweet basil downy mildew (SBDM) at 104 days after planting. SBDM severity was evaluated on a 0–100% scale, in which 0 = healthy plants and 100% = plants completely covered by disease symptoms. The best-fit regression formula is presented for each microelement. The Pearson regression values (*r*) are presented along with significance levels (*p*).

2.7. Effects of Foliar Spray or Fertigation Applications of Zn, Mn and Zn Combined with Mn under Commercial-like Conditions (Experiment C5, Autumn 2017)

In the Autumn 2017 commercial-like experiments, we tested the effects of increased Mn, Zn and their combination in the irrigation solution (Figure 10a,b) and in spray (Figure 10c,d) on SBDM, when the plants were either not sprayed or additionally sprayed with fungicides. At 90 days after planting, the concentration of Zn in the sweet basil shoots increased significantly when Zn was applied through the irrigation solution and by spray (Table 3). Similarly, Mn concentration significantly increased in the shoots following the application of Mn through the fertigation solution or by spray (Table 3).

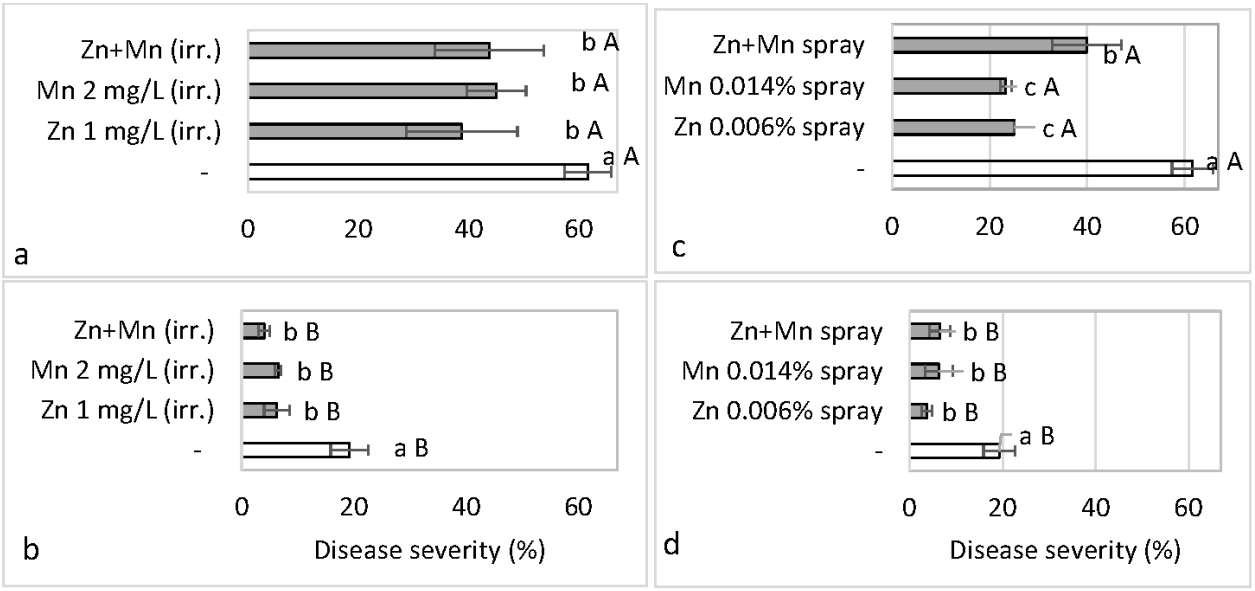

Figure 10. Effects of the addition of Zn-EDTA (1 mg/L) and Mn-EDTA (2 mg/L) to an irrigation solution containing 1.65 mM Mg (**a**,**b**) and spray of Zn-EDTA (0.006%) and Mn-EDTA (0.014%) (**c**,**d**) on the severity of sweet basil downy mildew (SBDM) under commercial-like conditions (Experiments C5, Autumn 2017–2018). The microelements were applied either alone or in combination; treatments were applied (**a**,**c**) without any fungicides or (**b**,**d**) with fungicides. SBDM severity at 94 days after planting was evaluated on a 0–100% scale, in which 0 = healthy plants and 100% = plants completely covered by disease symptoms. Values within each graph that are followed by a different lower-case letter and values followed by a capital letter in each pair of treatments without and with fungicides are significantly different according to one-way ANOVA with Tukey's HSD test. Default significance levels were set at $p \leq 0.05$. Bar = SE. DW—dry weight.

Table 3. Concentrations of nutritional elements in the shoots of sweet basil plants grown under commercial conditions and treated with Zn and Mn (applied through the irrigation solution) at 90 days after planting (Experiments C5, Autumn 2017, 2018).

Microelement			Concentration in Shoots (mg/kg DW)	
	In Irrigation Solution (mg/L)	**Spray (%)**	**Zn**	**Mn**
None	0	0	60.0 ± 3.90 c	71.4 ± 7.82 d
Zn	1		102.0 ± 6.03 a	70.5 ± 5.61 d
Mn	2		56.6 ± 4.65 c	94.2 ± 4.87 c
Zn + Mn	1 + 2		101.4 ± 11.73 a	102.1 ± 10.08 bc
Zn		0.006	76.0 ± 5.07 b	79.5 ± 4.37 d
Mn		0.014	59.5 ± 6.78 c	116.3 ± 14.06 ab
Zn + Mn		0.06 + 0.014	98.0 ± 4.77 a	124.8 ± 8.95 a

Values for each Mg treatment and each microelement treatment that are followed by a different letter are significantly different according to one-way ANOVA with Tukey's HSD test.

The addition of Zn and Mn to the irrigation solution significantly suppressed SBDM severity at 94 days after planting, but the combination of Zn with Mn did not result in additional SBDM

reduction (Figure 10). The fungicides treatment significantly reduced disease and the combination of microelements and fungicide reduced disease significantly, as compared with the fungicides treatment alone (Figure 10).

Synergism was obtained between each of the supplemental −Zn, −Mn and −Zn + Mn treatments, and the fungicides treatment (Table 4). Differences in shoot yield were insignificant (results not presented).

Table 4. Synergy factors (SF) of irrigation or spray treatments and foliar fungicides treatment at 94 days after planting.

Irrigation Supplement		Spray Treatment	
2 mg/L Mn	1.16	0.014% Mn	1.02
1 mg/L Zn	1.12	0.006% Zn	1.08
2 mg/L Zn + 1 mg/L Mn	1.20	0.006% Zn + 0.014% Mn	1.12

The combined effects of the microelements and fungicides were estimated using the Abbott formula; SF = the synergy factor achieved by the combined treatment. When SF = 1, the interaction between the control measures is additive; when SF < 1, the interaction is antagonistic and when SF > 1, the interaction is synergistic.

3. Discussion

We recently demonstrated that the addition of Ca and Mg to the irrigation solution can reduce the severity of SBDM. We also demonstrated that although increased K concentration in the fertigation solution increased SBDM severity, foliar applications of KCl and K_2SO_4 suppressed SBDM [21]. In the present study, the effects of the microelements Mn, Zn, Cu and Fe (individually and in combination) on SBDM were tested. We found that even under commercial-like conditions, Zn and Mn provided a consistent, effective control of SBDM. The microelements that were applied at very low concentrations (i.e., 0.006–0.014% in the foliar treatments, and 1–2 mg/L in the irrigation solution) provided effective disease control.

There are no examples of previous research regarding the effects of microelements on SBDM or downy mildew of other crops. However, the effects of microelements have been examined in other pathosystems. For instance, foliar applications of B, Mn and Zn were tested for the control of tan spot disease (*Drechslera tritici-repentis*) in field-grown winter durum wheat. Following the foliar application of these microelements, the flag leaves of the treated plants had significantly fewer tan spots than those of the untreated plants. In general, there were minor differences between the effects of the Mn and Zn treatments. Similar to the present study, the treatments did not significantly affect wheat yield components [26]. Research with coffee rust (*Hemileia vastatrix*) yielded similar results; B, Zn, and Mn supplied to coffee plant (*Coffea* sp.) seedlings, reduced the severity of coffee rust on the leaves [27]. Severity of potato early blight (*Alternaria grandis*) was decreased in the potato (*Solanum tuberosum*) canopy when plants were treated with Zn and B + Zn. The authors of that work suggested that Zn plays a critical role in potato tolerance to early blight, and should be considered as a method for the control of potato early blight [28].

Increased levels of iron sulfate have been shown to decrease the severity of take-all, wheat crown and root rot caused by the soilborne fungus *Gaeumannomyces graminis,* and to enhance the biomass of the aerial parts of infected wheat plants. The effects of Zn and Cu fertilizers on the suppression of disease in wheat were weaker than the effects observed for Fe [29]; thus, our results are only partially similar to those of the wheat take-all research. Interestingly, wheat plants grown from seeds with a higher Mn content in soils that were naturally infested with *G. graminis*, were generally more vigorous and had an average of 11% less take-all [30]. Earlier, Brennan [31] performed five field experiments using five different levels of $MnSO_4$ fertilizer, and found that take-all severity was decreased at two trial sites. In plots in which Mn levels were deficient, the application of Mn lowered the severity of take-all, had no effect on the incidence of take-all disease and increased the dry-matter and grain yields of the wheat plants. No beneficial effects were observed when Mn was added to soils that already contained adequate levels of Mn.

In another study involving bread wheat and durum wheat genotypes and another soilborne pathogen, *Fusarium solani*, the application of Zn had a positive effect on the plants' resistance to *F. solani*

root rot. Those researchers not only suggested that Zn nutrition can increase resistance to *F. solani*, but also that Zn deficiency should be avoided, in order to prevent susceptibility [32]. In contrast, a high foliar concentration of Zn, achieved through fertigation, increased the susceptibility of rice (*Oryza sativa*) to rice brown spot (*Bipolaris oryzae*) [33]. Nevertheless, in upland rice, low severity levels of panicle blast (*Pyricularia grisea*) were associated with higher concentrations of Zn and K in the plant tissue; no correlations were observed between the concentrations of other micronutrients in plant tissues and panicle blast severity [34].

In other research, cucumber (*Cucumis sativus*) plants were sprayed with $MnSO_4$ four and six days before they were inoculated with *Podosphaera fuliginea* (the causal agent of powdery mildew) [35] and cucumber plants in a hydroponic system were sprayed four days before inoculation with the same pathogen [36]. In both of those studies, the Mn salt reduced the severity of cucumber powdery mildew. In a later study, foliar applications of $MnSO_4$ to cucumber plants either before or after inoculation with *Colletotrichum lagenarium*, the causal pathogen of cucumber anthracnose disease, also suppressed the fungal infection of leaves and cotyledons. In that study, better disease control was observed when the treatment was applied three days prior to inoculation. All applied concentrations of Mn reduced disease severity to a similar degree [37].

The integrated effect of Zn applied with other nutrients on early blight (*Alternaria solani*) of tomato (*Solanum lycopersicum*) was also examined. In this patho-system, Zn applied in combination with Mg sometimes had no beneficial effect, and other times had a synergistic disease-control effect. Zn applied in combination with N, P and K had a significant synergistic effect on early blight [38]. In the present research, however, combining Mg with Zn and Mn in the irrigation solution did not provide any further reduction in disease severity. This is similar to the findings of our recent study, in which the combination of Ca and Mg did not provide improved SBDM control [21]. Moreover, in the present research, the combination of Zn and Mn did not provide better control than that provided by each microelement alone, regardless of whether the microelements were applied through the irrigation solution or as a foliar spray (Experiment C5). The combination of the tested microelements had a beneficial effect in only some of the commercial-like experiments; the disease control provided by Zn was synergistic with the fungicides treatment in Experiments C4 and C5. The control provided by Mn was synergistic with the fungicides treatment in Experiment C5. Therefore, a general conclusion regarding the benefit of this combination cannot be stated.

The application of Zn in combination with N, P and K for the potential control of early blight (*A. solani*) of tomato was associated with an increase in the total phenolic content and phenylalanine ammonia lyase (PAL) activity in plants. This suggests that these nutrients may have an additive effect on the production of salicylic acid, which may help to induce systemic resistance against a pathogen attack [38]. Similarly, in the coffee rust patho-system mentioned above, B, Zn and Mn significantly affected the concentration of total soluble phenols, while only Mn influenced the concentration of lignin [27]. The research that revealed that the foliar application of Mn could reduce the severity of cucumber powdery mildew [35,36] also involved a mechanistic study. A pre-inoculation foliar application of $MnSO_4$ increased phenol oxidase activity and polyphenol oxidase activity in the treated leaves [35]. Moreover, the increased lignin, cellulose and pectin contents of the cell walls improved the leaf water status, which led the authors of that work to conclude that Mn nutrition could control cucumber powdery mildew by reinforcing the cell-wall structure and reducing the loss of water from infected leaves [36]. Similarly, Mn treatment of cucumber plants in the absence of a pathogen promoted lignification and the accumulation of ROS. A pre-inoculation Mn treatment of cucumber plants infected with *Colletotrichum lagenarium* enhanced pathogen-induced lignification, callose or ROS production, and reduced pathogen-induced cell death [37], indicating a role for Mn in induced-resistance mechanisms.

Foliar-applied Mn ameliorated the negative effects of orange rust (*Puccinia kuehnii*) on sugarcane (*Saccharum officinarum*). This effect was accompanied by increased sugarcane biomass production, and, in that system, Mn treatment caused direct damage to the fungal spores and improved lignin deposition in the mesophyll of the plants. During pathogenesis, Mn-sprayed leaves exhibited lower levels of oxidative stress, in addition to an improved structural organization of xylem and phloem vessels, as compared to the untreated control [39]. Since induced resistance may have a role in disease suppression by nutritional elements, further research should be directed towards this mode of action in various plant species.

4. Materials and Methods

4.1. Plants, Pathogen and Growing Conditions

Sweet basil (*Ocimum basilicum*) cv. Peri [40] was used for all of our experiments. The seedlings were grown in a commercial nursery (Shorashim, Mivtahim, Israel) and transplanted for the experiments 3 to 4 weeks after seeding in the nursery. The 'Peri' cultivar is known to be susceptible to *Peronospora belbahrii* [24]. Plugs of sweet basil seedlings were used, each containing three to five plants; hereafter, one plug is referred to as a "plant" as is common practice [41]. The experiments involving potted plants were performed in an experimental greenhouse at the Volcani Institute, Rishon LeZion, Israel (Site A). Experiments were also carried out using plants grown in containers under semi-commercial greenhouse conditions at the Volcani Institute, Rishon LeZion, Israel (Site B) and at the Tzvi R&D Experimental Station, Jordan Valley, Israel (Site C).

The sites of experimental work, the host plant and disease, plants maintenance, experimental designs, nature of data collected and statistical analysis were the same as described in earlier publications about parallel research projects [21,22]. Since experiments of the different research projects were carried out in parallel to each other in similar greenhouses at the same locations with similar planting dates, then technical details of the experiments were similar. In order to keep the research presented here independent, all technical issues are described below in detail as was earlier described for the parallel research works and described by Elad et al. [21,22].

At Site A, experiments were carried out in 2 L pots, each containing one plant. At Site B, experiments were carried out in polystyrene containers that were 0.8 × 0.8 × 0.17 m in size, with 12 plants in each container. Each container served as a replicate. At Site C, the experiments were carried out in 0.8 × 1.0 × 0.17 m containers; each experimental plot consisted of three containers and 24 plants were planted in each container (i.e., 72 plants per replicate). Sweet basil plants were planted in the pots and containers, which were filled with perlite (medium size, 1.2 mm, Agrifusia, Fertilizers & Chemicals Ltd., Haifa, Israel). For the potted-plant experiments involving foliar treatments, we used a potting mixture consisting of coconut fiber:tuff (unsorted to 8 mm; 7:3 vol.:vol.). The plants were irrigated to excess via a drip system two to four times a day, depending on the season, at a volume calibrated to lead to >30% water leaching. The daily irrigation volume was determined after analyzing the irrigation and drainage solutions once every 2 weeks, to prevent over-salinization or acidification of the root-zone solution. Plants in pots and containers were maintained according to the local extension service's recommendations. All pot experiments were irrigated with fresh water (electrical conductivity (EC) < 1.0 dS/m). The tested elements were applied with the irrigation water (fertigation) or as a foliar spray, as described below and summarized in Table 5.

Table 5. Experimental setup, factors tested, application methods and growing seasons [1].

Site	Expt. Code	Growing Setting	Microelements Tested	Additional Treatment	Application	Season
A	A1	Pots	Mixture (Koratin)		Spray	Autumn, Spring
A	A2	Pots	Fe, Cu, Zn, Mn		Spray	Autumn, Spring
B	B	Containers (semi-commercial)	Zn, Mn		Spray	Autumn, Spring
C	C1	Containers (commercial-like)	Mixture (Koratin)	+/− Fungicides	Spray	Autumn
C	C2	Containers (commercial-like)	Zn, Mn	Fungicides	Spray	Spring
C	C3	Containers (commercial-like)	Zn, Mn Zn, Mn Zn, Mn	Mg +/− Fungicides +/− Fungicides	Irrigation (Irr.) Irr., Spray Spray	Autumn
C	C4	Containers (commercial-like)	Zn, Mn Zn, Mn Zn, Mn	Mg +/− Fungicides +/− Fungicides	Irrigation Irr., Spray Spray	Spring
C	C5	Containers (commercial-like)	Zn, Mn, Zn+Mn Zn, Mn, Zn+Mn	+/− Fungicides +/− Fungicides	Irr., Spray Spray, Spray	Autumn

[1] Experiments A were performed three times and B were performed twice with 5–7 replicates each time.

The oomycete inoculum originated from an experimental station in Rehov, Emek Hamaayanot, Israel [24] and was maintained on sweet basil plants. Conidia of *P. belbahrii* were collected in water by washing conidiating leaves of sweet basil plants that were kept in an experimental greenhouse at the Volcani Institute. The canopy of potted sweet basil plants was inoculated with a conidial suspension that contained 10^3 cell ml^{-1} during the afternoon of the inoculation date. For symptom development, the plants were incubated at high RH (>95%) in the dark in a growth chamber at 22 ± 1 °C for 12 h, incubated in a greenhouse chamber at 22 ± 2 °C for 1 week, incubated at high RH (>95%) in the dark in a growth chamber at 22 ± 1 °C for 12 h and then incubated in a greenhouse chamber at 22 ± 2 °C. Potted sweet basil plants subjected to this artificial inoculation served as inoculum sources to ensure even inoculum loads across the greenhouse in the potted-plant experiments (Site A) and the semi-commercial container experiments (Site B). Disease severity (i.e., chlorosis, dry necrotic lesions and/or sporulation on the lower leaf side) was evaluated in the sweet basil canopy according to a 0–100 scale, in which 0 = no signs or symptoms and 100 = entire surface displays signs and/or symptoms, while, for instance, 20 and 50 are 20 and 50% canopy coverage by symptoms, respectively [21,22,24].

In the commercial-like experiments (Site C), downy mildew epidemics developed naturally during every experiment. Disease-severity evaluation in the field plots included all plants except for those along the edges of each plot. Disease severity was determined every 2 to 3 weeks in each plot of each experiment on a scale of 0 to 100, in which 0 = all plants visually healthy and 100 = all leaves on all plants in the plot showing typical downy mildew symptoms/signs of chlorosis, dry necrotic lesions and/or conidiating *P. belbahrii* on their lower side. Twenty and 50 levels of severity are 20 and 50% canopy coverage by symptoms, respectively [21,22,24].

Shoots that were >15 cm long were harvested and weighed. In the potted-plants experiment, yield was measured for each pot. In the semi-commercial and commercial-like experiments, shoots were harvested from each plot separately. The harvested shoots were sorted by quality and calculated Grade A yield per m^2 figures are presented.

4.2. Foliar Applications of Microelement Chelates to Potted Sweet Basil Plants (Experiment A, Autumn and Spring 2014–2015)

The aim of these experiments was to study the effects of foliar applications of microelements on the development of SBDM in potted sweet basil plants. Treatments consisted of seven sweet basil plants that were grown in 2 L pots filled with potting mixture. The pot experiments were irrigated with fresh tap water (electrical conductivity (EC) < 1.0 dS/m). The plants were irrigated via a drip system three times a day at an excess rate (>30% leaching) using 2 L/h drippers. The daily irrigation volume was set after analysis of the irrigation and drainage solution once every 2 weeks to prevent over-salinization or acidification of the root-zone solution. All of the plants were fertigated with 5-3-8 fertilizer (N-P_2O_5-K_2O; Fertilizers and Chemical Compounds Ltd., Haifa, Israel) at 90 g/m^3 N. The water contained 0.7 mM Ca, 0.62 mM S and 0.95 mM Mg, as well as 0.023 mM B, 9.8 μM Fe, 4.9 μM Mn, 2.1 μM Zn, 0.31 μM Cu and 0.16 μM Mb (Fertilizers and Chemical Compounds Ltd.).

Foliar applications of microelements were made once a week with a 1 L hand sprayer. Plants were sprayed to runoff with the mixture of microelements 0.1–0.4% Koratin (Fertilizers and Chemical Compounds Ltd.). Koratin contains the following chelates: Cu-EDTA (0.2 g/L), Fe-EDTA (5.5 g/L), Zn-EDTA (1.35 g/L), Mn-EDTA (2.7 g/L) and Mo, as $(NH_4)_2MoO_4$) (0.15 g/L). In another set of experiments, the chelates of the same microelements were sprayed individually, after one shoot harvest, at the following concentrations: Cu-EDTA (0.0095 mg/L), Fe-EDTA (0.03 mg/L), Zn-EDTA (0.006 mg/L) and Mn-EDTA (0.014 mg/L). Following two shoot harvests, when the plants in the experiments were 45 to 50 cm tall, they were artificially inoculated with *P. belbahrii* as described above.

4.3. Foliar Applications of Microelement Chelates to Sweet Basil Plants Grown in Containers (Commercial-like Experiment B, Autumn and Spring 2015–2016)

Sweet basil plants grown under semi-commercial conditions were treated with Zn-EDTA and Mn-EDTA over two months. The aim of these experiments was to study the effect of foliar applications of microelements on the development of downy mildew in sweet basil plants grown in polystyrene containers. The sweet basil plants were planted in 0.8 × 1.0 × 0.17 containers filled with perlite (medium size, 1.2 mm, Agrifusia, Fertilizers & Chemicals Ltd., Haifa, Israel), 12 plants (plugs) per container, in five replicates (one container = one replicate). The plants were irrigated with water, as

mentioned above, via a drip-irrigation system three times a day at an excess rate (>30% leaching) using 2 L/h drippers. The daily irrigation volume was set after analysis of the irrigation and drainage solution once every 2 weeks to prevent over-salinization or acidification of the root-zone solution. All of the plants were fertigated with 5-3-8 fertilizer (N-P_2O_5-K_2O) at 90 g/m^3 N (Fertilizers and Chemical Compounds Ltd., Haifa, Israel). The water contained 0.7 mM Ca, 0.62 mM S and 0.95 mM Mg, as well as 0.03 mM B, 9.8 μM Fe, 4.9 μM Mn, 2.1 μM Zn, 0.31 μM Cu and 0.16 μM Mb (Fertilizers and Chemical Compounds Ltd.).

Foliar applications of microelements were conducted once a week with a backpack sprayer equipped with a conical nozzle. In one set of experiments, Koratin (0.1%) was sprayed to runoff. In a second set of experiments, Zn-EDTA (0.014 mg/L) and Mn-EDTA (0.006 mg/L) were applied (Fertilizers and Chemical Compounds Ltd., Haifa, Israel). Following two shoot harvests, when the plants in the experiments were 45 to 50 cm tall, they were artificially inoculated with *P. belbahrii* as described above.

4.4. Control of SBDM under Commercial-like Conditions (Experiments C)

At Site C, experiments were carried out in a polyethylene-covered greenhouse. Plants were irrigated daily according to local extension service recommendations. During the initial 5 days, plants were sprinkler-fertigated with 4.3 mM N (10% NH_4^+), 1.6 mM K and 0.65 mM P in the fertigation solution to aid their establishment. After that initial period, the plants were irrigated through drippers and fertilized with 8.57 mM N, 3.2 mM K and 0.65 mM P in water until the fertigation treatments were initiated, as described below. Throughout the growing season, the irrigation solution also contained 2 mM Ca, 1.65 mM Mg (unless supplemented to reach 4.94 mM Mg as mentioned below) and 0.09 mM B. Fertigation was performed from 1000 L tanks dedicated to each treatment, with a 17 mm drip-irrigation pipe that had a 2 L/h dripper embedded every 20 cm along its length. Spray treatments were made using a backpack sprayer equipped with a conical nozzle. Sprays were administrated until runoff once or twice a week. Experiments were carried out in the autumn (September–January) or spring (February–June) growing seasons. Each experiment was conducted in randomized blocks with four replicates. Each replicate consisted of three containers [21,22].

SBDM usually appeared 45–60 days after planting, reached a peak of severity and then began to become less severe. In the spring growing season, SBDM severity decreased to a low level due to the high temperatures and low humidity typical of the summer. In the autumn growing season, SBDM severity was low due to low temperatures [21,22]. The results from the greenhouse experiments refer to SBDM severity at a certain time after planting or to AUDPC over a certain period.

Chemical fungicides treatments included a rotation of two types of treatments. The first type of treatment was administered soon after harvest and consisted of a foliar application of a mixture of two fungicides: Canon (potassium phosphite 780 g/L, Luxembourg Industries (Pamol) Ltd. Tel Aviv, Israel applied at 0.3%) + Cabrio Duo (dimethomorph 72 g/L + pyraclostrobin 40 g/L) BASF, Ludwigshafen, Germany applied at 0.05%)) or, alternatively, Infinito ((fluopicolide 62.5 g/L + propamocarb-HCL 625 g/L), Bayer AG, Germany, applied at 0.1%). The second type of treatment was a foliar application of Canon at 1 week before harvest.

4.4.1. Effects of Foliar-Applied Microelements under Field Conditions (Experiment C1, Autumn 2015–2016)

To examine the effects of the microelements on SBDM severity during the autumn–winter season, sweet basil was planted on 1 September 2015. The fertilizer mentioned above for fertigation with irrigation water was used in all treatments. Sweet basil plants were spray treated to runoff with the mixture of microelements 0.2% Koratin (Fertilizers and Chemical Compounds Ltd., Haifa, Israel) once a week. Koratin contains the following chelates: Fe-EDTA (5.5 g/L), Mn-EDTA (2.7 g/L), Zn-EDTA (1.35 g/L), Cu-EDTA (0.2 g/L) and Mo, as $(NH_4)_2MoO_4$) (0.15 g/L).

Fungicides treatments were as described above. The EC of the fertigation solutions in the six treatments ranged from 1.91 to 2.45 dS/m and their pH values were 6.9 ± 0.1. Analysis of the nutritional elements was carried out at 94 days after planting (3 December 2013). Concentrations of the nutritional elements K, Ca, Mg and Cl in the shoots ranged between 3.34 and 4.90, 3.43 and 3.69, 0.42 and 0.52, and 0.66 and 0.82, respectively. Microelement concentrations in the shoots are presented in the Results

(Figure 3). The sweet basil shoots were harvested on 14 October 2015, 1 November 2015 and 7 January 2016. Analysis of nutritional elements in sweet basil shoots was carried out on 3.12.15.

4.4.2. Effects of Foliar-Applied Microelements under Commercial-like Conditions (Experiment C2, Spring 2016)

To test the effects of Zn and Mn on SBDM, sweet basil was planted on 23 February 2016. Fertigation started on 10 March 2016 with the basic fertilizer mentioned above and was the same in all treatments. Spray treatments with the microelement chelates Zn-EDTA (0.006%) and Mn-EDTA (0.014%) were applied weekly and fungicides were applied as described above; treatments were initiated on 24 April 2016.

Analysis of the nutritional elements was carried out at 69 days after planting (2 May 2016). The concentrations of N, P, K, Ca, Mg, Na and Cl in the shoots ranged between 4.06 and 4.31, 0.725 and 0.797, 4.75 and 5.53, 3.14 and 4.35, 0.80 and 0.85, 0.049 and 0.076, and 1.32 and 1.56%, respectively. The concentrations of Zn and Mn in the shoots are presented in the Results Section. The concentration of Fe in the shoots ranged between 110.5 and 117.6 mg/kg dry weight. The sweet basil shoots were harvested on 2 May, 19 May, 9 June, 5 July and 27 July.

4.4.3. Effects of Zn and Mn Applied as Foliar Sprays or in the Irrigation Solution under Commercial-like Conditions (Experiment C3, Autumn 2016)

To test the effects of Zn and Mn on sweet basil downy mildew in an autumn crop, sweet basil was planted on 21 September 2016. Fertigation treatments were started on 28 September 2016. The basic fertilizer mentioned above was used in all treatments. The basic Mg concentration was 1.65 mM (40 mg/L) and an additional treatment of 4.94 mM (120 mg/L) Mg was made by adding $MgCl_2$ to the irrigation solution [21]. The microelements were added to the irrigation solutions containing each of the Mg fertigation levels. Zn and Mn were added to the irrigation solutions at concentrations of 1 and 2 mg/L, respectively. Spray applications of 0.18% solutions of Zn and Mn were applied weekly to plots irrigated with a solution that contained 1.65 mM Mg. Fungicides treatments were as mentioned above and were applied in combination with the microelement fertigation treatments. The +/− fungicides treatments were located adjacent to one another. Fungicides were sprayed in the experimental plots starting from 16 November 2016. The sweet basil shoots were harvested on 26 October, 15 November and 19 December.

The EC of the fertigation solutions for the three Mg treatments ranged from 1.91 to 2.74 dS/m and their pH values ranged between 6.70 and 7.03. An analysis of nutritional elements in the shoots was carried out 55 days after planting (16 November 2016). The concentrations of N, P, K, Na and Cl in the shoots ranged between 4.57 and 5.67, 0.85 and 1.03, 4.13 and 4.70, 3.14 and 4.35, 0.07 and 0.08, and 0.77 and 1.47%, respectively. The concentrations of Zn and Mn in the shoots are presented in the Results Section (Table 1). The concentration of Fe in the shoots ranged between 121.1 and 151.6 mg/kg dry weight.

4.4.4. Effects of Zn and Mn Applied under Field Conditions as Foliar Sprays or through the Irrigation Solution (Experiment C4, Spring 2017)

To test the effects of Zn and Mn on sweet basil downy mildew in the spring season, sweet basil was planted on 28 February 2017. Fertigation treatments started on 25 April 2017. The basic fertilizer described above was used in all treatments. The basic Mg concentration was 1.65 mM and an additional treatment of 4.94 mM was made by adding $MgCl_2$ to the irrigation solution. The microelements added to the irrigation solution were 1 mg/L Zn-EDTA and 2 mg/L Mn-EDTA. Fungicides were sprayed in the plots from 30 May 2017 and Mn-EDTA (0.006%) and Zn-EDTA (0.014%) sprays were begun on 29 May 2017. The plots that received the +/− fungicides treatments were located adjacent to one another. Sweet basil shoots were harvested on 8 May, 29 May and 18 June.

The EC of the fertigation solutions for the two Mg treatments ranged between 1.85 and 2.87 dS/m and their pH values ranged from 6.95 to 7.12. An analysis of nutritional elements in the shoots was carried out at 90 days after planting (29 May 2017). The concentrations of N, P, K, Ca, Na and Cl in the shoots ranged between 4.68 and 4.95, 0.83 and 0.95, 4.90 and 6.02, 3.36, and 3.77, 0.07 and 0.08, and 1.15 and 2.03%, respectively. The concentrations of Fe and Cu in the shoots ranged between 95.6 and 106.7,

and 10.7 and 12.6 mg/kg dry weight. The concentrations of Zn and Mn in the shoots are presented in the Results Section (Table 2).

4.4.5. Effects of Zn and Mn Applied as Foliar Sprays or in the Irrigation Solution under Commercial-like Conditions (Experiment C5, Autumn 2017–2018)

To test the effects of Zn and Mn on sweet basil downy mildew in the spring season, sweet basil was planted on 26 September 2017. Fertigation treatments started on 25 October 2017. Fungicides sprays (as described above) were initiated on 9 November 2017 and microelements were applied individually or in combination (Mn-EDTA (0.006%) and Zn-EDTA (0.014%)), starting on 8 November 2017. The plots that received the +/− fungicides treatments were located adjacent to one another. The sweet basil shoots were harvested on 29 October 2017, 27 December 2017 and 24 January 2018.

The EC of the fertigation solutions were between 1.91 and 2.02 dS/m and their pH values ranged from 6.75 to 6.98. An analysis of the nutritional elements in the shoots was carried out at 92 days after planting (27 December 2017). The concentrations of N, P, K, Ca, Mg, Na and Cl in the shoots ranged between 3.97 and 4.73, 0.70 and 0.89, 4.83 and 6.05, 2.30 and 3.06, 0.47 and 0.80, 0.050 and 0.071, and 0.73 and 0.87%, respectively. The concentration of Fe and Cu in the shoots ranged between 87.4 and 106.1, and 10.4 and 11.9 mg/kg dry weight. The concentrations of Zn and Mn in the shoots are presented in the Results Section (Table 3).

4.5. Analysis of Elements

Shoots were sampled randomly in all experiments at harvest time from potted plants and from the commercial-like experiments for the determination of their mineral concentrations. The shoots were rinsed with distilled water and dried in an oven at 70 °C for 48 h. The dried plant material was ground and subjected to chemical analysis. The N and P concentrations in the shoots were determined after digestion with sulfuric acid and peroxide [42]. The concentrations of N and P were determined with an autoanalyzer (Lachat Instruments, Milwaukee, WI, USA). Concentrations of K, Na, Mg, Ca and microelements were analyzed with an atomic absorption spectrophotometer (Atomic Absorption PerkinElmer 460, Norwalk, CT, USA) after digestion with nitric acid and perchlorate spectrophotometry [43].

4.6. Data Analysis

The correlations between the concentration of a nutritional element and disease severity or between shoot concentrations of two selected nutritional elements were calculated using all individual pairs of data. Linear, exponential, logarithmic and polynomial correlations were examined. The formulas describing these correlations, correlation coefficient (r) values and α significance levels are noted in the Results Section.

Data in percentages were arcsine-transformed before further analysis. Area under the disease progress curve (AUDPC) values were calculated. Standard errors (SE) of means were calculated and are presented alongside with the number of degrees of freedom (df = $n - 1$ for controlled conditions experiments and df = $n - 2$ for correlations calculated for field conditions data). Disease-severity data and AUDPC data were analyzed by ANOVA and Tukey's HSD test. Statistical analysis was performed using JMP 14.0 software (SAS Institute, Cary, NC, USA).

Disease reduction was calculated as follows:

$$\%\ \text{disease reduction} = 100 - 100 \times (\text{disease severity}_{\ T}\ /\text{disease severity}_{\ \text{control}})$$

where T = the disease level in the treatment and control = the disease level in the untreated control.

The combined effect of the control measures used was estimated using the Abbott formula [44,45]. The expected disease reduction (control efficacy) and the combined suppressive activity were calculated as:

$$CE_{exp} = a + b - a \times b/100 \text{ and } SF = CE_{obs}/CE_{exp}$$

where a = disease reduction due to one measure when applied alone; b = disease reduction due to the other measure when applied alone; CE_{exp} = expected control efficacy of the combined treatment, if the two measures act additively; CE_{obs} = observed disease reduction of the combined treatment; SF = the

synergy factor achieved by the combined treatment. When SF = 1, the interaction between the control measures is additive; when SF < 1, the interaction is antagonistic and when SF > 1, the interaction is synergistic [44–46]. The same formula was used to calculate SF in the context of yield.

5. Conclusions

Zn and Mn, sprayed on plants or applied as part of the irrigation solution, contributed to the suppression of SBDM. The mode of action of this disease suppression merits further investigation. Similar to the further investigation of the suppression of SBDM by Mg, Ca and K [21], such future research should address possible host plant responses to the microelements and pathogen, including the upregulation of induced resistance pathways. The microelements are not toxic to the pathogen and the fact that only low concentrations of Zn and Mn are needed to reduce disease severity suggests that changes in plant host susceptibility may play a role in the observed effects.

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The Endophytic *Pseudomonas* sp. S57 for Plant-Growth Promotion and the Biocontrol of Phytopathogenic Fungi and Nematodes

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Abstract: Oregano from Socoroma (Atacama Desert) is characterized by its unique organoleptic properties and distinctive flavor and it is produced using ancestral pesticide-free agricultural practices performed by the Aymara communities. The cultivation in this zone is carried out under extreme conditions where the standard production of different crops is limited by several environmental factors, including aridity, high concentration of salts, and boron among others. However, oregano plants are associated with microorganisms that mitigate biotic and abiotic stresses present in this site. In this work, the S57 strain (member of the *Pseudomonas* genus that is closely related to *Pseudomonas lini*) was isolated from roots of oregano plants, which are grown in soils with high content of non-sodium salts and aluminum. This bacterium stimulates the growth of Micro-Tom tomato plants irrigated with saline-boric water. Moreover, it controls the growth of phytopathogenic fungi *Fusarium oxysporum* and *Botrytis cinerea* and the nematode *Meloidogyne incognita* under saline-boric conditions. Together with the high levels of bacterial biomass (~47 g/L), these results allow the establishment of the bases for developing a potential new agricultural bioproduct useful for arid and semiarid environments where commercial biological products show erratic behavior.

Keywords: Atacama Desert; endophyte; plant-growth promoting bacteria; biocontrol; pre-Andean agriculture

1. Introduction

Socoroma (in Aymara *Chukuruma*; running water) Valley (18°15′37″ S, 69°36′24″ W) is located at the Andean pre-mountain range of the Arica and the Parinacota Region at 3070 m.a.s.l. in the Commune of Putre in the extreme north of the Atacama Desert, Chile [1]. This valley is characterized by the cultivation of oregano [2], which possesses unique organoleptic properties, possesses geographical indication distinction and granted the seal of origin by the National Institute of Intellectual Property, INAPI, Chile [3]. Particularly, oregano cultivation is performed under ancestral agricultural practices, in which crops are maintained employing pesticide-free applications, harboring unexplored microbial genetics resources associated with this type of plants. Microbial communities associated to plant tissues (plant microbiome) are considered beneficial because they contribute to the plant by mitigating biotic and abiotic stress conditions [4,5]. Thus, this allow plants to survive under the extreme conditions that prevail in this region, including aridity, high concentrations of salts and boron, a wide thermal amplitude between the day and night, poor soil composition, and high UV radiation among other

extreme environmental conditions which are restrictive for agricultural activities in arid and semiarid regions [6,7].

Under the extreme conditions of this zone, plant-associated bacteria play a crucial role in maintaining the proper functioning of plants [8]. These bacteria are known as plant-growth-promoting rhizobacteria (PGPR) and correspond to a free-living soil bacteria with the ability to colonize the rhizosphere and roots, which promotes the growth through the release of metabolites that act directly in plants [9] including the production of phytohormones, such as indole-3-acetic acid (IAA), gibberelins, and cytokinins [10,11]; and the fixation of elemental nitrogen [12]. Furthermore, the solubilization of minerals also occur, such as inorganic phosphate and iron [13,14], and the biological control of phytopathogenic organisms through the production of inhibitory compounds and hydrolytic enzymes also occur [15].

Beneficial PGPR associated with oregano plants from Socoroma could increase interest due to the extreme conditions where they are cultivated. Under this type of conditions, bacteria could confer tolerance to the different stresses to which oregano crops are naturally subjected to, which allows plant survival [11]. Although different bacteria have been isolated from several plants [16], PGPR associated with oregano from Socoroma have been scarcely described and harbors a high potential to characterize the plant-microorganism interaction under extreme conditions of cultivation, to describe adaptation mechanisms to this kind of environment, and to exploit new natural compounds for biotechnological and agricultural purposes. This study was focused on the isolation, identification, and in vitro and in planta functional characterization of the *Pseudomonas* sp. strain S57, which was obtained from oregano roots from Socoroma. The S57 isolate was selected from the plant-associated bacterial culture collection of the Laboratory of Plant Pathology and Bioproducts (Universidad de Tarapacá, Chile) because it possesses promissory plant-growth promoting activities making it an excellent candidate for the development of a new bioproduct. This strain is closely related to *Pseudomonas lini* and qualitative functional analysis revealed that this bacterium possesses plant-growth-promoting activities and a wide range of antifungal properties against several phytopathogenic fungi, including under saline-boric conditions. Furthermore, bacterial cultures were optimized in a flask and bioreactor to produce high quantities of biomass to perform the experimental procedure in Micro-Tom tomato plants and not only to establish the baseline for the development of a new bioproduct tolerant relative to the extreme conditions present in the valley of Arica and Parinacota Region, Chile, but also relative to arid and semiarid environments.

2. Results

2.1. Soil Samples Characterization

Three samples were taken from different sites in Socoroma to characterize arable soils and to establish the extreme conditions where oregano plants are cultivated (Table 1). The S1 sample showed high levels of total nitrogen, phosphorous, potassium, boron, aluminum, and copper and accepted values of sulfur and sodium were found. For the S2 sample, excessive levels of total nitrogen, phosphorous, potassium, sulfur, aluminum, and copper were detected. Meanwhile, boron and sodium levels were in accepted values. S3 sample showed high levels of potassium and aluminum; deficient levels of total nitrogen, sulfur, and boron; and accepted values for phosphorous, copper, and sodium. The NPKS content in the three samples showed a direct relationship with the organic matter content. All the samples showed high electrical conductivity, suggesting non-sodium highly saline soils due to the low concentration of sodium. Measured pH values indicated the presence of strong acid soils for S1 and S2 samples and slightly acid soil for S3.

Pesticide residues analysis (not showed) included the detection of 163 insecticides, acaricides, and nematicides; 74 herbicides and plant growth regulators; and 93 fungicides. No pesticide residues were detected in the three samples, which confirms free-pesticides agricultural practices.

Table 1. Composition of soil samples from Socoroma.

Parameter	S1	S2	S3
GPS Location	**18°15′13.3″ S 69°36′36.0″ W**	**18°15′14.8″ S 69°36′46.3″ W**	**18°15′50.1″ S 69°35′°39.0″ W**
Altitude (m.a.s.l)	2909	2892	3090
Total N (%)	0.37	0.20	0.07
P (mg/kg)	77.30	57.00	21.49
K (mg/kg)	0.85	0.79	0.77
S (mg/kg)	24.00	44.50	16.00
B (mg/kg)	13.00	0.68	0.04
Al (cmol/kg)	11.90	5.12	0.12
Cu (mg/kg)	4.11	3.70	0.80
Na (cmol/kg)	0.28	0.33	0.30
pH	4,66	4.75	6.11
Organic matter (%)	4.87	2.58	0.79
Electrical conductivity (dS/cm)	87.6	116.2	102.2

2.2. S57 Strain Characterization and Identification

The S57 strain was isolated as an endophytic bacterium from roots of oregano plants from Socoroma. Microbiological characterization (Table 2) showed Gram negative straight rod cells, which are motile in semi-solid media and able to produce gas after the addition of 3% H_2O_2, which indicates the production of catalase enzyme. The colonies were yellowish, circular, and convex with regular margins after 2 days of growth on King's medium B agar. This bacterium produced acids from D-mannitol, D-glucose, D-fructose, D-galactose, D-rhamnose, D-melibiose, sucrose, and lactose, but no acid production was detected when D-sorbitol and D-cellobiose were used as carbon sources.

Table 2. Microbiological characterization of the S57 strain. A positive reaction is indicated as +; meanwhile, no reaction is indicated as −.

Parameter	S57
Gram staining	−
Morphology	Straight rods
Motility test	+
Catalase	+
Acid production from:	
D-Sorbitol	−
D-Mannitol	+
D-Glucose	+
D-Fructose	+
D-Galactose	+
D-Rhamnose	+
D-Melibiose	+
D-Cellobiose	−
Sucrose	+
Lactose	+

Antibiotic susceptibility of the S57 strain was performed by the disk diffusion method (Table 3), observing that this bacterium was sensitive to chloramphenicol (50 μg), ciprofloxacin (1 and 10 μg), and kanamycin (30 μg). S57 showed intermediate resistance

to chloramphenicol (10 μg), kanamycin (5 μg), and neomycin (10 and 30 μg). Differences in the susceptibility using chloramphenicol and kanamycin could be explained by a lesser diffusion of the antibiotic subjected to compound concentration. Moreover, this strain was resistant to amoxycillin, ampicillin, and penicillin G, which is expected due to β-lactam antibiotics being mainly active against Gram positive bacteria.

Table 3. Antibiotic susceptibility test of the S57 strain. Two antibiotic quantities were employed to determine differences in the behavior of S57 bacterium in the susceptibility test. Error corresponds to the standard deviation of the independent assays.

Antibiotic	Quantity	Inhibition Diameter (cm)	Susceptibility
Amoxicillin	2 μg	0.0	Resistant
	25 μg	0.0	Resistant
Ampicillin	2 μg	0.0	Resistant
	25 μg	0.0	Resistant
Chloramphenicol	10 μg	12.3 ± 0.4	Intermediate
	50 μg	24.3 ± 1.0	Sensitive
Ciprofloxacin	1 μg	32.0 ± 0.5	Sensitive
	10 μg	40.7 ± 0.3	Sensitive
Kanamycin	5 μg	15.0 ± 1.0	Intermediate
	30 μg	22.3 ± 0.4	Sensitive
Neomycin	10 μg	10.3 ± 0.4	Intermediate
	30 μg	14.0 ± 0.0	Intermediate
Penicillin G	1 U	0.0	Resistant
	10 U	0.0	Resistant

Sequencing and comparison of 16S rRNA gene to sequences deposited in GenBank revealed that the S57 strain is a member of *Pseudomonas* genus and the phylogenetic analysis using the Neighbor-Joining method (Figure 1) showed the S57 strain was closely related to the PGPR *Pseudomonas lini*.

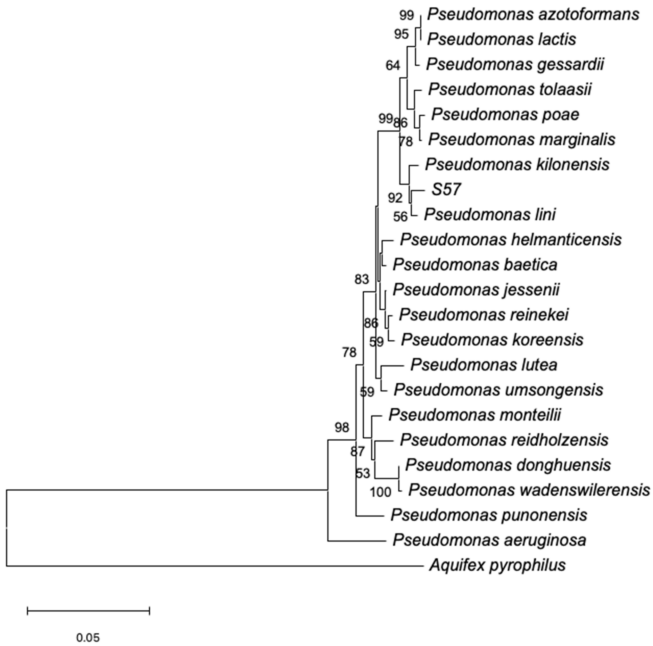

Figure 1. Neighbor-joining tree of the partial 16S rRNA gene sequence of the S57 strain and the closely related species of *Pseudomonas* genus.

2.3. Tolerance to NaCl and H_3BO_3 of the S57 Strain

A qualitative methodology was performed to determine the tolerance of the S57 strain to saline-boric conditions (Table 4). Abundant growth was observed in King's medium B supplemented with 8 g/L and 15 g/L of NaCl or 10–100 ppm of H_3BO_3 similar to the control conditions in the absence of NaCl and H_3BO_3. However, poor growth was detected when 20 g/L NaCl was added to the medium. When the liquid medium was supplement with 0.86 g/L NaCl and 114 ppm of H_3BO_3 (1× Lluta irrigation water), abundant growth of the S57 strain was detected. Furthermore, poor growth was observed using 9× Lluta irrigation water and no growth was perceived using higher concentrations of the NaCl- H_3BO_3 mixture.

Table 4. Tolerance of the S57 strain to NaCl and H_3BO_3**.** Bacterial growth was registered as ++: abundant growth, similar to control condition, and in the absence of NaCl and H_3BO_3; +: poor growth; and −: no growth.

Condition	Bacterial Growth
Control	++
8 g/L NaCl	++
15 g/L NaCl	++
20 g/L NaCl	+
10 ppm H_3BO_3	++
50 ppm H_3BO_3	++
100 ppm H_3BO_3	++
1× Lluta irrigation water	++
9× Lluta irrigation water	+
10× Lluta irrigation water	−

2.4. In Vitro PGP Traits and Antifungal Activity of the S57 Strain

In vitro PGP activities of the S57 strain are described in Table 5. S57 bacterium grew in NFb semisolid medium and formed a subsurface veil-like pellicle indicating the bacterial ability to fix elemental nitrogen. Furthermore, the S57 strain was capable of solubilizing phosphate in PVK solid medium as it was observed through the apparition of a clear halo rounding the colony. After 5 days of inoculation in fresh King's medium B, the S57 isolate was able to produce 7.9 µg/mL of IAA and 24.2 psu of siderophores.

Table 5. In vitro PGP traits of the S57 strain. A positive reaction is indicated as +. Error corresponds to standard deviation of the independent assays.

PGP Trait	Result
Nitrogen fixation	+
Phosphate solubilization	+
(IAA) (µg/mL)	7.9 ± 0.5
Siderophore production (psu)	24.2 ± 3.16

The antifungal activity of the S57 strain is shown in Table 6. Under standard conditions (absence of NaCl and H_3BO_3), S57 isolate showed antifungal activity against the phytopathogens *Fusarium oxysporum* (23.5%), *Botrytis cinerea* (48.3%), *Geotrichum candidum* (58.6%), and *Monilinia fructicola* (67.2%), reaching IMRG percentages $\geq$ 50% for the last three fungi. When 10 g/L NaCl and 110 ppm H_3BO_3 were added to the culture medium (saline-boric conditions), antifungal activities against *F. oxysporum* and *B. cinerea* were unaltered; meanwhile, the ability to control *G. candidum* and *M. fructicola* decreased to 29.6% and 52.3%, respectively. Due to *F. oxysporum* and *B. cinerea* being the most common

phytopathogenic fungi present in the Arica and Parinacota Region, a dual culture assay using irrigation water from the Lluta River instead of distilled water was performed and observed that the use of Lluta irrigation water did not affect the antifungal activity against these phytopathogenic fungi.

Table 6. In vitro antifungal activity of the S57 strain. Results are indicated as percentage of IMRG. Error corresponds to standard deviation of three independent assays. N.D.: not determined.

Fungus	IMRG (%)		
	Standard Conditions	Saline-Boric Conditions	Lluta Irrigation Water
B. cinerea	48.3 ± 5.8	42.8 ± 5.3	43.3 ± 4.4
F. oxysporum	23.5 ± 2.8	26.3 ± 3.2	22.8 ± 4.2
G. candidum	58.6 ± 7.0	29.6 ± 3.6	N.D.
M. fructicola	67.2 ± 8.1	52.3 ± 6.3	N.D.

2.5. Growth Optimization of the S57 Strain in Flask and Bioreactor Conditions

Three parameters (temperature, pH, and agitation) were modified independently to optimize the bacterial growth of the S57 strain (Table 7). No significant differences were observed in the generational time and the microbial growth rate when incubation temperature was varied from 25 °C to 35 °C, as is observed in Table 7; 35 °C being the temperature with the highest μ (2.98 h^{-1}) and the lowest g (0.33 h) and it is considered as the optimum growth temperature. A significant decrease in μ and increase in g were registered at higher temperatures.

Table 7. Optimal conditions for the growth of S57 bacterium. Each optimum parameter was determined by maintaining the other two parameters invariable. Error corresponds to the standard deviation of three independent assays.

Parameter	g (h)	μ (h^{-1})
Temperature (°C)		
25	0.35 ± 0.03	2.85 ± 0.24
30	0.34 ± 0.04	2.89 ± 0.34
35	0.33 ± 0.02	2.98 ± 0.18
40	1.26 ± 0.22	0.79 ± 0.14
pH		
5.0	0.48 ± 0.04	2.07 ± 0.17
5.5	0.30 ± 0.02	3.45 ± 0.23
6.0	0.30 ± 0.01	3.32 ± 0.11
6.5	0.31 ± 0.03	3.25 ± 0.31
7.0	0.32 ± 0.03	3.13 ± 0.29
7.5	0.31 ± 0.04	3.25 ± 0.42
Agitation (rpm)		
0	6.54 ± 0.50	0.15 ± 0.01
50	1.89 ± 0.17	0.53 ± 0.05
100	0.46 ± 0.09	2.16 ± 0.42
150	0.33 ± 0.04	3.06 ± 0.37

Higher microbial growth rates and lower generational times were determined when the S57 isolate was inoculated in a buffered culture medium in the pH range from 5.5 to 7.5 (Table 7). When King's medium B was buffered at pH 5.0, a decrease in μ (2.07 h^{-1}) was observed. The pH range between 5.5 and 6.0 was considered as optimum pH for bacterial growth in the bioreactor.

The lowest generational time (0.33 h) and the highest microbial growth rate (3.06 h^{-1}) were registered when the S57 strain was grown using 150 rpm of agitation (Table 7) and this is considered as the optimum agitation speed. Higher g values and lower μ rates were determined when the agitation speed is reduced.

Optimization in the bioreactor was performed using a two-variables experiment (Table 13), where aeration and impeller speed (agitation) were modified simultaneously to increase the cellular mass of the S57 strain. Optimal temperature and pH were selected from in flask assays. Two-variables experiment results are shown in Table 8. Higher CFU/mL (2.5×10^{27} CFU/mL) and biomass (47.1 g/L) were obtained when experiment number six was carried out (0.5 VVM of aeration and 75 rpm of agitation) and its parameters were considered as optimal parameters for the growth of the S57 strain in the bioreactor. However, no bacterial growth was observed when 150 rpm of impeller speed was used after 48 h post-inoculation (not showed).

Table 8. Optimization of growth of the S57 strain in bioreactor. Error represents standard deviation of the independent assays.

Experiment Number	Log (CFU/mL)	Biomass (g/L)
1	23.2	35.9 ± 5.40
2	21.3	33.0 ± 6.60
3	19.0	29.4 ± 7.35
4	22.1	34.5 ± 6.90
5	23.9	37.1 ± 4.50
6	27.4	47.1 ± 6.30
7	26.9	41.7 ± 6.30

2.6. Effect of the Temperature and the Addition of Stabilizing Agents to Formulations of the S57 Strain

Viability of the S57 strain in liquid culture medium in the presence and absence of stabilizing agents carboxymethylcellulose (CMC), cocamidopropyl betaine, Tween 20, and Triton X-100 at 0.1% final concentration was evaluated for 6 months when a dilution of ~5×10^9 CFU/mL was prepared and stored at room temperature and 4 °C (Figure 2). It was observed that viable bacterial counting decreased over the month for the five formulations stored at room temperature and 4 °C. The formulations reached values of ~1×10^7 CFU/mL after one month of storage at room temperature and kept constant for 6 months, except for 0.1% Triton X-100 for which viability was reduced to ~1×10^6 CFU/mL up to the fourth month. After this, the S57 viability was recovered reaching values similar to the other formulations. Similar behavior was observed at 4 °C where the viable count diminished to values close to 1×10^7 CFU/mL after six months of storage, except for 0.1% cocamidopropyl betaine stored at 4 °C, which possesses the lowest viability after six months (5.73×10^6 CFU/mL).

Auxin production by the S57 strain was variable according to the formulation employed (Figure 3). The highest values were obtained for formulations containing 0.1% of Triton X-100 stored at room temperature and 4 °C for six months and values reached 19.78 μg/mL and 15.31 μg/mL, respectively. These results suggest that this formulation produces a biostimulant based on the S57 bacteria and 0.1% of Triton X-100. Meanwhile, the lowest values were obtained for 0.1% of CMC after six months of storage in which IAA productions were 1.72 μg/mL (4 °C) and 2.10 μg/mL (room temperature).

Antagonistic activity against *B. cinerea* by the different formulations of the S57 strain was evaluated for six months to determine if this activity could be reduced due to storage (Figure 4). It was possible to observe that four formulations (Control, Tween 20, Triton X-100, and CMC) reduced the biocontrol activity against *B. cinerea* considerably during the first month, while cocamidopropyl betaine formulations did not show important losses for this activity. Furthermore, all formulation stored at the two temperatures exhibited a considerable increase in antagonistic activity in vitro and reached values ~75% of mycelial growth inhibition of *B. cinerea*, except for the 0.1% CMC formulation stored at room temperature in which mycelial growth inhibition reached ~66%. These results reveal that all formulations increased the antagonistic activity of S57 against *B. cinerea* during storage.

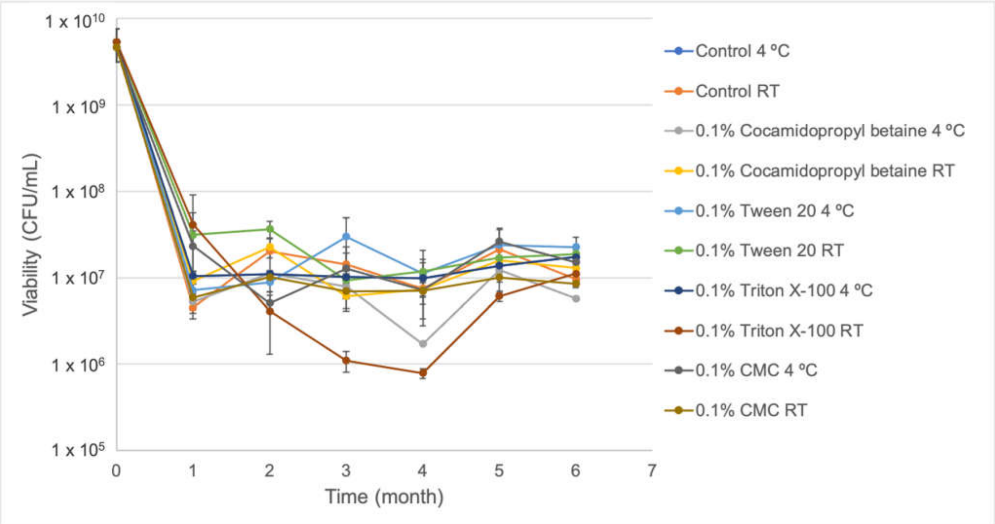

Figure 2. Viability of five formulations of the S57 strain stored at room temperature and 4 °C. Error bars represent the standard deviation of the independent assays.

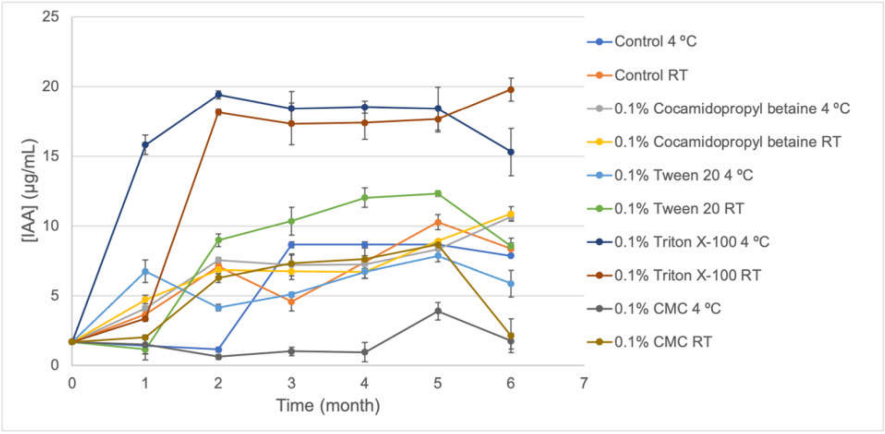

Figure 3. IAA production of five formulations of the S57 strain stored at room temperature and 4 °C. Error bars represent the standard deviation of the independent assays.

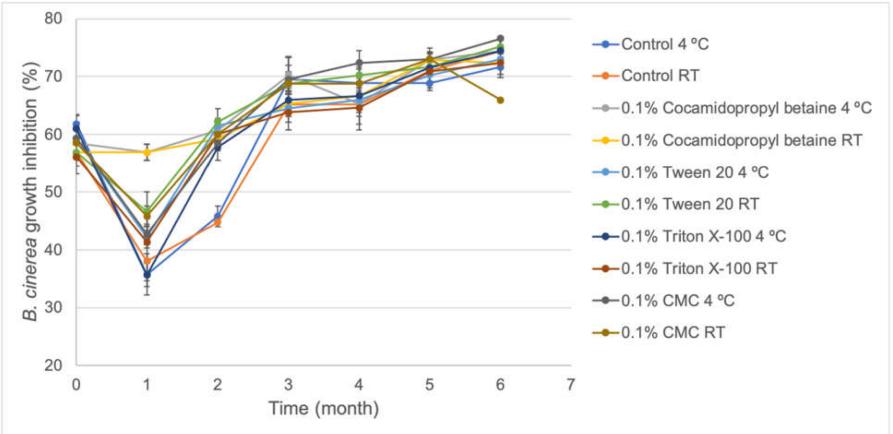

Figure 4. Antagonistic activity against *B. cinerea* of five formulations of the S57 strain stored at room temperature and 4 °C. Error bars represent the standard deviation of the independent assays.

2.7. PGP Activity of the S57 Strain in Micro-Tom Tomato Plants

In order to test the plant-growth promotion ability of the S57 strain, Micro-Tom tomato plants were inoculated with fresh 1×10^8 CFU of this bacterium (S57 treatment) and compared with uninoculated plants or control conditions (Table 9 and Figure 5). A higher development in the aerial part of the plant was measured when Micro-Tom tomato plants were treated with the S57 bacterium and an increase of 2.27-fold was observed in the wet weight and 1.33-fold in stem length compared to uninoculated plants. However, no significant differences were observed in the dry and wet weight of treated roots compared to the control.

Table 9. Effect of the application of the S57 strain in the Micro-Tom tomato plants growth. Error represents standard deviation of the five independent assays.

	Control	S57 Treatment	Fold
Stem length (cm)	11.8 ± 1.40	15.7 ± 1.10	1.33
Root wet weight (g)	2.3 ± 0.40	2.7 ± 0.50	1.18
Root dry weight (g)	0.18 ± 0.04	0.18 ± 0.04	1.00
Aerial wet weight (g)	2.36 ± 1.00	5.35 ± 1.00	2.27
Aerial dry weight (g)	0.57 ± 0.10	0.83 ± 0.10	1.46

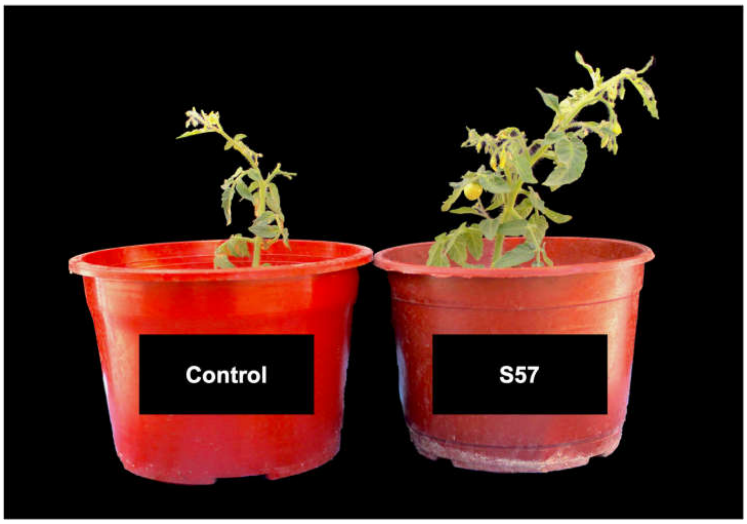

Figure 5. Representative image of Micro-Tom tomato plant growth treated with the S57 strain in comparison to untreated plants (control). Plants were irrigated with saline-boric water from Azapa Valley and grown in perlite as substrate.

2.8. Biocontrol Aactivity of S57 Strain in Plants Against Phytopathogenic Fungi

Table 10 shows the height of bell pepper plants after 30 days of cultivation. Considering the conditions of the evaluation, plants exposed to *B. cinerea* and *F. oxysporum,* statistical analysis showed significant differences between the treatments ($p \leq 0.05$; capital and lowercase letters, respectively), which allowed two level grouping: Control and Serenade (Gold Standard, GS) as statistical group one; and S57 as a statistical group two. For bell peppers plants in the presence of *B. cinerea*, the S57 treatment was higher than control and the GS; meanwhile, in the presence of *F. oxysporum*, S57 and GS treatments were equal.

Fresh weight was determined from the third leaf in bell pepper plants cv. Almuden (Table 11) after 30 days of cultivation in pots to determine the effect of the S57 treatment in the plant growth. For this evaluation, a random design was considered with four replicates; this allowed the definition of two statical groups: GS with lower weight; and S57 with the same weight as the control and grouped in a second superior level.

Table 10. Height of bell pepper plants cv. Almuden treated with S57 strain in comparison to control and GS treatments. Error represents the standard deviation of the three independent assays. The same letters represent statistical equality (Tukey test, $p \leq 0.05$).

Treatment	*B. cinerea*	*F. oxysporum*
S57	26.0 ± 1.0^{b}	26.0 ± 1.0^{A}
Serenade	21.7 ± 1.5^{a}	26.0 ± 1.0^{A}
Control	23.0 ± 1.0^{a}	27.7 ± 2.1^{B}

Table 11. Fresh weight of the third leaf of pepper plants cv. Almuden treated with the S57 strain in comparison to control and GS treatments. Error represents the standard deviation of the four independent assays. The same letters represent statistical equality (Tukey test, $p \leq 0.05$).

Treatment	Fresh Weight (g)
S57	1.68 ± 0.3^{b}
Serenade	1.34 ± 0.3^{a}
Control	1.66 ± 0.5^{b}

When evaluating the effect of the treatments on *B. cinerea* damage (Figure 6), no significant differences were detected between the treatments even though they effectively exert a mitigating effect equal to the GS. Despite this, the infection was limited in all treatments and the foliar development of the treatments was adequate according to the plant phenological state (Figure 5).

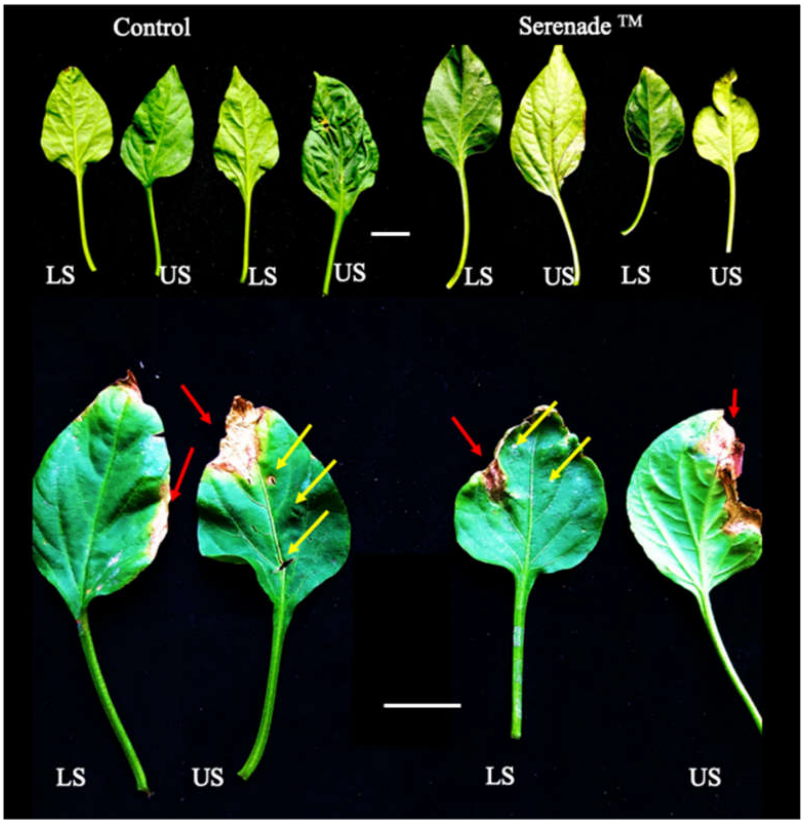

Figure 6. Development of the third leaf in bell pepper plants cv. Almuden after undergoing 5 treatments. Red arrows indicate *B. cinerea* damage points. Yellow arrows indicate inoculation wounds without infection. Abbreviations: LS: lower side; US: upper side. White scale bars represent 2 cm.

2.9. Nematicide Activity of S57 Strain in Plants Against Meloidogyne Incognita

As expected, the non-inoculated tomato plants cv. *Poncho Negro* did not present galls, while the plants with *M. incognita* inoculum (without any controller) presented 2.428 galls/100 g of roots. The

plants treated with fluopyram presented 2 galls/100 g of roots (Table 12) and presented data that validates the extensive use of this nematicide in commercial productions. From the treatment with the S57 strain, it was possible to count 689 galls/100 g of roots. This information allows consideration that the S57 bacterium exerts a moderate effect on the root-knot formation of *M. incognita* in tomato plants. By using 4–5 separate applications every 5 to 10 days, the inhibitory effect on the formation of galls could probably be higher.

Table 12. The number of galls of *M. incognita* per 100 g of roots of tomato plants cv. *Poncho Negro*. Error represents standard deviation of the four independent assays. The same letters represent the statistical equality (Tukey test, $p \leq 0.05$).

Treatment	N° Galls
Control without inoculate	0 ± 0 [a]
Control without controller	2428 ± 109 [b]
S57	689 ± 83 [c]
Fluopyram	2 ± 2 [a]

3. Discussion

Arid and semi-arid environments are widely distributed worldwide, occupying between 30% to 40% of the global terrestrial surface. Under these conditions, soil fertility is constrained by different environmental factors, including wide thermal amplitudes, low water availability, reduced bioavailable nitrogen and phosphorous, decreased water-holding capacity, and extreme pH values, low soil organic matter (ranging from 0.1% to 3%) among other specific limitations [17]. Socoroma soils have been maintained using ancestral agricultural practices and pesticides-free soil managements, which was confirmed by pesticides residues analysis for 330 types of agrochemicals (not showed). However, several plant limiting factors were detected (Table 1), including increased values in electrical conductivity higher than 80 dS/cm with a reduced quantity of Na (less than 0.33 cmol/kg), suggesting non-sodium highly saline soils. Salinity affects the productivity and yields of crops by reducing plant biomass, leaf area, and growth, which can be explained by the increase in soil osmotic pressure. The interference in the nutrient and water uptake; diminished CO_2 availability with reduced photosynthetic pigments content that directly affects photosynthesis; and salt accumulation in roots that promotes the development of osmotic stress and disrupt cell ion homeostasis by inhibiting the uptake of essential elements (K^+, Ca^{2+}, and $NO_3{}^-$) and favors the accumulation of Na^+ and Cl^- and promoting specific ion toxicities, which causes the inhibition of photosynthesis and protein synthesis, inactivate enzymes, and damages chloroplasts and other organelles [18].

Furthermore, high aluminum content was detected in Socoroma soils, which can exert a toxic effect on plants and diminishes their growth. As the concentration of aluminum increases, the soil pH decreases and the toxic effect of this metal appears, which have an important growth-limiting factor in acid soils below pH 5.0 (as it was observed in S1 and S2 soil samples, Table 1) where Al^{3+} ions predominate. Under these conditions, aluminum interferes with the elongation of root tips and lateral roots. Cell walls become more rigid by the formation of cross-linking pectins; inhibits DNA replication through the increase in DNA rigidity; bonds to phosphorous forming less available and insoluble compounds in soil and on root surfaces and thereby creating phosphorous deficiency; and affects the metabolism, disturbs the essential nutrient uptake and transport, and causes alteration in nutrient balance [19,20].

Plants possess mechanisms to reduce the toxic effect mediated by salinity and aluminum. In addition, a significant contribution to mitigating a variety of abiotic stress is carried out by plant-associated microorganisms at the plant phyllosphere, rhizosphere, and endosphere [21]. Several biomolecules are produced by PGPR, which act as plant growth regulators under saline conditions and allows plants to tolerate the adverse environments. Among these, molecules include the production of phytohormones, the enzyme 1-aminocyclopropane-1-carboxylate deaminase (reduces ethylene levels), siderophores, and microbial exopolysaccharides among others [11,21]. Meanwhile, aluminum toxicity can be diminished by plant-associated bacteria through the production of organic acids, such as malic acid, citric acid, oxalic acid, malonic acid, tartaric acid, and salicylic acid, which not only chelates Al^{3+} but also can bind to phosphorous and reduces the toxic effect of aluminum in roots [22–24].

Panhwar et al. [22] also described that exopolysaccharide production could mitigate the aluminum-induced acidification through the absorption of H^+, increasing pH values at the rhizosphere, and proving a protective layer in the roots, which could chelate metallic ions due to the presence of active functional groups. Aluminum detoxification was described by Mora et al. [25] through the production of siderophores by members of *Klebsiella*, *Stenotrophomonas*, *Enterobacter,* and *Serratia* genera. Iron and aluminum have a similar ionic radius, suggesting the formation of Al^{3+}-siderophore complexes and reducing the aluminum toxic effects.

Additionally, Farh et al. [26] suggest the activation of aluminum-stress related genes in *Arabidopsis thaliana* (*AtAIP*: Al-induced protein gene; *AtALS3*: Al-sensitive 3 gene; and *AtALMT1*: Al-activated maleate transporter 1 gene) when plants were treated with PGPR of *Pseudomonas*, *Chryseobacterium*, and *Burkholderia* genera that are isolated from Korean ginseng. The S57 strain has the ability to produce siderophores and IAA to fix elemental nitrogen and to solubilize inorganic phosphate (Table 5); stimulate Micro-Tom tomato growth using irrigation water from Azapa Valley (Table 9 and Figure 5); and can tolerate saline-boric conditions (Table 4); it would be interesting to determine the mechanisms associated to plant-growth promotion for this bacterium and to determine how this strain could increase the plant tolerance to abiotic factors, such as salinity, presence of boron, and aluminum.

The in vitro biocontrol activity of the S57 isolate against phytopathogenic fungi is promising because this property is maintained even under saline-boric conditions (Table 6). Moreover, biocontrol activity against *B. cinerea* was evidenced under the saline-boric conditions of the Azapa Valley using bell pepper plants (Table 10; Figure 6), but no statistical differences relative to control conditions were observed when *F. oxysporum* was used as phytopathogenic fungus. This difference was expected due to the lower in vitro biocontrol activity against *F. oxysporum* determined by the antagonistic assay (Table 6). Additionally, the S57 strain reduced the number of galls formed by *M. incognita* under saline-boric conditions and represents an interesting alternative for developing a new bioproduct with nematicide activity. These results show that the S57 bacterium possesses characteristics of an interesting biocontrol agent with antifungal and nematicide activities which require an exhaustive characterization.

The agricultural potential of the S57 strain renders it an excellent candidate for the development of a new biostimulant and biocontrol agent for agriculture in arid and semi-arid environments. Thus, it is gaining more importance considering that the increasing worldwide soil salinity process could reach 50% of arable soils by 2050 [18] and current commercial bioproducts show non-reproducible results when they are applied to crops maintained under these extreme conditions of cultivation [27].

For the development of bioproducts, it is important to generate a highly concentrated microbial culture for the production's success. Several factors are crucial for increasing bacterial cell biomass during the production process, including time-saving and cost-effective methods. It is possible to adjust some growth factors, such as pH, temperature, agitation, and incubation time [28], to improve bacterial biomass. For the S57 strain, pH, temperature, and agitation were performed in a flask to determine optimal parameters for inoculum preparation and bacterial scale-up under bioreactor conditions. This bacterium was able to grow optimally at 35 °C in a pH range from 5.5 to 6.0 using 150 rpm of agitation (Table 7) and producing ~12.0 g/L in 48 h, representing a significant increase in biomass in comparison to initial conditions, where less than 1 g/L was produced during 1 week under non-optimal conditions (not showed). Further improvements were achieved when the S57 strain was grown in a fermenter using 0.5 VVM of aeration and 75 rpm of impeller agitation (Table 8), reaching ~47.1 g/L of biomass (~2.5×10^{27} CFU/mL) in 24 h.

S57 viability showed to be very stable in the absence and presence of stabilizer compounds (0.1% cocamidopropyl betaine, CMC, Triton X-100, and Tween 20) after six months stored at 4 °C and at room temperature (Figure 2), ranging from ~1×10^6 CFU/mL to ~1×10^7 CFU/mL and increasing their biocontrol activity in vitro against *B. cinerea* (Figure 4) for all formulations. Meanwhile, IAA production (Figure 3) was notoriously increased in 0.1% Triton X-100, making this formulation a potential biostimulant product. Several formulations based on *Pseudomonas* bacteria have been reported. For *Pseudomonas fluorescens* AMB-8 where a notorious decrease in viable cell counting was detected when the bacterium was incubated at room temperature for 6 months without the addition of any protective agent to King's medium B or nutrient broth. The amendment of nutrient broth with 2% glycerol improved the viability of this strain to 10^7 CFU/mL compared to 10^5 CFU/mL in non-amended nutrient broth [29]. He et al. [30] also observed an improvement in the viability

of *Pseudomonas putida* RS-198 when bentonite, alginate, CMC, and polyvinyl alcohol were used as stabilizer agents to culture medium after 6 months of incubation. Particularly for the S57 strain, non-notorious improvements in viability were observed when protective agents were added to the bacterial formulation. A bacterium-based bioproduct must be formulated appropriately to guarantee that it will provide all the beneficial traits that it is supposed to afford. The incorporation of stabilizers substances to S57 liquid formulation allowed maintaining higher viable cells and functionally active bacteria during long-term storage. Several protective compounds have been employed to increased bacterial viability during time, including natural polymers (carrageenan, arabic gum, xanthan gum, gelatin, and alginate), synthetic polymers (polyvinyl alcohol and polyvinylpyrrolidone), horticultural oils, glycerol, and mono-saccharides and di-saccharides; all of these must protect the bacterium from abiotic stress generated during storage [31]. It is important to mention that solid formulations (granules and dry and wet powders) could extend the S57 strain stability over time. For example, *Pseudomonas tolaasii* IEXb maintained its viability after 6 months stored at 4 °C, when it was freeze-dried using whey as the carrier and sodium glutamate as the stabilizer agent; meanwhile, a minor reduction in viable bacterial cells from 10^9 CFU/mL to 10^6 CFU/mL was observed at room temperature [31]. The saline-boric tolerance, PGP traits, and biocontrol activities for the S57 strain are promising and reveal the possibility to develop a new biostimulant and biofungicide that are functional and active under arid and semi-arid conditions. Additionally, the produced biomass of the S57 strain is attractive compared to other bacterial products and allow high quantities of the bacterium to be obtained in a single fermentation step. However, further studies are necessary to improve the viability during storage and to characterize the mechanisms of plant-growth promotion and antifungal biocontrol activity.

4. Materials and Methods

4.1. Sampling and Bacterial Isolation

Sample collection was performed at three sites in Socoroma, Arica and Parinacota Region, Chile, in 2017 (Table 1). Samples consisting of entire oregano plants were collected aseptically using a metallic shovel disinfected with 70% (*v*/*v*) ethanol and stored in sterilized plastic bags (17 × 11 inch). Soil samples were taken for composition and pesticide residues analysis (Analab, Santiago, Metropolitan Region, Chile). Samples were kept at 4 °C in a cooler and immediately transported to the laboratory for processing.

The S57 strain was isolated following the procedure described by Muñoz et al. [6] and is part of the plant-associated bacterial culture collection of the Laboratory of Plant Pathology and Bioproducts belonging to the Universidad de Tarapacá. This collection comprises one hundred eighty bacteria, which is functionally characterized for the bacterial PGP traits. The S57 isolate was selected from the culture collection due to their promising in vitro PGP activities and obtained using the method described by Yang et al. [32] by cutting oregano roots into small parts and disinfected using 95% (*v*/*v*) ethanol for 2 min, 2% (*v*/*v*) sodium hypochlorite for 2 min, and 70% (*v*/*v*) ethanol for 2 min followed by two washes of autoclaved distilled water for 2 min each time. The disinfected portions were placed into plates of King's medium B [33] containing the following (per liter): 20.0 g peptone, 10.0 mL glycerol, 1.5 g K_2HPO_4, 1.5 g $MgSO_4 \cdot 7H_2O$, and 15.0 g agar (pH 7.0); and incubated at 25 °C for 1 week or until microbial growths were observed. Colonies were isolated using serial dilutions on King's B broth and streaking on plates of solid medium. Incubations were performed at room temperature. Isolated colonies were transferred to a liquid medium. These procedures were repeated until a single and homogeneous morphology was observed under the microscope.

The isolated S57 strain was deposited in the Chilean Collection of Microbial Genetics Resources under the accession number RGM2930.

4.2. Bacterial Identification

Genomic DNA from selected PGPR was obtained using the DNeasy UltraClean Microbial kit (QIAGEN, Germantown, Maryland, USA) according to the procedure described by the manufacturer. The 16S rRNA gene was amplified by PCR using bacteria-specific primers 27F and 1492R [34]. PCR reaction mix and PCR cycles were performed according to the procedure described by Muñoz et al. [6]. Amplification reactions were performed using a VeritiTM 96-well Thermal Cycler (Thermo Fisher

Scientific, Waltham, Massachusetts, USA). A band of ~1500 bp was observed after amplification on 1.0% (*w*/*v*) agarose gel prepared in 1X TAE buffer (40 mM Tris-acetate, 10 mM EDTA) and visualized under UV light using 1X GelRed (Biotium (San Francisco, LA, USA). The PCR products were sequenced using the primers described above (Macrogen (Seoul, Korea)) and manually edited using the ChromasPro software (http://technelysium.com.au/wp/chromaspro/ accessed on 1 April 2020) to remove low-quality bases. Forward and reverse sequences were assembled using the Megamerger tool (http://www.bioinformatics.nl/cgi-bin/emboss/megamerger accessed on 1 April 2020) to obtain a sequence length of 1448 bp. The partial sequence was compared to GenBank using BLAST software [35]. The partial 16S rRNA gene sequence was deposited in the GenBank nucleotide sequences databank under accession number MK883138.

The partial sequence of 16S rRNA obtained from the S57 strain and selected sequences belonging to the *Pseudomonas* genus retrieved from GenBank were aligned using the Clustal W software [36]. The alignment was manually edited to obtain sequences of similar length. Phylogenetic analysis was carried out using the MEGA7 software [37] and by considering the *Aquifex pyrophilus* strain Kol5a as the outgroup. The phylogenetic tree was inferred from the multiple sequence alignments by using the Neighbor-Joining method and by using a bootstrap analysis of 1000 replicates to determine the reliabilities of each node.

4.3. Bacterial Characterization

The phenotypic characterization of the S57 strain was performed according to the procedure described by Muñoz et al. [38].

Antibiotic susceptibility was carried out using the disk diffusion method according to Simirgiotis et al. [39] by using different antibiotics as observed in Table 3. Two dissimilar quantities of each antibiotic were employed to determine if the S57 strain had differences in its behavior in the susceptibility test. The assay was performed using five independent replicates.

4.4. Tolerance of the S57 Strain to NaCl and H_3BO_3

The tolerance of the S57 strain to NaCl was determined using King's medium B supplemented with 0–20 g/L of NaCl. The tolerance to H_3BO_3 was determined using King's medium B amended with 0–100 ppm of H_3BO_3.

A mixture consisting of King's medium B supplemented with 0.86 g/L NaCl and 114 ppm of H_3BO_3 (1×) was used to emulate the characteristics of the irrigation water of the Lluta River, which is the main source of water for crops in Socoroma [40], and to determine the tolerance of the S57 strain proving that this bacterium can grow under the extreme water conditions of this zone. Furthermore, the medium was amended with a higher concentration up to 10× the irrigation water of the Lluta River.

Bacterial growth was monitored each day during one weak of incubation through the measurement of OD_{600} using a spectrophotometer. Bacterial growth was registered as ++ for abundant growth, which is similar to control condition (absent of NaCl and H_3BO_3); + for poor growth; and – for no growth. All assays were carried out using three independent replicates.

4.5. In Vitro PGP Traits

Inorganic phosphate solubilization was detected in Pikovskaya (PVK) solid medium according to the method described by Pikovskaya [41]. The appearance of a clearing zone around the bacterial colony after incubation is indicative of phosphate solubilization.

Nitrogen fixation was determined using NFb semisolid medium using the procedure described by Rodríguez [42] in which nitrogen fixation is defined by the formation of a sub-superficial whitish 'veil-like' pellicle after incubation.

The production of IAA was quantified using the colorimetric Salkowski's method [43]. Bacterial supernatant was mixed with Salkowski's reagent (0.5 M $FeCl_3$ in 35% $HClO_4$) in the ratio of 1:2 (supernatant: Salkowski's reagent) in the dark at room temperature. A calibration curve was adjusted using an IAA standard using the concentration range 0–50 μg/mL and the absorbance at 530 nm was measured using a T60 UV/VIS spectrophotometer (PG Instruments Limited).

Siderophores production was quantified by the Chrome Azurol S (CAS) method described by Schwynand and Neilands [44], where a ratio of 1:1 of supernatant: CAS reagent was employed and the absorbance at 630 nm was registered using a UV/VIS spectrophotometer after 20 min of incubation. Siderophore production was in percent siderophore unit (psu) according to the following formula:

$$\text{psu} = (\text{Ar} - \text{As}) \times 100/\text{Ar}$$

where Ar is the absorbance of the reference (CAS solution and un-inoculated medium) and As is the absorbance of the supernatant of the S57 strain.

All experiments for in vitro PGP traits were carried out using three independent replicates.

4.6. Antifungal Activity

In vitro growth inhibition of phytopathogenic fungi was determined through a dual culture assay performed in potato dextrose agar (PDA) plates by using the phytopathogenic fungi *B. cinerea*, *F. oxysporum*, *G. candidum*, and *M. fructicola* inoculated at the center of each plate and aliquots of the S57 strain were inoculated surrounding the fungus according to the method described by Sepúlveda-Chavera et al. [45]. As controls, plates with only the fungus in the center were used. The inhibition of the mycelial radial growth (IMRG) of the fungus will be calculated using the following equation:

$$\text{IMRG} = [(\text{C} - \text{T})/\text{C}] \times 100$$

where C is the growth area of the fungus in the control plate and T is the fungal growth area using the treatment.

Moreover, the same experiment was performed using PDA plates amended with 10 g/L NaCl and 110 ppm H_3BO_3 (saline-boric conditions) to determine if biocontrol activity against phytopathogenic fungi is maintained under saline-boric conditions. Furthermore, dual culture assays against *B. cinerea* and *F. oxysporum* (common phytopathogenic fungi in the Arica and Parinacota Region) were also performed using PDA plates prepared with twice-autoclaved irrigation water from the Lluta River instead of distilled water.

All antifungal assays were carried out using three independent replicates.

4.7. S57 Culture Optimization

A growth curve was performed by taking 1 mL aliquots each 1 h during 48 h from the culture medium inoculated with the S57 strain. Bacterial growth was monitored by the increase in OD_{600} using a spectrophotometer and viable count was monitored busing serial dilutions on a solid medium.

Flask-experiments were conducted until the stationary phase using a final volume of 200 mL of bacterial culture. This strategy was used to characterize and model the behavior of the S57 strain by defining the variables of the process (agitation, pH, and temperature) to promote bacterial growth in the flask. The effect of each parameter was recorded by obtaining growth curves and calculating generational time (g) and microbial growth rate (μ). Two variables were maintained constant to determine each optimum parameter.

The S57 strain was inoculated in King's medium B and the temperature was maintained in a shaker incubator to determine the temperature effect. The range of temperature to be assayed was 25–40 °C.

The S57 strain was inoculated in King's medium B and pH was buffered to 5.0–6.0 using 50 mM MES and 6.5–7.5 using 50 mM HEPES in order to determine the pH effect and adjusted to their respective values using NaOH or HCl.

The effect of agitation was determined in a shaker incubator at room temperature by using different agitation speeds between 0–150 rpm.

The S57 strain culture was scaled up in a one-liter bioreactor (BioFlo®/CelliGen® 115 fermenter) under optimal flask-conditions. Optimization in the bioreactor was performed by using the design of a two-variables experiment where aeration and agitation were modified simultaneously according to Table 13. The experimental temperature was maintained at 35°C and pH was controlled to 5.5–6.0 using 5% (*w*/*v*) H_3PO_4 and 5% (*w*/*v*) NaOH. Biomass and viable count were determined to select optimal growth conditions of the S57 strain in the bioreactor.

Table 13. Experimental design to optimize S57 strain culture conditions in bioreactor.

Experiment Number	Aeration (VVM)	Agitation (rpm)
1	0.5	100
2	1.0	100
3	1.5	100
4	0.5	50
5	1.5	50
6	0.5	75
7	1.5	75
8	0.5	150
9	1.0	150
10	1.5	150

For viable counts, samples of 1 mL were taken after 24 h of incubation and serial dilutions were prepared in sterile King's medium B. Each dilution was inoculated in a solid medium and colonies counting was performed after incubation to determine colony-forming units per mL (CFU/mL).

Furthermore, bacterial viability was determined once a month using dilutions of the S57 strain to ~5×10^9 CFU/mL and stored at room temperature and 4 °C for six months. For this purpose, four formulations were generated using the stabilizing agents carboxymethylcellulose (CMC), cocamidopropyl betaine, Tween 20, and Triton X-100 at 0.1% final concentration.

Samples of 1 mL were taken once a month and serial dilutions in sterile King's medium B were performed. Each dilution was inoculated in a solid medium and colony counting was determined after incubation as described above. Experiments were executed using three replicates. Moreover, in vitro antifungal activities against *B. cinerea* and auxin production were monitored monthly according to the procedure described above.

4.8. PGP Activity of the Strain S57 in Micro-Tom Tomato Plants

PGP activity was also evaluated in Micro-Tom tomato plants (Tomato Genetics Resource Center, University of California Davis). Micro-Tom tomato seeds were disinfected using 95% (*v*/*v*) ethanol for 2 min, 2% (*v*/*v*) sodium hypochlorite for 2 min, and 70% (*v*/*v*) ethanol for 2 min followed by two washes with sterile distilled water for 2 min each time. Seeds were germinated in a sterile dark wet chamber at 25 °C for ten days. After their germination, Micro-Tom seedlings were transferred to pots containing twice autoclaved perlite as the only substrate and were kept in a greenhouse. Plants were treated once a week for a month using 1×10^8 CFU of the S57 strain by the application of the bacterium at the stem's base. The fresh inoculum was prepared and diluted in sterile water. Micro-Tom tomato plants treated only with sterile King's medium B were used as a control. Plants were watered daily with irrigation water from the Azapa Valley. After 4 weeks of inoculation, the Micro-Tom plants were removed from the substrate and washed and the length of the stem, wet weight, and dry weight of the roots and the aerial parts were measured separately. Experiments were carried out using five independent replicates.

4.9. Biocontrol Activity of the S57 Strain in Planta Against Phytopathogenic Fungi

In order to determine the biocontroller effect of the S57 bacterium, a planta assay was designed in pots with plants of bell pepper cv. Almuden (Syngenta®). The plants were obtained from a commercial nursery and transplanted when they had the first true expanded leaf (two weeks of germination). The experimental design corresponded to a completely randomized design with uniform management. The application of bacteria suspended in water was the only source of variation. A positive control treatment (Gold Standard, GS) corresponding to *Bacillus subtilis* (Serenade®-Syngenta) was considered. Plants that were not inoculated and treated only with water were used as a negative control. The plants were grown in commercial peat as substrate and fertilized after the first week of transplantation and watered daily. The experimental design considered three replicates. After 10 days of transplantation, two phytopathogenic fungi were incorporated: *B. cinerea* and *F. oxysporum*. For the inoculation of bell pepper plants, 2 mL of a suspension (10^6 CFU/mL) of *B. cinerea* conidia was applied to each plant. *F. oxysporum* was inoculated in conidial suspension (10^6 CFU/mL) by applying 2 mL per plant at a location close to the neck of the plants. Each fungus was applied in independent pots.

The S57 strain and the GS were applied three times: 5, 15, and 25 days after transplantation. The development of foliar symptoms and plant height were evaluated. For each application, an independent suspension of each bacterium was prepared in a liquid proteose peptone medium and it was cultivated at 35 °C for 48 h using 150 rpm of shaking (obtaining a concentration of 1×10^9 CFU/mL). For the application in plants, 1 mL of the medium with bacterial growth was taken and it was suspended at a rate of 1 mL of broth in 1 L of sterile distilled water. Three mL of bacterial suspension were applied to each plant using a manual spray pump.

4.10. Nematicide Activity of the S57 Strain in Planta

4.10.1. Nematode Isolation

The nematode *M. incognita* was obtained from an axenic population maintained in tomato plants cv. *Poncho Negro*. The nematode collection was performed by the method described by Hussey and Barker [46] and modified by Bonetti and Ferraz [47]. The roots of tomato plants infested by the nematode were gently washed to release the adhered soil and macerated in the presence of 0.5% NaClO. The nematode suspension was filtered using a 500-mesh sieve to retain the eggs; the total count of the eggs was conducted in a Peters chamber using a stereomicroscope. The suspension was calibrated to 5000 eggs/mL.

4.10.2. In Planta Nematicide Assay

In order to determine the nematicide effect of the S57 strain, tomato plants cv. *Poncho Negro* were transplanted to 3 L pots using a mixture of peat: perlite (2:1) as soil substrate. Four treatments were considered for the assay: tomato plants without *M. incognita* eggs (Control without inoculating), tomato plants inoculated only with *M. incognita* eggs (Control without controller), tomato plants inoculated with *M. incognita* eggs and treated with fluopyram at commercial dosses (3 mg/plant diluted in irrigation water) (Chemical control), and tomato plants inoculated with *M. incognita* eggs and treated with the S57 strain. After two days of transplantation, one mL of *M. incognita* eggs (~5000 eggs/mL) was inoculated to each respective plant in the roots. For the S57 strain, one mL of bacterial suspension (1×10^6 CFU/mL) was added twice with ten days of separation. Tomato plants were cultivated for 35 days. The experiment represented a completely randomized design with 4 replicates. After cultivation, 100 g of roots were obtained and the number of galls was determined for each treatment.

5. Conclusions

The S57 strain is an endophytic bacterium associated with oregano roots from Socoroma (the Atacama Desert) and it is a member of the *Pseudomonas* genus that is closely related to *P. lini*. This bacterium has PGP traits, including the ability to fix nitrogen, solubilize phosphorous, produce IAA and siderophores, and promote the growth of aerial parts of Micro-Tom tomato plants. Furthermore, the S57 strain can tolerate saline boric conditions. It can inhibit the mycelial growth of phytopathogenic fungi in vitro, even in the presence of NaCl and H_3BO_3 which renders it a good candidate for the development of a new bioproduct functional and active under desertic conditions. Moreover, the S57 bacterium possesses biocontroller activity in planta against the fungus *B. cinerea* and the nematode *M. incognita* under saline-boric conditions. This bacterium can be produced in high quantities and remains viable and functionally active after 6 months of storage. However, it is necessary to improve its viability over time and further characterize the mechanisms associated with PGP and biocontrol activities.

6. Patents

Two patent applications were requested to INAPI under the accession number 202000346 and 202000348.

Author Contributions: All authors contributed to the study conception and design. Material preparation, sampling, and experimental procedures were performed by P.M.T., M.A.M., S.C., N.H., W.H.-M., D.C. and G.F.S.C. Data analyses were executed by P.M.T. and G.F.S.C. The first draft of the manuscript was written by P.M.T. and G.F.S.C. All authors have read and agreed to the published version of the manuscript.

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plants

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Field Efficacy of *Steinernema* sp. (Rhabditida: Steinernematidae) on the Colorado Potato Beetle Overwintering Generation

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Abstract: Colorado potato beetle (CPB) is an economic pest of potato that has developed resistance to all classes of chemical insecticides, thus requiring alternative control measures. As a potential solution, entomopathogenic nematodes (EPNs) have proven effective in suppressing this pest, but their efficacy against overwintering generations of CPB in Croatia has not been sufficiently researched. The aim of this two-year (2018–2019) field study was to determine the efficacy of *Steinernema feltiae* and *Steinernema carpocapsae* applied to overwintering CPB adults. EPNs were applied at three doses (7.5 mil./10 m^2, 5.0 mil./10 m^2 (the recommended dose) and 2.5 mil./10 m^2) by watering the soil where the adults were overwintering. The first-year results were satisfactory for both EPNs: the efficacy of *S. feltiae* ranged from 79.03% to 100.00%, while the efficacy of *S. carpocapsae* ranged from 77.32% to 96.22%. In the second year, the highest efficacy (69.57%) was obtained using the recommended dose of *S. feltiae*. Although the results are not consistent across the two years of our study and suggest further research, they indicate that EPNs have great potential in controlling overwintering CPB generations to reduce first generation abundance and damage, and also to prevent the spread of new generations to surrounding potato growing areas.

Keywords: biological control; entomopathogenic nematodes; *Leptinotarsa decemlineata*; *Steinernema carpocapsae*; *Steinernema feltiae*

1. Introduction

The Colorado potato beetle (*Leptinotarsa decemlineata* (Say); CPB) is the most important defoliating pest of potatoes [1]. It is widely distributed in North America, Europe, and Asia, covering an area of about 16 million km^2, and continues to spread [2]. In the 20th century, this pest has become a major problem throughout Europe, Asia, and China [3]. For successful potato production, CPB was controlled with insecticides. Initially, insecticides had great efficacy against CPB. The first recorded case of resistance was in the 1950s, against DDT [2]. Over time, CPB developed resistance to the newly registered insecticides which were initially effective [1]. Insecticide resistance is a major problem in crops, landscapes, and indoor environments [4,5]. To date, more than 300 cases of CPB resistance to 56 insecticidal active ingredients have been detected [6].

Resistance to chlorinated hydrocarbons (DDT, aldrin, and dieldrin) was first reported in EU countries [7]. Resistance of CPB to the active ingredients DDT and lindane was detected in Croatia in 1967, and resistance to organophosphorus insecticides, carbamates and pyrethroids was also reported in the late 1980s [7]. The main reason for the rapid spread of CPB resistance was improper use, i.e., exceeding prescribed doses, use of insecticides with the same mode of action, and frequent application [7]. Similar factors affecting the development of CPB resistance were previously reported by Alyokin et al. [2]. The problem with developing new insecticides and creating new products that are effective

and prevent the emergence of resistance is that it is time-consuming and costly to do so [2]. In order to prevent the emergence of resistance (anti-resistance strategies), insecticides with a different mode of action should definitely be used, non-chemical protection measures should be applied and economic thresholds should be taken into account in control decisions [2,8–10]. This study, among others [2,11,12] explores the possibility of suppressing CPB through other measures, especially those that do not pose a threat to the environment, humans, and animals. Crop rotation, among the other cultural practices, is always recommended, and biological control measures can also be used.

Due to the resistance problem, there is a growing need for biological control for CPB. This pest has been shown to have a large number of natural enemies, for example the species *Perillus bioculatus* (Fab.) and *Podisus maculiventris* (Say) of the family Pentatomidae, which feed on CPB larvae. *Coleomegilla maculata* (DeGeer) attacks CPB eggs and larvae in the early stages of development. The species *Picromerus bidens* (L.) has also been successful in controlling CPB [13]. The species *Lebia grandis* (Hentz) of the family Carabide feeds on eggs, but also on the older larval stages, and develops an ectoparasitization on pupae. The parasite *Edovum puttleri* Griss. has successfully parasitized CPB eggs on eggplant [2]. The entomopathogen *Beauveria bassiana* (Bals.) Vuill. has an important role in the biological control of CPB. It can be applied directly to the soil or leaf surface [1].

Entomopathogenic nematodes (EPNs) can play an important role as biological control agents in integrated pest management strategies (IPM). Their natural habitat is soil and they live in moist conditions [14]. For biological control of CPB, the most promising EPN species belong to the Steinernematidae family. Today, the species *Steinernema carpocapse* (Weiser, 1955) and *Steinernema feltiae* (Filipjev, 1934) are commercialized for effective control of flies, weevils, mosquitoes, beetles, caterpillars, and other pests and are available as biological control agents [15].

The effectiveness of nematodes in controlling insect pests depends on biotic factors such as natural enemies and competition for resources. Among the natural enemies of EPNs, the most important are nematophagous fungi, Collembola, mites, and predatory nematodes may also be important [14]. Abiotic factors include their sensitivity to extreme temperatures, soil moisture, osmotic pressure, soil texture, pH and UV radiation [15].

Compared to conventional chemical insecticides, the use of EPNs has several advantages: fast and effective action, high reproductive potential, broad host list, they can be cultivated relatively easily, and they are not harmful to other living organisms or the environment [16]. In addition, there is no risk of residues, they are easy to apply, no waiting time is required from application to sowing or planting, they are compatible with other chemical products, and no protective equipment is required during application [9,17,18]. However, like any insecticide, EPNs have some disadvantages: broad host range (may include some beneficial insects), low tolerance to environmental conditions (e.g., moisture requirement), not suitable for long-term storage, non-persistent, and more expensive compared to chemical products [14,19,20].

The potential of using EPNs to control CPB and other pests has not yet been fully exploited. In Croatia, there are data on the potential use of *S. carpocapsae* for the control of codling moth (*Cydia pomonella* (L.)) [16] and the species *S. feltiae* for control of western corn rootworm (*Diabrotica virgifera virgifera* LeConte) [21], but there is not much information on their efficacy against CPB. Since they are ecotoxicologically safe, act quickly and efficiently over a long period of time, and are easy to apply [9], it would certainly be worthwhile to explore the possibility of suppressing Colorado potato beetle using EPNs, thus contributing to anti-resistance strategies to control this pest. Therefore, this study aimed to determine the efficacy of two nematode species (*Steinernema feltiae* and *Steinernema carpocapsae*) on adult Colorado potato beetles that had overwintered in the field where potatoes were grown the previous year.

2. Results

In spring 2018 and 2019, emergence of overwintered CPB adults was monitored in entomological cages for treatments with different doses of *S. feltiae* and *S. carpocapsae* and for the untreated control in fields where potatoes were grown in the previous year. In 2018, a total of 34 Colorado potato beetles were recorded, of which 20 individuals (58.82%) were found on the control, corresponding to an average infestation of 6.33 beetles/m^2 or 63,300 beetles/ha, suggesting that pest intensity was high in the field studied.

The number of CPBs that emerged on the untreated control was similar in 2019 (23 individuals). However, the number of emerged beetles on treated plots was higher in 2019 than in 2018, suggesting that the efficacy of the applied treatments was lower in 2019 than in 2018.

2.1. Weather Conditions

Weather data from the Croatian Meteorological and Hydrological Servicer recorded in March, April, and May 2018 and 2019 differed from year to year (Figures 1 and 2). The average monthly air temperature in April and May was higher in 2018 than in 2019, which is also true for the soil temperature at 10 cm depth. Total monthly precipitation in April and May was almost half as much in 2018 as in 2019.

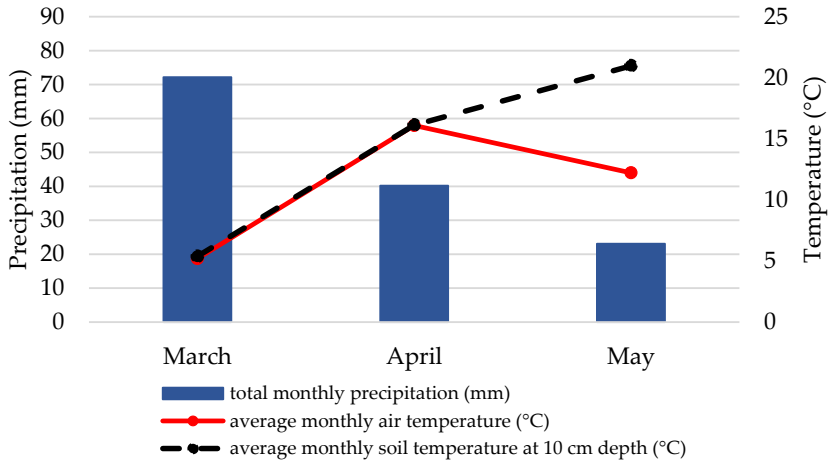

Figure 1. Weather conditions (the average monthly air temperature, total monthly precipitation and average monthly soil temperature at 10 cm depth) in March, April and May 2018, Zagreb. Data are presented up to 10 May 2018.

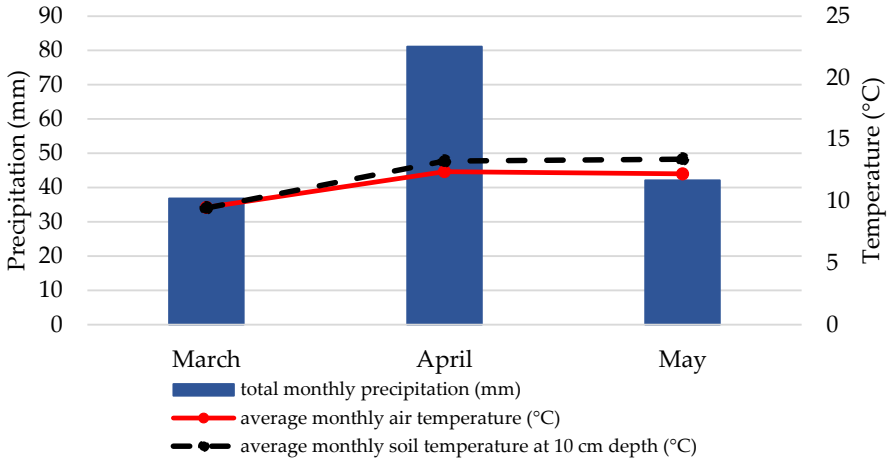

Figure 2. Weather conditions (the average monthly air temperature, total monthly precipitation and average monthly soil temperature at 10 cm depth) in March, April and May 2019, Zagreb. Data are presented up to 10 May 2019.

2.2. Efficacy of EPN Treatments

In 2018, both *S. feltiae* and *S. carpocapsae* showed good average efficacy on the overwintered CPB population, and no significant differences were observed between the different nematodes and the different dosages (Figure 3). Both EPNs had the highest efficacy at the highest dose applied, e.g., 100.00% for *S. feltiae* and 96.22% for *S. carpocapsae*. The recommended dose (5.0 mil./10 m^2) for *S. feltiae* showed the same efficacy as the highest dose of *S. carpocapsae*, while the lowest dose of *S. feltiae* was slightly more effective (79.03%) as the recommended dose of *S. carpocapsae* (77.32%). The lowest dose of *S. carpocapsae* showed greater efficacy than the recommended dose, but the differences were not statistically significant.

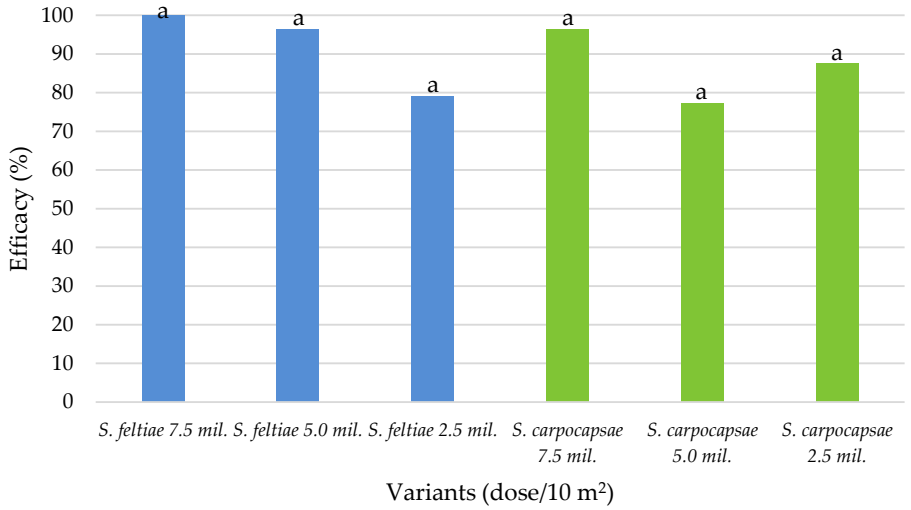

Figure 3. Average EPN efficacy on overwintered Colorado potato beetle populations in the 2018 field trial, Zagreb. Bars with the same letters are not significantly different ($p < 0.05$, Duncan's New MRT test).

Statistical analysis of the results in the 2019 field trial showed significant differences in mean EPN efficacy between treatments (Figure 4). The highest efficacy (69.57%) was obtained with the recommended dose of *S. feltiae*, followed by the lowest dose of *S. carpocapsae* (65.22%). All other treatments showed significantly lower effect on reducing the number of overwintered adult CPB, as their efficacy ranged from 17.39% to 56.52%.

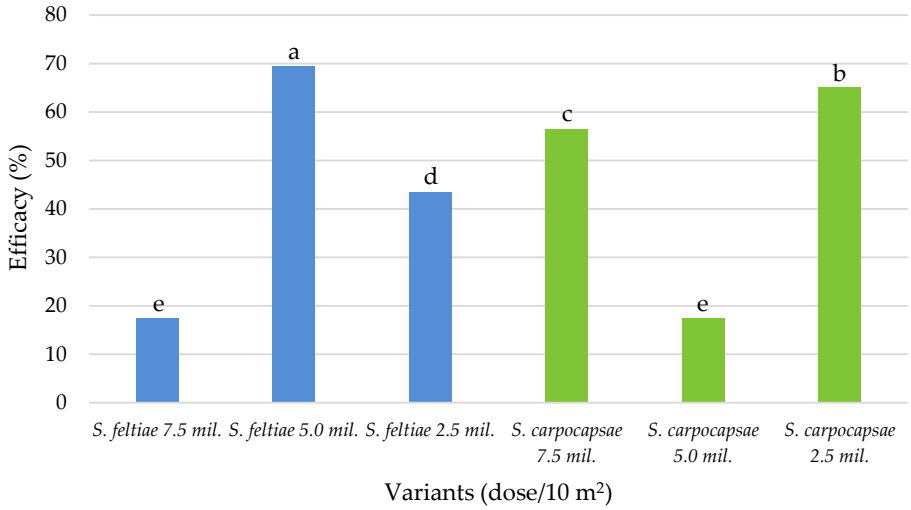

Figure 4. Average EPN efficacy on overwintered Colorado potato beetle populations in the 2019 field trial, Zagreb. Bars with different letters are significantly different ($p < 0.05$, Duncan's New MRT test).

3. Discussion

In the literature review, several studies have shown that the nematode *S. feltiae* effectively suppresses CPB, but these are mainly studies of efficacy on larval stages rather than adults [22–24]. A study by Wright et al. [25] found high efficacy of different strains and doses of *S. feltiae* on larvae (80% to 90%) and overwintering CPB adults (88% to 100%). Kepenekci et al. [24] used the EPNs *S. feltiae, S. carpocapsae* and *H. bacteriophora* Poinar to control CPB larvae. *S. feltiae* caused 96% mortality, *H. bacteriophora* 75%, while *S. carpocapsae* caused only 36% mortality at the highest dose at 25 °C. Prishchepa et al. [26] studied the efficacy of S. *feltiae and S. carpocapsae* species on CPB and the results showed high efficacy of *S. feltiae* species while *S. carpocapsae* gave poorer results. A study conducted by Laznik et al. [27] showed that *S. feltiae* can be as effective as the chemical insecticide thiamethoxam on CPB larvae under field conditions. A similar study to ours, using entomological cages, was conducted by Toba et al. [28], in which no effect of *S. feltiae* on CPB adults was found, but later infection and mortality of larvae (59% to 71%, depending on the dose) were determined. Our study showed that there were no significant differences in efficacy between *S. feltiae* and *S. carpocapsae*. However, *S. feltiae* provided the highest efficacy value at the recommended dosages. The data also showed a large variability in efficacy from year to year.

Trdan et al. [29] conducted a study with four EPNs (*S. carpocapsae, S. feltiae, H. bacteriophora*, and *H. megidis* Poinar, Jackson & Klein) in CPB control. Mortality rate was influenced by temperature, nematode species, dose, and developmental stage of the pest. The highest mortality of adult individuals at temperatures between 20 °C and 25 °C was caused by *S. feltiae* and *S. carpocapsae*. At lower temperatures (15 °C), the highest dose of *S. feltiae* applied to overwintering adults showed high efficacy. *S. carpocapsae* was more effective at higher temperatures and is therefore recommended for control of first generation adult CPB. Similar results were obtained in the same study with larvae, as EPNs penetrate larval bodies more easily [29].

In addition to CPB, EPNs are also effective in controlling many other pests. For example, Canhilal and Carner [30] successfully suppressed the pest *Melittia cucurbitae* (Harris) with *S. feltiae, S. carpocapsae*, and *S. riobrave* Cabanillas, Poinar & Raulston, where *S. carpocapsae* and *S. feltiae* showed long infectivity in the soil after application. Batalla-Carrera et al. [31] showed that early larval stages of tomato leaf miner (*Tuta absoluta* (Meyrick)) were highly sensitive to the application of *S. carpocapsae, S. feltiae*, and *H. bacteriophora*. Even at a lower dose, *S. feltiae* showed the highest efficacy (100%), but other nematodes were also effective (*S. carpocapsae* 85.7%, *H. bacteriophora* 78.6%).

In our study, there was a difference in treatment efficacy between years that may have been influenced by weather conditions in the spring of 2018 and 2019 (Figures 1 and 2). Although there are not many studies on the effects of weather conditions on CPB, Tauber et al. [32] and Lefevere and De Kort [33] found that precipitation has an impact on the occurrence of CPB. Their analyses showed that when precipitation decreases, CPB emergence from the soil slows down or stops altogether and can resume after sufficient precipitation and restoration of high soil moisture. The experimental field area received about half as much precipitation in April and May 2018 as in April and May 2019, and these conditions may have contributed to the low number of CPB beetles found in 2018. Another important factor that may have influenced the effectiveness of the treatments is soil temperature. In April and May 2018, the soil temperature ranged from 16 °C to 21 °C, while lower soil temperatures (13 °C in both April and May) were recorded in 2019. Radová and Trnková [34] studied the effect of soil temperature and moisture on the pathogenicity of the same two EPN species and reported that both *S. feltiae* and *S. carpocapsae* were significantly more efficient at temperatures between 15 °C and 25 °C, while at 10 °C their efficacy was very low. A similar effect was observed in our study, where efficacy was much higher in 2018 when soil temperature conditions were more favorable for the nematodes. In relation to precipitation (i.e., soil moisture content), *S. carpocapsae* is considered a species with better adaptation to dry conditions [35]. However,

in studies [34], *S. feltiae* was found to tolerate dry conditions equally as well (in some cases even better) as *S. carpocapsae*. This may also help explain why both EPNs were not affected by the lower precipitation in 2018 and performed with high efficacy against CPB.

Considering that numerous studies on the control of CPB by these nematodes have been conducted primarily on larvae, our research provides a valuable contribution by demonstrating the possibility of suppressing the first occurrence of these important pests in the season. Often, overwintering adult CPB are not suppressed because they do not usually cause major damage to potatoes. However, suppression is justified when potatoes are weak and there are more than two beetles on a plant [8]. If this is the case, CPB should be controlled in fields where potatoes were grown the previous year and where CPB adults have overwintered, in order to reduce the abundance of the first generation and prevent the spread of new generations to surrounding potato growing areas. Such efforts could pose an organizational problem, since the same field may change ownership each year and the new owner may not need to use pesticides against overwintering Colorado potato beetle. Potato fields in Croatia are often smaller and not very far from each other, so overwintering CPB generations can easily spread to neighboring potato fields regardless of crop rotation. For control of overwintering CPB to be effective, an area-wide (AW) approach should be adopted, including larger potato growing areas. Drmić et al. [36] found area-wide mass trapping very helpful in reducing insecticide use and in establishing an integrated pest management plan to deal with sugar beet weevil. AW mass trapping in combination with other control measures also offers great potential for reducing codling moth damage levels [37]. In addition, insecticides with different modes of action should be used to reduce the potential for resistance to develop. More importantly, biological measures and biotechnical insecticides should be increasingly included. The use of locally available nematode strains should also be explored, as these are better adapted to local climatic conditions than commercial products and could provide better results.

Although a major disadvantage of entomopathogenic nematodes is their higher cost compared to chemical products [38], there are many other advantages: they act quickly and efficiently over a long period of time, have a broad host range, and can be easily applied. According to Oštrec [9], EPNs are not harmful to other living organisms and the environment, there is no risk of residues, there is no need to wait from application to sowing or planting, and no protective equipment is required during application. Based on the above facts, we can say that entomopathogenic nematodes are an environmentally friendly alternative to classical chemical control of CPB. As this research was carried out only on overwintering adults, it would be interesting and very valuable to investigate the EPN effect on other developmental stages of CPB in the course of potato cultivation, or, for example, to combine nematodes with other chemical or biological products.

4. Materials and Methods

4.1. Meteorological Data

Data collection on average daily air temperature, total daily precipitation and average daily soil temperature at 10 cm depth was obtained from the Croatian Meteorological and Hydrological Servicer in the period between March 1 and May 10 in both years for the weather station Maksimir, Zagreb.

4.2. Field Trial Setting and Treatments

This two-year (2018–2019) study was conducted at the Experimental Station Maksimir at the Faculty of Agriculture in Zagreb. The size of the field used in 2018 was 1800 m^2, while the trial in 2019 was established on a field of 800 m^2. The fields were located about 500 m apart. Potatoes were grown in both fields in the previous year, which had a natural infestation with Colorado potato beetle.

Two commercial products of entomopathogenic nematodes (EPNs) were used in the experiment, both manufactured by e-nema GmbH (Schwentinental, Germany) and

supplied by Pro-eco (Varaždin, Croatia): Nemaplus® containing the nematode *Steinernema feltiae*, and Nemastar® containing *Steinernema carpocapsae*. The products were stored in a refrigerator (4–8 °C) for a few days until they were applied in the field.

The study included a total of seven treatments. The two commercial products were administered at three different doses: 7.5 million/10 m^2, 5.0 million/10 m^2 (the recommended dose), and 2.5 million/10 m^2. Treatment 7 was the control in which only pure water was used. Each treatment was applied to an area of 2 m^2 in three repetitions in a randomized block design.

The nematodes were applied as a liquid suspension on 4 April 2018 and 2 April 2019 by watering the soil early in the morning while the soil was still moist. The amount of water used was 1.5 L/m^2. After treatment, the soil was watered with an additional 1 L of water/m^2 as recommended by the manufacturer. To collect and monitor the number of overwintering adult Colorado potato beetles, an entomological emergence cage (1 m × 1 m × 1 m) was placed on each treatment on 6 April 2018 and 4 April 2019.

4.3. Assessment of Treatments and Statistical Analysis

Between 10 April and 10 May 2018, and between 6 April and 10 May 2019, the number of CPB adults that emerged in the entomological cages was recorded for each treatment. Surveys were conducted every 3–4 days and all collected beetles were removed from the cage during each inspection. Based on the total number of CPB that emerged on each variant, the efficacy of EPN treatments was calculated using the formula of Abbott [39].

To determine the effectiveness of EPNs between treatments in the study, the results were statistically analyzed by analysis of variance (ANOVA) and ranked by Duncan's multiple range test (DMRT), using the statistical program ARM 9® (GDM Solutions, Inc., Brookings, SD, USA) [40]. To assess normality, equality of variances was tested with Levene's test and reaches with equal variances ($p > 0.05$). Efficacy calculations were available only for those treatments and assessment time points for which a statistically valid difference was found between the untreated control and the treatments in the study. In the case of uneven data distribution, data were transformed by log(x + 1) transformation.

5. Conclusions

The results of our two-year study show the potential use of EPNs for the control of Colorado potato beetle in Croatia. The EPNs *Steinernema feltiae* and *Steinernema carpocapsae* showed good overall efficacy in the field, although results in the second year of the trial were not as consistent as in the first year. Therefore, the study should be repeated on a larger number of fields and the efficacy should also be evaluated in different climatic areas of potato cultivation. Nevertheless, due to many advantages over chemical insecticides and the increasing problem of CPB resistance, EPNs represent a valuable tool and ecotoxicologically advantageous alternative to classical chemical control and their use may reduce the potential for CPB resistance development in potato growing areas in Croatia.

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Effects of Agronomic Practices on the Severity of Sweet Basil Downy Mildew (*Peronospora belbahrii*)

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Abstract: Downy mildew (caused by *Peronospora belbahrii*) is a severe disease of sweet basil (*Ocimum basilicum*) crops around the world. We examined cultural methods for reducing the severity of sweet basil downy mildew (SBDM) under commercial conditions in greenhouses and walk-in tunnels. The effects of the orientation of walk-in tunnels, air circulation in greenhouses, plant density, and soil mulch were tested. SBDM was less severe in the tunnels that were oriented north-south than in those oriented east-west, but the yields in both types of tunnels were similar. Increased air circulation reduced SBDM severity, but did not affect yield. Gray or transparent polyethylene mulch reduced SBDM severity and, in most cases, increased yield relative to bare soil/growth medium. Yellow polyethylene mulch provided a smaller amount of control. The combination of increased air circulation and yellow polyethylene mulch provided synergistic SBDM control, whereas no synergism was observed when we combined increased air circulation with the other two types of mulch. Planting at half the usual density reduced disease severity. The reduced plant density was associated with reduced yield in the greenhouses, but not in the tunnels. All of the tested methods provided an intermediate level of SBDM control that varied among the different experiments.

Keywords: agrotechnical control; cultural control; integrated management; downy mildew; greenhouse crop; plant disease; *Ocimum basilicum*; *Peronospora belbahrii*

1. Introduction

Cultural methods (i.e., agricultural practices) can play important roles in preventing or minimizing many plant diseases. Different practices can be implemented during seeding/planting, as the crop is growing or even after harvest. Cultural techniques aimed at minimizing disease include the choice of altitude at which to plant a particular crop, the means of preparing and cultivating the soil, the use of particular cultivars, the treatment of propagation material, the choice of particular planting times and depths, the exposure to air movement, the direction of plant rows, weed-management and irrigation practices, changes in plant nutrition, and general sanitation [1–3]. In covered crops, farmers can also consider the type of cover spread over the crop, heating, ventilation, and soil mulch [4]. Cultural methods that affect the environmental conditions inside the greenhouse and minimize the presence of water on the canopy can be effective ways of controlling diseases that are promoted by high humidity [5].

Sweet basil (*Ocimum basilicum* L.) is an economically important annual herb crop from the Labiatae family that is grown in polyethylene-covered structures (i.e., greenhouse structures or walk-in tunnels). Sweet basil greenhouses are common along the ridge above the Syrian-African Rift, south of the Sea of Galilee, and around and north of the

Dead Sea. Crops are planted from September on, so winter and spring crops are common, but are challenged by humidity-promoted diseases [6–9].

Downy mildew of basil (*Peronospora* sp.) was first identified in Uganda [10]. The causal agent was identified as an oomycete pathogen and named *Peronospora belbahrii* Thines [11,12]. This pathogen mainly infects the leaf blades of sweet basil [12]. Infected leaves are distorted, asymmetric chlorosis develops on the leaf blades, and dark spores (on sporangiophores) form on the underside of the leaves [13,14]. Sweet basil downy mildew (SBDM) was found to be particularly severe when plants were kept wet for at least 6 to 12 h immediately after inoculation; the pathogen was most active at 20 °C, whereas at 12 and 27 °C, the disease was suppressed [13]. Sporulation occurs when infected plants are incubated for at least 7.5 h in a dark, moisture-saturated atmosphere at 10 to 27 °C and is suppressed by light [14]. The effects of temperature and relative humidity on SBDM have been confirmed under field conditions. Passive heating has been shown to increase root temperatures and induce resistance in sweet basil [9].

Methods for controlling SBDM may involve fungicides, seed treatments, and/or breeding for resistance [15–17]. Seeds have been found to be vehicles for pathogen transmission. It was, therefore, recommended that a seed-certification scheme be established and that the pathogen be controlled on the seeds [18]. In terms of fungicide treatments, mefenoxam with copper hydroxide, azoxystrobin, and mandipropamid effectively suppress SBDM, but the economic value of those treatments has not been demonstrated [19]. The development of resistance [15] to chemical fungicides and the need to avoid any residues on the harvested shoots limit the use of fungicides in sweet basil.

Previous studies have examined other humidity-promoted diseases of sweet basil: *Botrytis cinerea*-induced gray mold and *Sclerotinia sclerotiorum*-induced white mold. Gray mold initially infects stem wounds after harvest and white mold initially infects the stem base. Both of those pathogens are necrotrophs that benefit from the film of water present on the plant organs. Increased plant spacing, the use of polyethylene mulch, and increased greenhouse aeration have been shown to reduce the incidence of both gray mold and white mold in sweet basil [7,8]. *P. belbahrii* is an oomycete biotroph that initially infects leaves. This makes it different from the two necrotrophic pathogens, but it is similar to those pathogens in that it depends on water present on the leaf for infection. Our hypothesis was that the conditions in sweet basil greenhouses are favorable for SBDM infection and that we could affect the severity of disease by manipulating those conditions. Thus, the aim of the present research was to test cultural means of controlling SBDM in greenhouses and walk-in tunnels under commercial-like conditions. The cultural means examined included increased air circulation, manipulation of the direction of the walk-in tunnels, decreased planting density, and the use of polyethylene mulch.

2. Results

2.1. Tunnel Orientation

The effect of tunnel orientation on SBDM could only be evaluated at Site 2. We found that SBDM was less severe in the walk-in tunnels that were oriented north-south, as opposed to east-west. The decrease in severity varied with the experiment, soil cover, and year (Table S1). In terms of the area under disease progress curve (AUDPC), the difference in SBDM severity between east-west and north-south tunnels was between 25 and 33% in the first year and was about 63% in the second year (Table 1 and Table S1). The shoot yields in the two types of walk-in tunnels were significantly similar to each other (Table 1). Thus, the north-south tunnel direction is associated with lower SBDM severity as compared with east-west-oriented tunnels.

2.2. Effect of Increased Air Circulation

The effect of increased air circulation (AC) was evaluated only in the greenhouses at Site 1. We increased the air circulation in those greenhouses by operating fans during the night. This increased air circulation reduced SBDM severity (Table 2 and Table S2).

Disease reduction reached up to 72.5% in all experiments conducted over three seasons (Table 2 and Table S2), but did not affect shoot yield (Table 2). Therefore, AC decreases SBDM. Additionally, the results for the combination of increased air circulation with polyethylene mulch are described below.

Table 1. Effect of tunnel orientation on the severity of sweet basil downy mildew (SBDM) in walk-in tunnels at Site 2.

Experiment	AUDPC over the Growing Season (% × Days) [b]			Cumulative Yield (g/m^2)		
	Period (Days)	East-West	North-South	Period (Days)	East-West	North-South
TD1	114	2447 ± 232.3 [a]	1834 ± 213.6 [b]	93	3280 ± 327.4 [a]	3213 ± 422.1 [a]
TD2	114	3063 ± 272.3 [a]	2053 ± 260.9 [b]	93	2973 ± 164.4 [a]	3062 ± 154.9 [a]
TD3	112	3605 ± 68.2 [a]	1327 ± 122.9 [b]	80	1843 ± 210.4 [a]	2107 ± 192.5 [a]

[a] Experiments were carried out with sweet basil plants grown in sand in walk-in tunnels during spring 2014 (experiments TD1 and TD2) and winter 2014–2015 (experiment TD3) growing seasons. Beds were covered with transparent polyethylene (TD1 and TD3) or left uncovered (TD2). [b] SBDM severity was evaluated on a 0–100% scale, in which 0 = healthy plants and 100% = plants completely covered by SBDM symptoms/signs, and the value of the area under disease progress curve was calculated for each replicate. Averages ± SE. Values in each pair followed by a different letter are significantly different according to one-way ANOVA with Tukey's HSD. Default significance levels were set at $\alpha = 0.05$.

Table 2. Effect of increased air circulation on the severity of sweet basil downy mildew (SBDM) in greenhouses at Site 1a.

Experiment	AUDPC over the Growing Season (% × Days) [b]			Cumulative Yield (g/m^2)		
	Period (Days)	Without Fans	With Fans	Period (Days)	Without Fans	With Fans
AC1	134	838.9 ± 75.42 [a]	311.8 ± 85.12 [b]			
AC2	109	223.0 ± 42.4 [a]	61.3 ± 20.8 [b]	91	3381 ± 179.5 [a]	3327 ± 214.1 [a]
AC3	151	1151 ± 93.6 [a]	730 ± 97.1 [b]	91	2820 ± 279.5 [a]	2420 ± 234.1 [a]

[a] Field experiments were carried out with sweet basil plants grown in detached growth medium covered by gray polyethylene at Site 1 during the autumn 2013, spring 2014, and winter 2015 seasons (experiments AC1, AC2, and AC3, respectively). [b] SBDM severity was evaluated on a 0–100% scale, in which 0 = healthy plants and 100% = plants completely covered by SBDM symptoms/signs, and the value of the area under disease progress curve was calculated for each replicate. Averages ± SE. Values in each pair followed by a different letter are significantly different according to one-way ANOVA with Tukey's HSD. Default significance levels were set at $\alpha = 0.05$.

2.3. Polyethylene Mulch

In greenhouses at Site 1, covering the beds with gray or transparent polyethylene decreased SBDM severity (Table 3 and Table S3). Disease severity was significantly reduced (by up to 65%) in six out of the seven evaluation comparisons (Table S3). A whole-season evaluation based on AUDPC revealed 13-64% disease reduction in the covered beds (Table 3 and Table S3). The use of polyethylene mulch significantly increased yield in five of the six greenhouse experiments (Table 3 and Table S3). Therefore, polyethylene mulch can reduce SBDM and increase yield.

Table 3. Effect of polyethylene mulch on the severity of sweet basil downy mildew (SBDM) and yield of sweet basil in greenhouses at Site 1a.

Experiment	AUDPC over the Growing Season (% × Days) [b]			Cumulative Yield (g/m^2)		
	Period (Days)	No Mulch	Polyethylene	Period (Days)	No Mulch	Polyethylene
M2	114	554.6 ± 67.03 [a,c]	272.2 ± 68.95 [b]	100	4570 ± 108.8 [b]	4862 ± 99.3 [a]
M4	93	1005 ± 118.3 [a]	866.7 ± 97.04 [b]	94	3042 ± 108.7 [b]	3628 ± 125.8 [a]
M5	93	288.0 ± 60.44 [a]	197.0 ± 33.42 [a]	83	2776 ± 154.8 [a]	3523 ± 170.8 [a]
M6	123	2820 ± 211.7 [a]	1012 ± 143.7 [b]	91	2820 ± 211.7 [b]	4013 ± 143.7 [a]
M7	123	1089 ± 98.8 [a]	572.8 ± 82.33 [b]			
	151	1802 ± 120.3 [a]	1031 ± 125.6 [b]	91	1010 ± 420.3 [b]	2820 ± 402.5 [a]

[a] Field experiments were carried out with sweet basil plants grown in detached growth medium at Site 1 during the autumn 2013 (M2), spring 2014 (M4–M6), and winter 2015 (M7) growing seasons. Beds were covered with gray polyethylene (M2, M4, and M5) or transparent polyethylene (M6 and M7). [b] SBDM severity was evaluated on a 0-100% scale, in which 0 = healthy plants and 100% = plants completely covered by SBDM symptoms/signs, and the value of the area under disease progress curve was calculated for each replicate. [c] Averages ± SE. Values in each pair followed by a different letter are significantly different according to one-way ANOVA with Tukey's HSD. Default significance levels were set at $\alpha = 0.05$.

Three types of polyethylene mulch were compared in two growing seasons. The gray and the transparent polyethylene mulch provided more consistent and effective disease control (Table 4). The combination of increased air circulation and polyethylene mulch was examined in a third experiment (Figure 1, Table 5), which revealed no interaction between those treatments. Disease was significantly reduced by the increased air circulation and the gray polyethylene. The results observed for the transparent polyethylene were significantly different from those observed for the yellow polyethylene (Figure 1, Table 5). Calculating the synergy factor (SF) of the reduction in severity provided by each of the polyethylene mulches and the air-circulation treatment revealed no synergism between increased air circulation and either the gray or the transparent polyethylene. However, there was a significant synergistic effect between the increased air circulation and the yellow mulch (SBDM severity: SF = 1.04; AUDPC: SF = 1.12).

Table 4. Effect of the type of polyethylene mulch on the severity of sweet basil downy mildew (SBDM) in greenhouses at Site 1a.

Polyethylene Mulch	Experiment M1			Experiment M3	
	Severity (%) [b] at Day 114	Severity (%) at Day 131	AUDPC (% × Days) over 131 days	Severity (%) at Day 93	AUDPC (% × Days) over 93 Days
None	13.4 ± 2.09 [a,c]	21.5 ± 2.66 [a]	378.8 ± 40.64 [a]	13.1 ± 2.29 [a]	318.3 ± 53.37 [a]
Yellow	7.6 ± 2.02 [a,b]	7.1 ± 2.38 [b]	205.1 ± 54.64 [b]	4.6 ± 1.36 [b]	228.1 ± 34.99 [a,b]
Gray	3.8 ± 1.16 [b]	4.0 ± 1.37 [b]	103.4 ± 27.96 [b]	7.5 ± 1.13 [b]	167.8 ± 66.31 [b]
Transparent	4.1 ± 0.83 [b]	4.5 ± 100 [b]	117.2 ± 22.43 [b]	6.5 ± 2.73 [b]	149.6 ± 21.70 [b]

[a] Experiments were carried out with sweet basil plants grown in detached growth medium at Site 1 during the autumn 2013 (experiment M1) and spring 2014 (experiment M3) growing seasons. [b] SBDM severity was evaluated on a 0–100% scale, in which 0 = healthy plants and 100% = plants completely covered by SBDM symptoms. [c] Averages ± SE. Values in each pair followed by a different letter are significantly different according to one-way ANOVA with Tukey's HSD. Default significance levels were set at $\alpha = 0.05$.

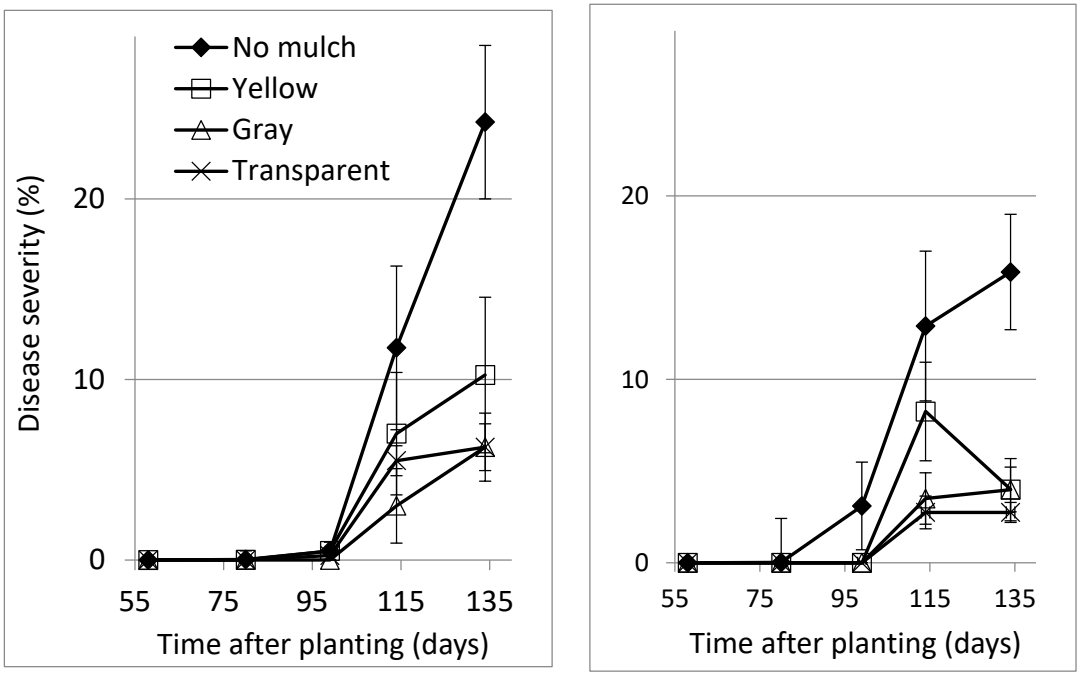

Figure 1. Effect of the type of polyethylene mulch on the development of sweet basil downy mildew (SBDM) on plants grown in tuff growing medium at Site 1 (experiment M8). Plants were grown with no supplemental air circulation (**left**) or with extra air circulation (**right**). SBDM severity was evaluated on a 0–100% scale, in which 0 = healthy plants and 100% = plants completely covered by SBDM symptoms/signs. SBDM severity is presented as percentage ± SE. An analysis of these results is presented in Table 5.

Table 5. Results from experiment M8 (Figure 1).

Mulch (M) Treatment	Severity Parameters and Air Circulation (AC)			
	Day 134 (% Severity)		AUDPC, 134 Days (% × Days) [a]	
	Without AC	With AC	Without AC	With AC
No mulch	24.3 ± 4.42	15.9 ± 3.41	458.7 ± 79.81	437.5 ± 79.81
Yellow	10.3 ± 4.11	4.0 ± 2.11	234.2 ± 53.24	185.3 ± 53.24

The effect of polyethylene mulch was also evaluated at Site 2. SBDM severity was significantly reduced in six out of eight comparisons (20-52% disease reduction) and in seven out of the eight epidemic (AUDPC) comparisons (17-51% reduction; Table 6 and Table S6). Shoot yield was increased (8-13%) by the soil mulch in two of the three comparisons (Table 6 and Table S6). Thus, polyethylene mulch was effective also in Site 2, where experiments were carried out in walk-in tunnel greenhouses.

Table 6. Effects of polyethylene mulch on the severity of sweet basil downy mildew (SBDM) and yield of sweet basil in walk-in tunnels at Site 2a.

Experiment	AUDPC over the Growing Season (% × Days) [b]			Cumulative Yield (g/m^2)		
	Period (Days)	No Mulch	Polyethylene	Period (Days)	No Mulch	Polyethylene
M8	114	2131 ± 128.5 [a,c]	1047.8 ± 111.5 [b]	110	3425 ± 128.8 [a]	3473 ± 95.5 [a]
M9	114	3063 ± 242.4 [a]	1833.5 ± 197.6 [b]	110	3359 ± 122.7 [b]	3802 ± 146.4 [a]
M10	114	2054 ± 124.7 [a]	1615.4 ± 112.4 [b]	101	4628 ± 139.7 [b]	4986 ± 117.2 a
M11	112	2040 ± 243.1 [a]	1708 ± 147.5 [a]			
M12	112	2863 ± 212.7 [a]	2369 ± 224.1 [b]			
M13	112	3156 ± 204.3 [a]	2319 ± 243.7 [b]			
M14	112	197.6 ± 47.98 [a]	102.6 ± 27.64 [b]			

[a] Experiments were carried out with sweet basil plants grown in detached growth medium at Site 2 during the spring 2014 (M8–M10) and winter 2014–2015 (M11–M14) growing seasons. Mulch-treatment plots were covered with transparent polyethylene. [b] SBDM severity was evaluated using a 0–100% scale, in which 0 = healthy plants and 100% = plants completely covered by SBDM symptoms/signs, and the value of the area under disease progress curve was calculated for each replicate. [c] Averages ± SE. Values in each pair followed by a different letter are significantly different according to one-way ANOVA with Tukey's HSD. Default significance levels were set at $\alpha = 0.05$.

2.4. Plant Spacing

The effect of plant density was evaluated at both locations. We compared a density of 24–30 plants/m^2, which is commonly used in both areas, with a reduced density of 14–15 plants/m^2. In the greenhouses at Site 1, significant reductions in SBDM were observed in all of the reduced-density plots (Table 7 and Table S7). The reduction in SBDM severity ranged between 32% and 68%. AUDPC was reduced by 22% to 63% (Table 7 and Table S7). The relationship between the SBDM severity data and the AUDPC data was calculated. The relation between the severity of disease at each evaluation date and the calculated % disease reduction at the evaluation date or between the values of AUDPC and the calculated % disease reduction were assessed. A negative correlation was found between the severity of disease and % reduction in both cases, single-day disease level, or AUDPC ($p < 0.01$ in both cases; Table 7), pointing to the fact that under lower disease pressure, the less-dense plant spacing provides more pronounced disease suppression. In three experiments, yield was decreased by 12% to 31% by sparse planting compared to dense planting (Table 7 and Table S7).

Table 7. Effect of planting density (PD) on the severity of sweet basil downy mildew (SBDM) in greenhouses at Site 1a.

Experiment	AUDPC over the Growing Season (% × Days) [b]			Cumulative Yield (g/m^2)		
	Period (Days)	Dense (24)	Sparse (14)	Period (Days)	Dense (24)	Spars (14)
PD1	114	654.6 ± 62.42 [a,c]	383.3 ± 28.75 [b]			
PD2	114	48.0 ± 3.53 [a]	17.5 ± 3.72 [b]	100	4570 ± 158.9 [a]	3985 ± 162.4 [b]
PD3	134	169.1 ± 20.14 [a]	63.9 ± 18.05 [b]			
PD4	93	1053 ± 48.6 [a]	821 ± 70.5 [b]	91	3110 ± 167.8 [a]	2130 ± 224.9 [b]
PD5	93	1005 ± 118.3 [a]	664 ± 79.8 [b]			
PD6	151	805 ± 74.6 [a]	503 ± 69.3 [b]	128	2426 ± 109.6 [a]	2140 ± 107.1 [b]
PD7	151	909 ± 81.3 [a]	604 ± 71.1 [b]			
Disease severity—Disease reduction relation						
		Equation	y = −25.4x + 1712			
		r	0.9766			
		n	5			
		p	<0.01			

[a] Experiments were carried out with sweet basil plants grown in detached growth medium in greenhouses at Site 1 during the autumn 2013 (experiments PD1-PD3), spring 2014 (experiments PD4–PD6), and winter 2015 (experiment PD7) growing seasons. Beds were left bare (experiments PD1, PD4, and PD6) or covered with polyethylene (experiments PD2, PD3, PD5, and PD7). [b] SBDM severity was evaluated using a 0–100% scale, in which 0 = healthy plants and 100% = plants completely covered by SBDM symptoms, and the value of the area under disease progress curve was calculated for each replicate. [c] Averages ± SE. Values in each pair followed by a different letter are significantly different according to one-way ANOVA with Tukey's HSD. Default significance levels were set at $\alpha = 0.05$. Equations for the relation between disease severity values of dense PD and significant disease reduction values by the sparse PD are presented and the

The reduced planting density, which was examined in six experiments at Site 2, resulted in a significant (19.5–52.5%) reduction in SBDM severity in six of the eight evaluations. AUDPC was reduced by 21.7% to 50.8%. The relationship between SBDM severity and disease reduction was negative ($p < 0.1$, Table 8). No effect on yield was recorded in the two experiments in which we measured yield (Table 8 and Table S8). Thus, in both locations, disease severity was reduced by diluted planting density, but yield was not increased.

Table 8. Effect of planting density (PD) on the severity of sweet basil downy mildew (SBDM) in walk-in tunnels at Site 2a.

Experiment	AUDPC over the Growing Season (% × Days) [b]			Cumulative Yield (g/m^2)		
	Period (Days)	Dense (30)	Sparse (15)	Period (Days)	Dense (30)	Sparse (15)
PD8	98	2131 ± 128.5 [a]	1048 ± 111.5 [b]	110	3581 ± 116.4 [a]	3317 ± 98.7 [a]
PD9	114	2776 ± 331.9 [a]	2121 ± 252.4 [b]	110	3652 ± 242.4 [a]	3508 ± 221.3 [a]
PD10	114	2058 ± 164.7 [a]	1611 ± 147.7 [b]			
PD11	98	197.6 ± 51.42 [a]	108.5 ± 22.44 [b]			
PD12	112	3156 ± 232.1 [a]	2319 ± 117.4 [b]			
PD13	112	3254 ± 212.4 [a]	2421 ± 112.1 [b]			
	Disease severity—AUDPC relation					
			insignificant			

[a] Experiments were carried out with sweet basil plants grown in sand in walk-in tunnels at Site 2 during the spring 2014 (experiments PD8–PD10) and winter 2014–2015 (experiments PD11-PD13) growing seasons. Beds were covered with polyethylene. [b] SBDM severity was evaluated using a 0–100% scale, in which 0 = healthy plants and 100% = plants completely covered by SBDM symptoms/signs, and the value of the area under disease progress curve was calculated for each replicate. Averages ± SE. Values in each pair followed by a different letter are significantly different according to one-way ANOVA with Tukey's HSD. Default significance levels were set at $\alpha = 0.05$.

3. Discussion

Cultural measures can be used to suppress SBDM. The following factors were examined in the present work: tunnel orientation, air circulation in a greenhouse, polyethylene mulch, and plant spacing. The level of disease control did not vary with the different growing seasons and intermediate-level disease control was observed in both locations. Studies of cultural means of controlling downy mildews are scarce; therefore, it is only possible to compare the currently studied means with similar methods that have been examined in other patho-systems.

Nighttime air circulation in the greenhouse was increased in an effort to reduce the amount of water on the aerial plant organs and the amount of time that water was present. Humidity-promoted pathogens such as *P. belbahrii* thrive in the film of water that primarily appears at night and depend on it for their reproduction and their ability to infect the plant [9,13]. Increased air circulation can reduce the presence of water at the boundary layer over the plant organs even under humid conditions [20] and can limit humidity-promoted diseases [4]. Indeed, the test carried out over three growing seasons revealed that increased air circulation at night can suppress SBDM in greenhouses. Similarly, Papas [21] concluded that *B. cinerea* in out-of-season tomato (*Solanum lycopersicum*) plants grown in unheated glasshouses in Greece can be limited by adequate indoor air circulation. Similarly, in-bed air circulation has been shown to reduce *B. cinerea* gray mold in lisianthus (*Eustoma grandiflorum*) [22].

In the sweet basil-growing regions in Israel, walk-in tunnels are traditionally oriented east-west. The tunnel orientation affects the microclimate in the tunnel, since it relates to the direction of the sun [6,9,23] and prevailing winds. Surprisingly, the north-south orientation was associated with lower SBDM severity in all of our experiments. Nevertheless, the yields from north-south and east-west tunnels were similar. These results suggest that the temperature × hours gain in the two types of tunnels are similar, but the moisture duration was different. We are not aware of similar results in other patho-systems.

Polyethylene soil cover has been suggested for the control of gray mold induced by *B. cinerea* [24]. A plastic cover with a white upper surface reduced the incidence of *B. cinerea* infection in strawberry (*Fragaria × ananassa*), compared to bare soil [25]. Similarly, in lisianthus, the use of a polyethylene barrier between the lower leaves and soil that prevented the lower leaves from coming into contact with the wet soil reduced the development of *B. cinerea* along the leaves toward the stem and prevented

plant mortality [22]. The prevention of contact between the canopy and the wet soil is not relevant in the case of SBDM. However, polyethylene mulch effectively suppressed SBDM in both locations, congruent with the effects such mulch has been shown to have on *B. cinerea*-induced gray mold [8] and *S. sclerotiorum*-induced white mold [7] in sweet basil. As expected [7,8], polyethylene mulch increased shoot yield in two of three experiments.

In addition to decreasing evaporation from the growth medium, the polyethylene mulch also increases the bed temperature. Shtienberg et al. [26] showed that the polyethylene mulch causes irradiation flux across the canopy and the drying of the leaves and fruits of greenhouse tomato and cucumber (*Cucumis sativus*) plants. This helps to control tomato late blight (*Phytophthora infestans*) and downy mildew (*Pseudoperonospora cubensis*) in cucumber [26]. Polyethylene soil cover is associated with increased yields, thanks to the accumulation of heat in the root zone and higher soil temperatures [27]. It was suggested that passive greenhouse warming increases sweet basil's resistance to downy mildew by warming the root zone [9]. Recently, Gupta et al. [28] demonstrated that warming the root zone induces systemic resistance in plants. Indeed, warming the root zone of sweet basil under field conditions resulted in shoot resistance to the necrotrophic fungi *B. cinerea* and *S. sclerotiorum* that continued after harvest [7,8]. It may be assumed that polyethylene mulch may reduce the susceptibility of harvested leaves also to SBDM.

Reducing the density of sweet basil plants reduced SBDM, as previously demonstrated for gray mold and white mold [7,8]. Reduced plant density has also been shown to suppress disease in other patho-systems. Vieira et al. [29] reported decreased incidence of white mold and increased soybean yields when within-row densities were reduced. Lower plant density has also been shown to reduce stem gray mold in lisianthus [22]. Reducing the number of blond psyllium (*Plantago ovata*) seeds sown per unit area reduced the incidence of downy mildew (*Peronospora alta*) in that crop [30]. The reduction from 12 to 6 bean plants/m^2 decreased the severity of bean white mold (*S. sclerotiorum*) in one of two experiments, but did not decrease yield [31]. The severity of soybean stem canker (*Diaporthe phaseolorum* var. *meridionalis*) decreased proportionately to a decrease in plant densities [32]. In downy mildew of rose (*Peronospora sparsa*), reducing the density of container-grown plants had a measurable effect on the progress of downy mildew [33].

The reduction in sweet basil planting density resulted in reduced canopy volume at the beginning of the season. But, after the second harvest, the canopy was dense. Nevertheless, SBDM levels were lower in the reduced planting despite the dense canopy. The mechanism of this control could not be studied with the biotroph *P. belbahrii*, but our experience with *B. cinerea* and *S. sclerotiorum* pointed to reduced shoot susceptibility to pathogens [7,8]. We hypothesize that the reduction in planting density also affects the plants' susceptibility to SBDM.

We calculated the correlation between disease severity values and the intensity of disease reduction across experiments. There was a negative correlation between the disease in the denser plots and disease reduction in the plots in which plants were planted at the lower density, pointing to the fact that under conditions of lower disease pressure, increased plant spacing provides more pronounced disease suppression. Surprisingly, such a negative correlation was not found for the polyethylene mulch practice. In some experiments, we also examined the possibility of combining polyethylene mulch with reduced planting density, but that combination did not provide synergistic disease control (results not shown). When applied in combination with chemical fungicide, neither reduced plant density nor the use of polyethylene provided synergistic disease control (results not shown). As described, there was also no synergistic effect between increased air circulation and transparent or gray polyethylene mulch.

4. Materials and Methods

Experiments were carried out at two experimental stations (Sites 1 and 2, described in detail below) under semi-commercial conditions during the years 2013–2015. Sweet basil cv. Peri [34] plants were used in all of these experiments. Plugs were prepared in a commercial nursery (Hishtil, Ashkelon, Israel) and transplanted 3 to 4 weeks after seeding. Each plug contained 3 to 5 plants, but the plugs are usually referred to as plants. "Peri" is susceptible to *P. belbahrii* [9]. The experiments were carried out in greenhouses (Site 1) and in walk-in tunnels (Site 2). Downy mildew epidemics occurred naturally at the field sites, following the placement of infected basil plants next to the plots as described below.

4.1. Inoculation with P. belbahrii and Disease Evaluation

Spores of *P. belbahrii* were harvested in water by washing sporulating leaves of sweet basil plants that were kept in an experimental greenhouse at the Volcani Center, Agricultural Research Organization, Israel. The suspension was then filtered through cheesecloth. The concentration of spores was determined using a hemocytometer and a light microscope, and adjusted to 1×10^3 cells ml^{-1}. Potted sweet basil plants were inoculated by spraying with a spore suspension (5 mL $plant^{-1}$), incubated at high RH (>95%) in the dark in a growth chamber at 22 ± 1 °C for 12 h and then incubated in a greenhouse chamber at 22 ± 2 °C for 1 week, and incubated at high RH (>95%) in the dark in a growth chamber at 22 ± 1 °C for 12 h and then incubated in a greenhouse chamber at 22 ± 2 °C for symptom development [9]. The potted sweet basil plants subjected to this artificial inoculation served as a source of inoculum to ensure even inoculum loads across the greenhouses and walk-in tunnels. The plants were placed at the borders of each plot.

The evaluation of the severity of sweet basil downy mildew (SBDM) in the plots included all plants except those along the 1 m edges of each plot. The severity of SBDM was determined periodically in all plants of each plot in each experiment on a scale of 0 to 100, in which 0 = all plants visually healthy, 10 = 10% of the leaf area in the plot covered by typical downy mildew symptoms of chlorosis and/or dry necrotic lesions or *P. belbahrii* spores on the undersides of the leaves, and 100 = all leaves on all plants in the plot show typical downy mildew symptoms/signs [9].

4.2. Shoot Weight

In selected experiments, shoots longer than 15 cm were harvested and weighed three to five times during the growing season, as detailed below. The yield was collected separately for each plot, sorted for quality, and calculated per m^2 bed. The cumulative yield figures for the various harvests were calculated and those figures are presented.

4.3. Site 1—Eden Experimental Station

Experiments were conducted at the Eden Experimental Station (32°46′79 N, 35°48′88 E; 120 m below mean sea level) at the Emek Hamaayanot Research and Development Center. The regional climate is Mediterranean, semiarid with winter rains and a dry, hot summer. At this site, experiments were carried out in two 400 m^2 greenhouses. The structures were covered with 150 µm-thick Sunsaver Clear IR AV polyethylene (Ginegar Plastic Products, Kibutz Ginegar, Israel). The greenhouses were aerated during the day and closed during the night (18:00 to 07:00). At night, the greenhouses were heated to 12 °C to prevent physiological damage to the leaves. There were five bays in each greenhouse and the bays were separated with 1.8 m-high transparent polyethylene.

The potting material was tuff (volcanic gravel; 3 to 6 mm particles) placed in plastic containers that were 1 m wide × 15 cm deep × 20 m long (Mapal, Mevo Hama, Israel). Plants were irrigated daily according to local extension service recommendations, allowing 30% drainage, and fertigated proportionally with 5-3-8 N-P-K fertilizer at a rate of 2 L/1000 L water. The nutrient concentrations were therefore 8.6, 1.0, and 4.0 mM N, P, and K, respectively. Fertigation was performed using a 17 mm drip-irrigation pipe with a 1 L/h dripper embedded in the pipe every 20 cm. Plots were 5 m long each, containing 108–125 plants/plot at the higher plant density (24–25 plants/m^2) mentioned below.

Experiments were carried out over three consecutive growing seasons, with planting dates of 9 September (fall 2013), 24 February (spring 2014), and 19 January (winter 2015). Treatments consisted of different cultural methods, as detailed below and in Table 9. Plots consisted of one bed (1 m wide and 4.5 to 5.0 m long) and there were 4–8 plot replicates.

Table 9. Experiments conducted with sweet basil downy mildew in greenhouses at Site 1.

Growing Season	Expt. No.	Tested Factors	Other Agronomic Parameters		
			Polyethylene Mulch	Plant Spacing (Plugs/m^2)	Air Circulation (AC)
Fall 2013	M1	Polyethylene mulch (PM)	Gray, yellow, transparent PE	25	No
	M2	PM	Gray PE	25	No
	PD1	Planting density (PD)	Bare soil	25 vs. 15	No
	PD2	PD	Gray PE	30 vs. 15	No
	PD3	PD	Gray PE	25 vs. 15	No
	AC1	AC	Gray PE	25	AC
Spring 2014	M3	PM	Gray, yellow, transparent PEs	24	No
	M4, M5	PM	Gray PE	24	No
	M6	PM	Transparent PE	24	No
	PD4, PD6	PD	Bare soil	21 and 24 vs. 14	No
	PD5	PD	Gray PE	24 vs. 14	No
	AC2	AC	Gray PE	24	AC

Fall 2013 experiments: SBDM was first observed on 27 November 2013, 80 days after planting. There were five shoot harvests, starting 7 October 2013 (29 days after planting).

Spring 2014 experiments: SBDM was first observed on 20 March 2014, 24 days after planting. There were six shoot harvests, starting 26 March 2014 (30 days after planting).

Winter 2015 experiments: SBDM was first observed on 14 April 2015, 90 days after planting. Shoots were harvested five times, starting 1 March (45 days after planting).

4.4. Cultural Methods Applied at Site 1

Air circulation: Four fans (60 cm diam., Adirom Heating and Ventilation Engineering Ltd., Ashkelon, Israel) were installed 2 m above the beds, facing the canopy of plants that were planted at the area of one third of a greenhouse bay toward the north or south edge of the greenhouse. The fans were operated once every hour for 15 min from 19:00 until 08:00. Beds were covered with gray polyethylene mulch and the planting density was 24–25 plants/m^2.

Planting density: Sweet basil plants were planted at two densities: 24–30 plants/m^2, as is customary in the area, and 14–15 plants/m^2. The higher planting density was also used in experiments in which planting density was not a tested parameter. The beds were left bare or covered with gray or transparent polyethylene (Table 9).

Polyethylene mulch: The beds were either left uncovered (bare growing medium) or covered with sheets of polyethylene. Several types of polyethylene were examined: (1) transparent 30 μm-thick Sunsaver Clear IR polyethylene (Ginegar), (2) gray-black 30 μm-thick Mulch-More polyethylene (Ginegar) with the gray-colored side visible and the black-colored side facing the ground, and (3) yellow-brown 30 μm-thick Mulch-More polyethylene (Ginegar) with the yellow side visible and the brown side facing the ground. The plant density was 24–25 plants/m^2 (Table 9).

4.5. Site 2—Zohar Experimental Station

This research station is located in the Sedom area south of the Dead Sea and is part of the Northern Arava Research and Development Center. It is located at 30°94′656.2 N, 35°40′341.7 E at 354 m below mean sea level. The weather at the Zohar Station is arid. In the winter, rain is rare and the mean daytime temperature is 22 °C. The summers are dry and hot, with an average daily temperature of 33 °C. The work at the Zohar Experimental Station was carried out in 10 walk-in tunnels. Each tunnel was 40 m long and 5 m wide (200 m^2). The structures were covered with 100 μm-thick Sunsaver Clear IR AV polyethylene (Ginegar Plastic Products, Ginegar, Israel). The front and back openings of each tunnel were covered with 50-mesh netting. Five round aeration openings (50 cm diam.) were cut along the length of the tunnels and covered with 50-mesh netting. One-meter-wide sandy soil beds were planted with 30 plants/m^2, unless otherwise noted. Plants were irrigated with local brackish water (4 decisiemens per meter), according to the local extension service recommendations, and fertigated with 1.0 L/1000 L 8-2-4 N-P-K fertilizer. Nutrient concentrations were therefore 6.9, 0.33, and 1.0 mM N, P, and K, respectively. Fertigation was performed using a 17 mm drip-irrigation pipe with a 1.2 L/h dripper embedded in the pipe every 20 cm. Each plot consisted of two beds that were each 9 m long, unless otherwise mentioned.

Experiments were carried out over two consecutive growing seasons, with planting dates of 19 February (spring 2014 season) and 11 November 2014 (winter 2015 season). Treatments consisted of different cultural methods, as detailed below and in Table 10. Plots consisted of two beds (1 m wide and 9 m long) containing 270 plants/plot of 30 plants/m^2 and there were 5–10 plot replicates.

Spring 2014 experiments: SBDM was first observed on 20 March 2014, 31 days after planting. There were six shoot harvests, beginning 15 March 2014 (36 days after planting).

Winter 2015 experiments: SBDM was first observed on 21 January 2015, 71 days after planting. Shoots were harvested four times starting 8 December 2014 (27 days after planting).

Table 10. Experiments conducted with sweet basil downy mildew in walk-in tunnels at Site 2.

Growth Season	Expt. No.	Tested Factors	Other Agronomic Parameters		
			Polyethylene Mulch	Plant Spacing (Plugs/m^2)	Tunnel Direction (TD)
Spring 2014	M8, M10	Polyethylene mulch (PM)	Transparent vs. bare	30	East-West (EW)
	M9	PM	Transparent vs. bare	30	North-South (NS)
	PD8, PD9	Plant density (PD)	Transparent	30 vs. 15	EW
	PD10	PD	Transparent	30 vs. 15	NS
	TD1	Tunnel direction (TD)	Transparent	30	NS vs. EW
	TD2	Tunnel direction (TD)	Bare	30	NS vs. EW
Winter 2014-2015	M11, M14	PM	Transparent vs. bare	30	NS
	M12, M13	PM	Transparent vs. bare	30	EW
	PD11	PD	Transparent	15 vs. 30	NS
	PD12, PD13	PD	Transparent	15 vs. 30	EW
	TD3	TD	Transparent	30	NS vs. EW

4.6. Cultural Methods Applied at Site 2

Tunnel direction: Walk-in tunnels were oriented north-south or east-west with either bare soil or with transparent polyethylene mulch. The planting density in the plots was 30 plants/m^2 (Table 10).

Planting density: Sweet basil plants were planted at two densities: 30 plants/m^2, as is the common local practice, or 15 plants/m^2. The higher planting density was also used in experiments in which the planting density was not a tested parameter. The soil was covered with transparent polyethylene (Table 10).

Polyethylene soil mulch: The beds were either left uncovered (bare growing medium) or covered with transparent polyethylene (30 µm-thick Sunsaver Clear IR polyethylene; Ginegar). The beds were planted with 30 plants/m^2 in tunnels oriented either north-south or east-west (Table 10).

4.7. Experimental Design and Statistical Analysis

Treatments in each year and each field experiment were replicated 4–10 times. Replicates of each treatment were arranged randomly. Disease severity was evaluated in each plot (replicate). Area under the disease severity progress curve (AUDPC) values were also calculated. Data in percentages were arcsine-transformed before further analysis. Disease severity (%) and AUDPC (% × days) data were analyzed using ANOVA and Tukey's HSD test. Standard errors (SE) of the means were calculated and disease levels were statistically separated following a one-way analysis of variance. Treatments in experiments with combined two-treatment factors were statistically separated following a two-way analysis of variance. Statistical analyses were performed using JMP 5.0 software (SAS Institute, Cary, NC, USA).

Disease reduction was calculated as follows:

$$\%\ \text{disease reduction} = 100 - 100 \times (\text{disease severity}_{TT}/\text{disease severity}_{control}). \quad (1)$$

The combined effect of the control measures used was estimated using the Abbott formula [35,36]. The expected disease reduction (control efficacy) and the combined suppressive activity were calculated as:

$$CE_{exp} = a + b - a \times b/100 \text{ and } SF = CE_{obs}/CE_{exp}, \quad (2)$$

where a = disease reduction due to one measure when applied alone, b = disease reduction due to the other measure when applied alone, CE_{exp} = expected control efficacy of the combined treatment if the two measures act additively, CE_{obs} = observed disease reduction for the combined treatment, and SF = the synergy factor achieved by the combined treatment. When SF = 1, the interaction between the control measures is additive. When SF < 1, the interaction is antagonistic, and when SF > 1, the interaction is synergistic [26,35,36]. The same formula was used to calculate SF in the context of yield.

5. Conclusions

Increased air circulation, reduced plant density, polyethylene mulch, and the north-south orientation of walk-in tunnels moderately reduced SBDM under commercial conditions. These practices can contribute to efforts to reduce the dependence on chemical fungicides in sweet basil crops that commercially, because of demands of minimized chemical residues, can tolerate only limited use of such chemicals at application times that are temporally far from harvest.

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