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Improving the Biocontrol Potential of Bacterial Antagonists with Salicylic Acid against Brown Rot Disease and Impact on Nectarine Fruits Quality

Nadia Lyousfi [1,2], Rachid Lahlali [1,*], Chaimaa Letrib [1], Zineb Belabess [3], Rachida Ouaabou [4], Said Ennahli [5], Abdelali Blenzar [2] and Essaid Ait Barka [6,*]

1 Phytopathology Unit, Department of Plant Protection, Ecole Nationale d'Agriculture de Meknès, Km10, Rte Haj Kaddour, BP S/40, Meknès 50001, Morocco; nadia.lyousfi@usmba.ac.ma (N.L.); cletrib@enameknes.ac.ma (C.L.)
2 Laboratory of Plant Protection and Environment, Faculty of Sciences, Moulay Ismail University, P.O. Box, Zitoune, Meknès 11201, Morocco; ablenzar@yahoo.fr
3 Plant Protection Laboratory, INRA, Centre Régional de la Recherche Agronomique (CRRA), Qualipole de Berkane, Berkane 63300, Morocco; belabess.zineb@gmail.com
4 Faculty of Science Semlalia, Cadi Ayyad University, Bd. Prince My Abdellah, B.P. 2390, Marrakech 40000, Morocco; rachaouaabou@gmail.com
5 Department of Arboriculture-Viticulture, Ecole Nationale d'Agriculture de Meknès, Km10, Rte Haj Kaddour, BP S/40, Meknès 50001, Morocco; ennahlisaid@gmail.com
6 Unité de Recherche Résistance Induite et Bio-Protection des Plantes-EA 4707, Université de Reims Champagne-Ardenne, 51100 Reims, France
* Correspondence: rlahlali@enameknes.ac.ma (R.L.); ea.barka@univ-reims.fr (E.A.B.); Tel.: +212-55-30-02-39 (R.L.); +33-3-2691-3441 (E.A.B.)

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Abstract: The main objective of this study was to evaluate the ability of both antagonistic bacteria *Bacillus amyloliquefaciens* (SF14) and *Alcaligenes faecalis* (ACBC1) used in combination with salicylic acid (SA) to effectively control brown rot disease caused by *Monilinia fructigena*. Four concentrations of salicylic acid (0.5%, 2%, 3.5%, and 5%) were tested under in vitro and in vivo conditions. Furthermore, the impact of biological treatments on nectarine fruit parameters' quality, in particular, weight loss, titratable acidity, and soluble solids content, was evaluated. Regardless of the bacterium, the results indicated that all combined treatments displayed a strong inhibitory effect on the mycelial growth of *M. fructigena* and disease severity. Interestingly, all SA concentrations significantly improved the biocontrol activity of each antagonist. The mycelial growth inhibition rate ranged from 9.79% to 88.02% with the highest reduction rate recorded for bacterial antagonists in combination with SA at both concentrations of 0.5% and 3.5%. The in vivo results confirmed the in vitro results with a disease severity varying from 0.00% to 51.91%. A significant biocontrol improvement was obtained with both antagonistic bacteria when used in combination with SA at concentrations of 0.5% and 2%. The lowest disease severity observed with ACBC1 compared with SF14 is likely due to a rapid adaptation and increase of antagonistic bacteria population in wounded sites. The impact of all biological treatments revealed moderate significant changes in the fruit quality parameters with weight loss for several treatments. These results suggest that the improved disease control of both antagonistic bacteria was more likely directly linked to both the inhibitory effects of SA on pathogen growth and induced fruit resistance.

Keywords: *Monilinia fructigena*; biological control; *Bacillus amyloliquefaciens* (SF14); *Alcaligenes faecalis* (ACBC1); salicylic acid; nectarines

1. Introduction

Fresh nectarines are much-appreciated fruits by consumers worldwide for their high nutrition and juicy and agreeable flavor [1]. Unfortunately, they are often subjected to rapid

deterioration during pre- and postharvest, especially when storage conditions are not well respected. Indeed, most of these losses are caused by spoilage fungi. Brown rot, caused by *Monilinia* spp., is a fungal disease that causes considerable pre- and postharvest economic losses of rosaceous fruit trees [2–4]. The genus *Monilinia* includes several economically important and aggressive pathogens on *Rosaceae*, *Ericaceae*, and *Empetraceae* [5]. Loss (10%) due to brown rot disease was estimated to be 2.1 M €/year [2,4,6] for a global production of peaches and nectarines of 25 MT in the growing season of 2019 [7]. Under some circumstances, the disease can lead to 80% of postharvest fruit loss, mainly when environmental conditions are suitable for disease growth in the orchard.

In general, postharvest fungal diseases limit the storage period and the marketing of fruits [8]. They cause significant losses in fruits and even vegetables, but they can be effectively controlled by applying synthetic chemical fungicides before and during storage [9]. Nevertheless, the frequent use of synthetic fungicides has prompted the appearance of resistant pathogen strains in the most markedly effective active substances [10–12]. Moreover, the strict requirements of sustainable agriculture based on integrated crop management and organic production have led to the need to develop alternative methods for brown rot disease control. To reduce fungicide residues in fruits and vegetables, decrease their subsequent environmental and toxicological risks, and protect consumers who have become more demanding of natural and organic products fungicides-free, the use of postharvest chemical treatments is increasingly limited due to rigorous regulation, carcinogenicity, long degradation period, and high and acute residual toxicity [12,13]. Furthermore, in addition to the environmental pollution, there is increasing public concern regarding chemical residues in fruits.

Therefore, several global research programs have focused on the development of effective eco-friendly alternative strategies for controlling fruit postharvest diseases that are safer to human health and the environment. Therefore, several biological control agents (BCAs) have been used against a large range of postharvest fungal pathogens, including *Botrytis cinerea*, *Monilinia* spp., *Penicillium* spp., and *Geotrichum candidum*, on many perishable agricultural products [12,14,15]. For example, numerous bacteria belonging to the *Bacillus* genus have shown good biocontrol potential against major postharvest disease pathogens of fruits [8,15]. However, BCAs have allowed a significant reduction of disease incidence but have not completely controlled the disease [16–19]. To overcome this gap, researchers have attempted their use in combination with other salts, additives, and fungicides at low doses [14,20,21]. Salicylic acid (SA) is among the additives widely used with bacterial antagonists and yeasts for the control of postharvest fruit diseases [17,22–25]. SA, a naturally occurring phenolic compound, is involved in mediating local and systemic resistance to plant pathogens [26–28]. In recent years, SA has become widely used as an alternative strategy for fruit fungal disease control [24,25,28,29].

As elsewhere, in Morocco, few attempts have been made to develop eco-friendly strategies for brown rot control [8,19,30]. In this regard, two antagonistic bacteria, *Alcaligenes faecalis* (ACBC1) and *Bacillus amyloliquefaciens* (SF14), were recently selected for their higher efficacy in reducing brown rot disease [8] and fire blight disease [31] on apple fruits. Although there is little information on SA-mediated resistance to various plant diseases [22,32–34], there is still an important gap to fill about the effect of bacterial antagonists associated with SA for postharvest disease control. Recently, the use of *Bacillus subtilis* in combination with SA was proven to be of great interest [16,17]. Therefore, to strengthen their biocontrol activity, their use in combination with SA will be evaluated under in vitro and in vivo conditions. Additionally, the impact of these combinations on the quality parameters of nectarine fruits, such as weight loss (WL), total soluble solids (TSSs), titratable acidity (TA), and maturity index (MI), would be evaluated as well.

2. Materials and Methods

2.1. Fungal Pathogen Preparation

The fungal pathogen *M. fructigena* (VPBG) causing the brown rot disease on numerous rosaceous fruit trees was used in this study. This pathogen was originally isolated from sweet cherry in Serbia in 2010 [8]. For long-term storage, this fungus was maintained in 25% glycerol at −80 °C. For frequent use, the fungus was subcultured on PDA (potato dextrose agar) medium for 7 to 12 days at 25 °C in darkness.

The conidial suspension of *M. fructigena* was prepared from a 10-day-old colony by adding 3 mL of sterile distilled water (SDW) containing Tween 20 (0.05%) and gently scraping the surface of the colony with a fine scalpel to separate the mycelium and spores from the PDA medium. The obtained yield was then filtered with sterilized Whatman paper to remove mycelial debris and to recover spores. The final concentration of the conidial suspension was adjusted to 1×10^4 spores/mL under an optical microscope (Ceti Microscopes NLCD-307B, Chalgrove, UK) with a Malassez cell (Roche, Meylan, France).

2.2. Antagonist Preparation

The bacterial antagonists ACBC1 and SF14 were respectively isolated from the soil and flowers of apple trees in Morocco [31]. These bacteria were chosen due to their higher displayed efficacies against *M. fructigena* on apple fruit during postharvest storage [8]. Both antagonists were stored at the Plant Pathology Laboratory (ENA-Meknes) in liquid Luria–Bertani (LB) medium amended with 20% glycerol in Eppendorf tubes at −20 °C until further use. Before the experiment, each antagonist was recovered, subcultured on LB medium, and incubated at 28 °C in the darkness. The bacterial suspension of each bacterium was prepared from a 24 h old culture grown on LB medium. For each bacterium, Petri dishes contained bacterial colony cultures in streaks were flooded with 10 mL of SDW, scraped off gently with a sterile dropper, recovered in Falcon tube (15 mL), and homogenized by vortexing. The final concentration of each bacterial antagonist was adjusted to ~2 OD (1×10^8 CFU/mL) using a Spectronic 20 spectrophotometer (Bausch & Lomb Incorporated, Rochester, NY, USA).

2.3. Chemical Substances

SA (AppliChem, Darmstadt, Germany) was assayed alone and in combination with each bacterial antagonist at different concentrations of 0.5%, 2%, 3.5%, and 5% (*w*/*v*). The fungicide methyl-thiophanate (500 g/L) has served as a positive control for in vivo experiments and has been applied to wounded fruit sites at a concentration of 1 ppm.

2.4. Fruit Preparation

The citrus fruits, nectarine (*Prunus persica* var. *zincal*) fruits, used in this study were harvested at their maturity stage from a commercial orchard near Sefrou City, Morocco, and had not received any postharvest treatment. The fruits were selected based on their size and the absence of physical injuries and infection. All fruits were surface-disinfected by dipping them into 2% sodium hypochlorite solution for 5 min, rinsed twice with SDW, and allowed to air-dry for 1 h, before their use in the various in vivo tests.

2.5. In Vitro Effects of SA, Antagonists, and Their Combined Treatments on Fungal Mycelial Growth

Different concentrations of SA were prepared by serial dilution in SDW, and then added to the PDA medium. The culture of both antagonistic bacteria was carried out using a 24 h old culture. To evaluate the effect of the bacteria on the pathogen, a circular disc (5 mm in diameter) of filter paper was placed on both sides of the PDA medium containing 2.5 µL of the bacterial suspension as previously described by Liu et al. [35]. To assess the combined effect, a suspension (2.5 µL) from each bacterium was added to the sterilized filter paper and placed on modified PDA media with SA at different concentrations (0.5%, 2%, 3.5%, and 5%). For all treatments, a mycelial plug (5 mm in diameter) of the *M. fructigena*

was placed at the center of each petri dish (90 mm) containing the PDA medium with/out SA and with/out either bacterium. Plates containing only PDA served as controls. All plates were arranged in a randomized complete design and incubated at 25 °C in darkness. For each treatment, four petri dishes were used. Mycelial growth was then evaluated and recorded for each treatment 5 and 10 days after incubation periods by measuring the diameter (mm) of the fungal colonies [8]. The inhibition rate (IR) was calculated according to the following formula:

$$IR = (D_C - D_T)/D_C \times 100$$

where D_C means the colony diameter of the pathogenic fungus in the control treatment (PDA medium without biological treatments/SA treatments), and D_T means the colony diameter of the pathogenic fungus in bacterial treatments, SA treatments, and their combinations.

Besides, microscopic examinations of the mycelial structure and shape of *M. fructigena* under different treatments (SA, antagonistic bacteria, and their combinations) were conducted 10 days after incubation periods using a light microscope (Ceti Microscopes NLCD-307B, Chalgrove, UK).

2.6. In Vivo Effects of SA, Antagonists, and Combined Treatments on Brown Rot Disease

Disinfected nectarines were wounded twice at their equatorial zone and treated with 50 µL/wound of SA (0.5%, 2%, 3.5%, and 5%) alone or in combination with either antagonist ACBC1 or SF14 (1×10^8 CFU/mL). The treated fruits were then inoculated 4 h afterward with a conidial suspension of the pathogen (50 µL/wound) concentrated at 1×10^4 spores/mL. The untreated control was inoculated only with 50 µL of SDW instead of biological treatments, while the negative control received 50 µL of the fungicide methyl-thiophanate (1 ppm). All fruits were then placed in a growth room chamber at 25 °C for 10 days [36]. The experiment was repeated twice over time with five replicates (of 5 fruits, 10 wounds) for each treatment. The disease severity was measured after 5 and 10 days after fruit infection. The lesion diameters were recorded using a caliper, and disease severity (DS) was calculated according to the following formula [8]:

$$DS\ (\%) = (D_T)/(D_C) \times 100$$

where D_T means the averaged diameter (mm) of treated wounds with biological treatments/SA/fungicides, and D_C means the averaged diameter (mm) of wounds in the untreated control (inoculated only with pathogenic fungus).

2.7. Effect of Treatments on Fruit Quality Parameters

2.7.1. Weight Loss

Nectarine fruit weight loss experiments were monitored with two replicates of three fruits per treatment. Fruits without defects or injuries were selected, then numbered, and subjected to appropriate treatment as described above. The weight of each fruit was recorded just after the treatment, and then after 10 days. For each fruit, the weight loss was calculated as % weight loss referenced to the initial weight of the fruit, immediately after treatment [100 × (initial weight − weight)/initial weight] [37].

2.7.2. Total Soluble Solids

The total soluble solids (TSSs) content was determined as previously described by Qin et al. [32]. Briefly, TSSs content was evaluated by recording the refractive index of the fruit after 10 days of incubation with a Model PAL-1 digital refractometer (Atago, Tokyo Tech., Tokyo, Japan) at room temperature, and the result was expressed as % Brix.

2.7.3. Titratable Acidity

Titratable acidity (TA) was measured 10 days after incubation by titration of 10 mL of fruit juice diluted with 50 mL of SDW with 0.1 mM NaOH at pH 8.1 and phenolphthalein as an indicator. The results were expressed in grams of malic acid per liter of juice [38].

TA is expressed as a percentage of citric acid anhydride per liter of juice by following the Association of Official Analytical Chemists (AOAC) 942.15 method [39].

2.7.4. Maturity Index

Maturity index (MI) was determined as the ratio of TSSs to TA as previously described [40,41].

2.8. Population Dynamics of Antagonists (SF14 and ACBC1) in Fruit Wounds

The most effective concentration for controlling postharvest fruit/vegetable diseases is generally considered to be 10^7–10^8 CFU/mL. The population dynamics of both antagonistic bacteria were studied in wounded nectarine fruits. Twenty µL of the bacterial suspension (1×10^8 CFU/mL/injury) of ACBC1 or SF14 was placed at the wound site of each fruit, which then was kept at 25 °C. The population of each antagonist was monitored 24, 48, 72, and 96 h after incubation. The bacterial cell number in each wound (CFU/wound) was determined by injecting 200 µL of SDW into each wound. The content of each wound was then recovered by pipetting and transferred to an appropriate tube. The tubes were then homogenized by vortexing for 10 min. The resulting suspension was 10-fold serially diluted in SDW, and an aliquot (100 µL) of each dilution was spread on LB medium. All plates were then incubated at 25 °C for 48 h before colony count. The population density of each antagonistic bacterium was expressed as log CFU/wound. This experiment was twice repeated over time with five fruits for each incubation period.

2.9. Statistical Analysis

All in vitro and in vivo datasets were subjected to the analysis of variance (ANOVA) procedure of the statistical software SPSS (SPSS 20.0, SPSS Inc., Chicago, IL, USA). When a significant effect was revealed, the least significant difference (LSD) test was performed for means separation at $p < 0.05$.

3. Results

3.1. In Vitro Effects of SA, Antagonists, and Their Combined Treatments on Fungal Growth

Regardless of the bacterial antagonist, the in vitro effects of the different treatments of SA, antagonistic bacteria, and their combinations on the mycelial growth of *M. fructigena* were significant (Table 1). All treatments significantly reduced mycelial growth relative to the control. Besides, the mycelial growth inhibition rates (%) were significantly influenced by the treatments and incubation periods (after 5 and 10 days). After 5 days of incubation, the highest inhibitions were recorded by the treatments SF14, 0.5 SA + SF14, and 3.5 SA + SF14 with inhibition rates of 78.60%, 74.18%, and 72.03%, respectively (Table 1), while ACBC1 recorded the highest inhibition rate (84.80%) when combined with SA at 0.5% and 3.5% (Table 1). After 10 days of incubation, the highest inhibition rate (88.02%) was obtained with the combined treatment 3.5 SA + ACBC1 (Table 1).

The results also underline that the inhibition rates obtained with SA increased by increasing the concentration of SA (Table 1). Furthermore, Figure 1 shows the form and structure of mycelium in treatments with ACBC1 alone or in combination with SA concentration (0.5 SA and 2 SA). Microscopic observations reveal that mycelium from inhibition zones showed a significant deformation and degradation in hyphal structures, cellular lysis, hyphal swelling, and vacuolation when compared with normal mycelium from the untreated control.

Table 1. Inhibition rate of mycelial growth (%) of *M. fructigena* obtained by salicylic acid, antagonistic bacteria (*Bacillus amyloliquefaciens* SF14), antagonistic bacteria (*Alcaligenes faecalis* ACBC1), and combinations thereof after 5 and 10 days of incubation at 25 °C.

Treatments	5 Days of Incubation		10 Days of Incubation	
	Colony Diameter (mm)	IR (%)	Colony Diameter (mm)	IR (%)
Untreated Control	54.03 ^{j}	0.00	82.75 ^{j}	26.28
0.5 SA	48.74 ^{i}	9.79	61.00 ^{i}	26.28
2 SA	45.43 ^{h}	15.92	55.18 ^{h}	33.32
3.5 SA	34.10 ^{g}	36.89	44.30 ^{g}	46.47
5 SA	12.25 ^{b}	77.33	35.35 ^{f}	57.28
SF14	11.56 ^{b}	78.60	16.74 ^{c}	79.77
0.5 SA + SF14	13.95 ^{bc}	74.18	15.55 ^{bc}	81.21
2 SA + SF14	23.57 ^{e}	56.38	24.52 ^{e}	70.37
3.5 SA + SF14	15.11 ^{c}	72.03	16.82 ^{c}	79.67
5 SA + SF14	16.63 ^{d}	69.22	17.64 ^{c}	78.68
ACBC1	18.00 ^{f}	67.34	20.13 ^{de}	75.82
0.5 SA + ACBC1	8.38 ^{a}	84.80	13.71 ^{b}	83.53
2 SA + ACBC1	17.68 ^{e}	67.92	18.97 ^{d}	77.21
3.5 SA + ACBC1	8.36 ^{a}	84.83	9.97 ^{a}	88.02
5 SA + ACBC1	12.25 ^{b}	77.78	15.46 ^{bc}	81.43

The data are the average of two independent trials with four replicates for each pathogen treatment combination. The mean diameters with the same letter are not significantly different according to the least significant difference (LSD) test ($p < 0.05$). SA: salicylic acid; SF14: *Bacillus amyloliquefaciens*; ACBC1: *Alcaligenes faecalis*; and IR (%): inhibition rate.

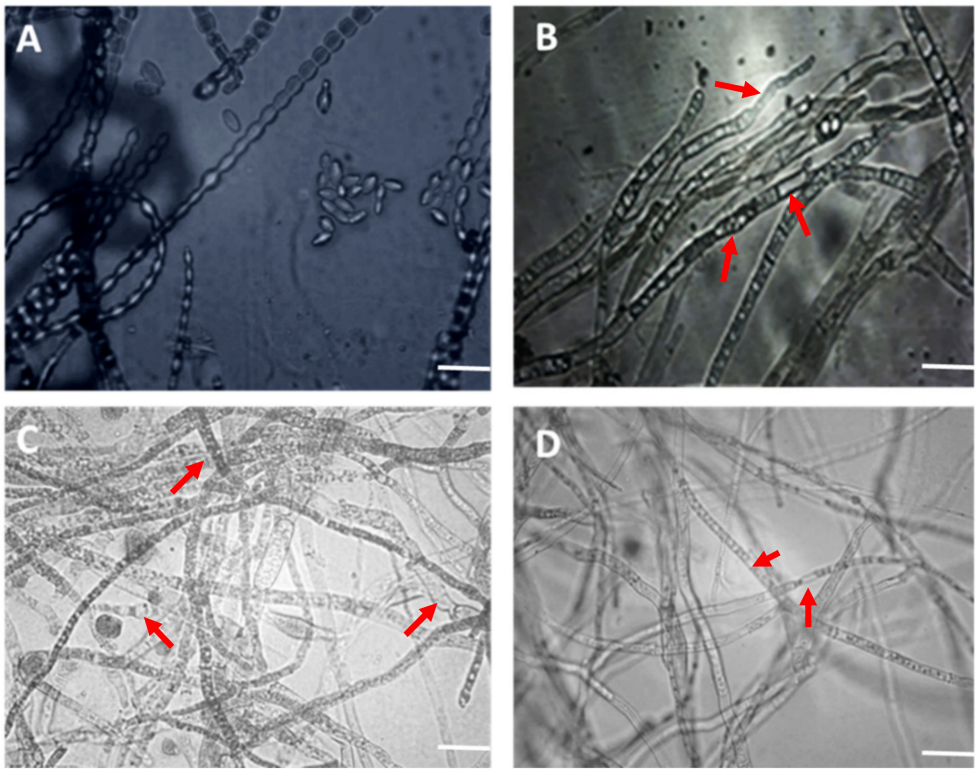

Figure 1. Microscopic observation of hyphal abnormalities of *M. fructigena* (10 × 40× magnifications) after 10 days of incubation at 25 °C. Normal hyphae in the untreated control (**A**,**B**) altered hyphae cell wall and swelling (ACBC1 alone), (**C**) swelling and vacuolation and discontinuation of hyphae (ACBC1 + 0.5 SA), and (**D**) fine swelling hyphae (ACBC1 + 2 SA). Arrows indicate changes occurring in the hyphae and mycelia. Scale bar = 50 µm.

3.2. In Vivo Effects of SA, Antagonists, and Their Combined Treatments on Brown Rot Disease

A significant difference between the control treatment and other treatments, including SA alone, antagonist, and their combinations, is highlighted (Figure 2). The lesion diameters of the brown rot disease due to *M. fructigena* in different treatments and their resulting disease severities are listed in Table 2 for both ACBC1 and SF14. The maximum severity (51.91%) was recorded for the 0.5 SA treatment at 10 days after incubation, where the lowest disease severity was recorded for both SA treatments at 3.5% and 5% with 9.36% and 3.53%, respectively. The lowest disease severities (less than 20%) were obtained for the treatments 3.5 SA (0–9.36%) and 5 SA (0–3.53%). When the SA was combined with the antagonists, the most effective combination was observed in 0.5 SA + ACBC1 (8.60–14.99%) and 0.5 SA + SF14 with severities of about 13.31% and 21.14% at 5 and 10 days after inoculation. However, for SF14 an increase of disease severity was seen at both SA concentrations of 3.5% and 5%.

The disease severity of brown rot increased with increasing incubation periods from 5 to 10 days. Statistical analysis showed a significant difference between treatments and control. The results underline that the disease severity was significantly reduced whatever was the duration of the incubation period (5 or 10 days) in the treated fruits.

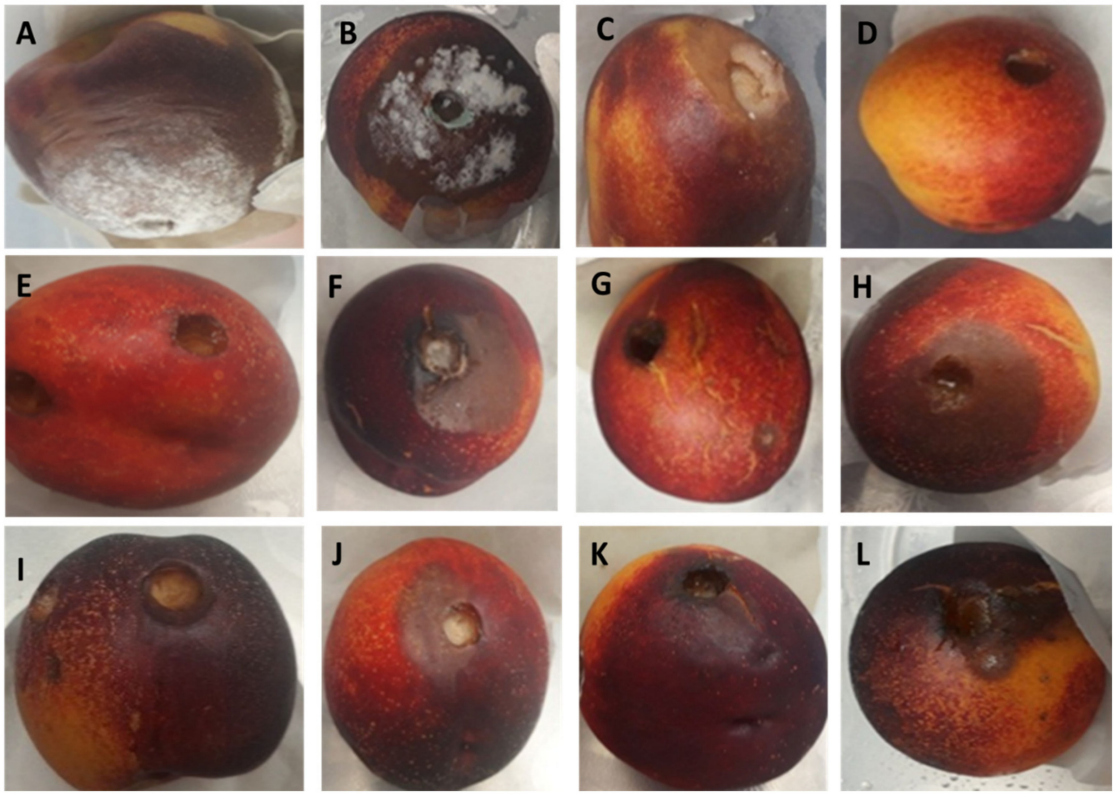

Figure 2. Brown disease development on nectarine fruits as affected by antagonists and their combined treatments with salicylic acid (SA) after 10 days of incubation at 20 °C. Each wound received 50 µL of each treatment and inoculated 4 h later with 50 µL of the pathogenic fungus (1×10^4 spores/mL). Untreated control (**A**), 0.5 SA (**B**), 2 SA (**C**), 3.5 SA (**D**), 5 SA (**E**), SF14 alone (**F**), SF14 + 0.5 SA (**G**), SF14 + 2 SA (**H**), methyl-thiophanate (**I**), ACBC1 alone (**J**), G: ACBC1 + 0.5 SA (**K**), and H: ACBC1 + 2 SA (**L**).

Table 2. Disease severities (DS, %) of brown rot on nectarines obtained by salicylic acid, antagonistic bacteria (*Bacillus amyloliquefaciens* SF14), antagonistic bacteria (*Alcaligenes faecalis* ACBC1), and combinations thereof after 5 and 10 days of incubation at 25 °C.

Treatments	5 Days of Incubation		10 Days of Incubation	
	Lesion Diameter (mm)	DS (%)	Lesion Diameter (mm)	DS (%)
Untreated control	58.08 ^{j}	100.00	68.27 ^{f}	100.00
0.5 SA	21.46 ^{i}	36.95	35.44 ^{e}	51.91
2 SA	11.04 ^{f}	19.01	21.08 ^{d}	30.88
3.5 SA	0.00 ^{a}	0.00	6.39 ^{ab}	9.36
5 SA	0.00 ^{a}	0.00	2.41 ^{a}	3.53
SF14	11.49 ^{f}	19.78	18.16 ^{cd}	26.60
0.5 SA + SF14	7.73 ^{d}	13.31	14.43 ^{bc}	21.14
2 SA + SF14	10.72 ^{f}	18.46	15.19 ^{bc}	22.25
3.5 SA + SF14	16.01 ^{h}	27.57	26.71 ^{de}	39.12
5 SA + SF14	15.42 ^{h}	26.55	22.27 ^{d}	32.62
ACBC1	5.35 ^{c}	9.21	10.3 ^{b}	15.08
0.5 SA + ACBC1	5.00 ^{c}	8.60	10.24 ^{b}	14.99
2 SA + ACBC1	9.78 ^{e}	16.83	14.07 ^{bc}	20.60
3.5 SA + ACBC1	13.27 ^{g}	22.84	18.91 ^{cd}	27.70
5 SA + ACBC1	10.96 ^{f}	18.87	16.92 ^{c}	24.78
MT	3.23 ^{b}	5.56	5.75 ^{ab}	8.42

The data are the average of two independent trials with five replicates (5 fruits, 10 wounds) for each pathogen treatment combination. The mean diameters with the same letter are not significantly different according to LSD test ($p < 0.05$). SA: salicylic acid; SF14: *Bacillus amyloliquefaciens*; ACBC1: *Alcaligenes faecalis*; DS: disease severity; and MT: methyl-thiophanate.

3.3. Effect of Treatments on Fruit Quality Parameters

3.3.1. Weight Loss

The results show that there were significant effects of the different treatments on weight loss (Table 3). Interestingly, all treatments had a loss lower than that of the control (0.16) and were significantly different from the control accordingly, except for the treatments 5 SA + ACBC1, 5 SA + SF14, 3.5 SA + ACBC1, 2 SA + ACBC1, and 0.5 SA.

3.3.2. Total Soluble Solids

TSSs were determined for all treatments (Table 3). The obtained results underline a significant difference between treatments. The treatment with a high concentration of SA and its combination with ACBC1 or SF14 at 0.5% and 3.5% displayed almost similar TSSs as the control. However, the antagonist SF14 alone was the only treatment to exhibit TSSs lower than those of the control.

3.3.3. Titratable Acidity

The acidity of nectarine fruits, as affected by different treatments, is listed in Table 3. At 10 days after incubation, the combined treatments with SA resulted in higher TA in fruits compared with the other treatments. They also showed higher TA values compared with the antagonistic bacteria when applied alone.

3.3.4. Maturity Index

In general, the results underline slight changes of MI between treatments. The maturity index ranged from 0.77 to 1.62 for both of the treatments 0.5 SA and 3.5 SA (Table 3).

Table 3. Effect of SA, antagonists, and their combined treatments on the quality parameters of nectarine fruits at 10 days after incubation at 25 °C.

Treatments	Weight Loss (WL)	Total Soluble Solids (TSSs)	Titratable Acidity (TA)	Maturity Index (MI)
Untreated control	0.16 ± 0.01 ^{xc}	10.6 ± 0.17 ^{cde}	10.18± 0.00 ^{fg}	1.04
0.5 SA	0.15 ± 0.00 ^{d}	8.06 ± 0.07 ^{a}	10.49 ± 0.51 ^{g}	0.77
2 SA	0.14 ± 0.00 ^{c}	10.2 ± 0.39 ^{c}	11 ± 0.06 ^{h}	0.93
3.5 SA	0.14 ± 0.00 ^{c}	13 ± 0.00 ^{g}	8 ± 0.04 ^{a}	1.62
5 SA	0.13 ± 0.00 ^{b}	10.5 ± 0.69 ^{cd}	8 ± 0.18 ^{a}	1.31
SF14	0.14 ± 0.00 ^{c}	9.1 ± 0.11 ^{b}	9.62 ± 0.87 ^{de}	0.95
0.5 SA + SF14	0.14 ± 0.00 ^{c}	11.7 ± 0.46 ^{f}	10.11 ± 0.07 ^{f}	1.16
2 SA + SF14	0.14 ± 0.00 ^{c}	12.9 ± 0.51 ^{g}	9.47 ± 0.15 ^{cd}	1.36
3.5 SA + SF14	0.12 ± 0.00 ^{a}	9.9 ± 1.39 ^{bc}	8 ± 0.11 ^{a}	1.24
5 SA + SF14	0.16 ± 0.00 ^{e}	10.7 ± 0.73 ^{cde}	9.49 ± 0.19 ^{cd}	1.13
ACBC1	0.14 ± 0.01 ^{c}	10.3 ± 0.65 ^{c}	10.11 ± 0.13 ^{f}	1.02
0.5 SA + ACBC1	0.14 ± 0.01 ^{c}	11.6 ± 0.00 ^{ef}	9.01 ± 0.04 ^{b}	1.29
2 SA + ACBC1	0.16 ± 0.01 ^{e}	12.1 ± 0.20 ^{fg}	9.18 ± 0.07 ^{bc}	1.32
3.5 SA + ACBC1	0.16 ± 0.00 ^{e}	10.4 ± 0.56 ^{c}	9.89 ± 0.06 ^{ef}	1.05
5 SA + ACBC1	0.16 ± 0.00 ^{e}	11.4 ± 0.58 ^{def}	9.40 ± 0.14 ^{bcd}	1.21
MT	0.14 ± 0.00 ^{c}	13 ± 0.73 ^{g}	9.53 ± 0.10 ^{d}	1.36

^{x} Data are the mean of two replicates of three nectarine fruits over time. SA: salicylic acid; SF14: *Bacillus amyloliquefaciens*; ACBC1: *Alcaligenes faecalis*; and MT: methyl-thiophanate. The mean diameters with the same letter are not significantly different according to the least significant difference (LSD) test ($p < 0.05$).

3.4. Population Dynamics of Antagonists on Fruit Wounds

The populations of both antagonists *B. amyloliquefaciens* (SF14) and *A. faecalis* (ACBC1) were determined after incubation at 25 °C for 5 days (Figure 3). In general, the population densities of both antagonistic bacteria increased with increasing incubation time. Furthermore, it was seen that the population of *B. amyloliquefaciens* (SF14) remained more or less stable between 48 and 72 h incubation time with concentrations (log) of 7.48 and 8.67 CFU/wound, respectively. In contrast, the antagonist ACBC1 growth had a linear evolution pattern of its population in fruit wounds.

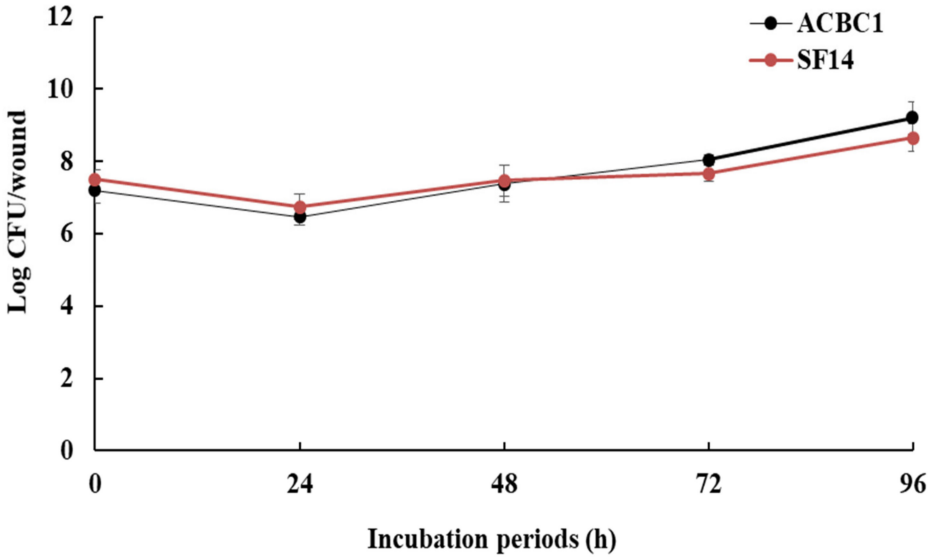

Figure 3. Population densities of *Bacillus amyloliquefaciens* (SF14) and *Alcaligenes faecalis* (ACBC1) as functions of time in-wounded nectarine fruits incubated at 25 °C. Data are the mean of five replicates.

4. Discussion

SA is a natural phenolic compound considered a plant hormone growth regulator that may contribute to improving disease resistance in some emerging plants [25–27,42]. In this study, the use of SA alone at different concentrations was proven effective in controlling brown rot disease. Complete control was achieved at a concentration of 5%. It was reported that the exogenous application of SA to highly susceptible plants could induce resistance to several pathogens [33,34]. These SA-mediated defenses are likely involved in the upregulation of several defense genes, particularly pathogenesis-related (PR) proteins, such as chitinase, glucanase, and peroxidase [26]. Srivastava and Dwivedi [42] underscored that fruit softening, sugar content, respiration rate, and the major cell wall degrading enzymes cellulase, polygalacturonase, and xylanase in banana fruit decreased with SA treatment [42]. In our study, the effect of SA on mycelial growth was weaker when compared with its efficiency on the disease on the fruit. These results are in agreement with the findings of Rasmussen et al. [43], who pointed out that SA had little direct activity on the in vitro mycelial growth of pathogens; thus SA probably acts by directly activating host defense mechanisms [26]. Yao and Tian [44] found that preharvest treatments with SA concentration significantly reduced the in vitro mycelial growth of *M. fructicola* and significantly inhibited the lesion diameter of brown rot on sweet cherries caused by the pathogen compared with untreated control. In our study, the highest in vitro mycelial growth was seen at both highest SA concentrations of 3.5% and 5%, which were positively correlated with those obtained in vivo. These results emphasize the possibility of using SA alone to induce fruit resistance to diseases. Ahima et al. [45] showed a significant reduction in the green mold incidence on oranges treated with salicylic acid. However, our results showed an increase of disease severity when SA was used at higher concentrations with antagonistic bacteria, compared with SA alone. This result indicates that at higher levels, SA might not be able to stimulate the growth of antagonists or to trigger the defense reaction of fruits to disease, which might also facilitate protection. Conversely, at a lower concentration of SA, both antagonists showed a moderate activity in reducing the disease severity. This may be due to the various susceptibilities of antagonists and nectarine fruits to SA. Furthermore, the use of SA with bacteria probably resulted in its antagonistic effect on jasmonic acid (JA), as previously shown for *Alternaria brassicicola* on Arabidopsis thaliana by Spoel et al. [46] and Bürger and Chory [27]. Therefore, further studies are needed to elucidate the biochemical responses of antagonistic bacteria and nectarine fruits to varying levels of SA and other phytohormones, including JA. Further, Kouzai et al. [47] reported that the effectiveness of phytohormone application on induced resistance is dependent on the pathogen's infection strategy.

This study investigated the feasibility of using microbial antagonists (ACBC1 or SF14) in combination with SA for the control of the brown rot disease on nectarine fruits. Results clearly indicate that the antagonists ACBC1 and SF14 were able to significantly reduce the mycelial growth of *M. fructigena*. Furthermore, the observed reduction of the brown rot disease by the bacterial antagonists significantly improved with SA. The highest inhibition rates (>90%) were obtained by combining ACBC1 and SF14 with SA at concentrations of 0.5% and 3.5%. The greatest disease severity reduction was achieved by the use of antagonists with SA at 0.5% and 2%.

Treatments combining antagonistic bacteria and SA induced several modifications of the mycelial structure. Lahlali et al. [8] found that both bacterial isolates provoked deformation of hyphae and vacuolation mainly due to the ability of the bacteria to secrete metabolites. It was noticed that the *B. amyloliquefaciens* LY-1 treatment delayed the progression of pericarp browning, reduced fruit rot and weight, and maintained the superior quality of harvested lychees during storage, suggesting that this bacterial antagonist is a promising effective biological control method for extending the shelf life of harvested lychee fruits [48]. *B. subtilis* CPA-8 has demonstrated its ability to control *Monilinia* spp. in peaches. Growth inhibition tests using supernatants of the bacterium showed a strong antifungal activity against *Monilinia laxa* and *M. fructicola* [49]. Dihazi et al. [50] pointed

out that *B. amyloliquefaciens* can control *Fusarium oxysporum* f. sp. *albedinis* by degrading fungal cell walls. They provided evidence that treatment with SA induces structural and biochemical changes in date palm roots in response to fungal infection. In accordance with our data, Ahima et al. [45] found that SA increased the antagonistic potency of the antagonist yeast *Rhodotorula mucilaginous* in comparison with other higher concentrations. In another study, the use of *Cryptococcus laurentii* (ST4-E14) and *Metschnikowia pulcherrima* (FMB-24H-2) with sodium bicarbonate (SB) reduced blue mold and bitter rot on apple fruit caused, respectively, by *Penicillium expansum* and *Colletotrichum acutatum* [51]. A complete disease control of bitter rot was achieved by a combination of these two BCAs. In addition, applying SB either alone or in combination with the antagonists reduced the rot incidence caused by *P. expansum*. Obagwu and Korsten [52] evaluated the effect of three *Bacillus* isolates, F1, L2, and L2-5, used in combination with SB for the control of green and blue molds in citrus. A significant increase in the biocontrol activity of all the isolates was observed when the bacterial isolates were combined with 1% SB. The combination of antagonists with additives and inorganic salts could be an effective method to improve their effectiveness. Several studies have underscored the improvement of biocontrol when antagonists are used in combination with salts, such as bicarbonates, and natural compounds, such as chitosan and SA [15,32]. Results highlight that both SA concentrations of 0.5% and 2% significantly improved the biocontrol of the efficacy of both antagonists with a disease severity of less than 20%. Several studies have demonstrated the ability of SA to trigger systemic resistance in plants and protect against pathogen attacks [29,53].

The successful use of bacteria as BCAs for fungal postharvest diseases might be attributed to their high inhibiting capacity, fast colonization in fruit lesions, and simple nutritional requirements [15]. The study of the population dynamics of antagonistic bacteria in nectarine wounds reveals an increase of their population density with a slight difference between the two antagonists, which might explain the strong inhibitory effect of ACBC1 compared with SF14. Such rapid growth in wounds indicates that these two antagonists are well adapted to the wound's environment, and they might be considered as potential BCAs. Several studies have shown that the colonization of host plants represents the mode of action of most BCAs [54–57]. Janisiewicz and Korsten [20] reported that the antagonistic activity of *B. subtilis* was mainly based on colonization and competition for nutrients. Other studies have also shown that the ability of the antagonist to colonize host plants is an important factor in disease control [16,54,58,59], while unsuccessful colonization of host plants leads to variable biocontrol efficacy [60]. For this reason, the BCA must be applied before the arrival of the pathogen so that it can colonize first the fruit surface, multiply, and compete for nutrients. Kong et al. [61] evidenced that the marine bacterium *Bacillus megaterium* has great potential for the control of postharvest decay of peanut kernels caused by *Aspergillus flavus*.

The in vivo results on the impact of different treatments on the fruit quality parameters show that all treatments have a significant effect on the weight loss of nectarine fruits when compared with the control. The lowest weight loss was, therefore, recorded in treatments with SA alone applied at 2%, 3.5%, and 5% or in combination with bacterial antagonists at both concentrations of 0.5% and 2%. We might suggest that SA has preservative properties. Further, as a potential inhibitor of the biosynthesis and the action of ethylene, SA might slow down the ripening and aging processes, thus preserving the quality of the fruit. Furthermore, treatment with higher concentrations of SA and its combination with antagonists at 0.5% and 3.5% reveals similar TSSs as the control. In treatments, the titratable acidity was reduced by 1.11 and 2.12 in comparison with the control. These results indicate that applying ACBC1 or SF14 in combination with SA did not alter the quality parameters of nectarine fruits, including titratable acidity, TSSs, and MI, in addition to reducing the severity of brown rot on nectarines. Further, interestingly, the combinations delayed the postharvest maturation of nectarine fruits and subsequently reduced weight loss. These results are in line with the findings of Qin et al. [32], who reported that applying the integration of chemicals with SA significantly reduced weight loss while maintaining the

fruit appearance, total soluble solids content, and titratable acidity of grapes during the storage period [32]. These results corroborate our findings, highlighting the efficiency of the integration of antagonist microbe with SA in controlling postharvest diseases of fruits [22,23,38]. Obviously, the reported ability of *B. amyloliquefaciens* SF14 and *A. faecalis* ACBC1 to effectively inhibit the development of the brown disease on nectarine fruits when these bacteria were used jointly with the SA may be explained by the fact that as a natural and safe signal molecule, SA improves and hastens the spread of the systemic immunizing effect of SF14 and ACBC1 in fruit tissues. Further, potentially, SA might trigger supplementary protective mechanisms responsible for preventing the brown disease and prolonging the life of products while maintaining freshness. Salicylic acid (SA) and several components of the SA pathway, including the methylated derivative of SA, are known to be among the signals contributing to systemic acquired resistance (SAR) [62]. Recently, the role of SA in SAR and its relationship with various SAR signals have been well reviewed [29,62].

5. Conclusions

The consumption of fresh fruits and vegetables has significantly improved as a health concern of consumers. To attenuate postharvest losses and to preserve the fresh like quality of horticultural crop during postharvest storage, several management technologies have been applied. This study highlights the potential use of the antagonistic bacteria *A. faecalis* ACBC1 and *B. amyloliquefaciens* SF14 in combination with SA to manage the brown rot on nectarine fruits. The in vitro results show that the combination of the antagonists with SA at 0.5% and 3.5% gave the best inhibition rate of mycelial growth at 10 days after incubation periods in comparison with the antagonists alone or SA. Conversely, the in vivo results underline that the antagonists used in combination with SA at 0.5% and 2% gave the lowest disease severity. Furthermore, treatments based on ACBC1 alone displayed higher efficiency than those based on SF14 alone, likely due to the rapid growth of ACBC1 in fruit wounds, as demonstrated in this study. Interestingly, the combination of the antagonists with SA at 0.5% and 2% did not alter the fruit quality parameters, suggesting that these treatments present a promising and reliable alternative to preventively control the brown rot disease after harvest. Moreover, this work provides insights into the feasibility of using SA in combination with antagonistic bacteria to enhance the biocontrol potential against the postharvest diseases of fruits.

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