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Research Article

Biocontrol and Biostimulant Effect of a Fungal-Derived Extract in Commercial Varieties of Strawberry (*Fragaria x ananassa* Duch.)

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Abstract

Objective: Strawberry postharvest fruits and plants are mainly affected by the fungus *Botrytis cinerea* (*B. cinerea*), which causes grey mould rot. Biological control agents and biostimulants can effectively control this fungus and promote plant growth. This study aimed to evaluate the capacity of a novel fungal-derived extract called culture filtrate (CF) to biocontrol grey mould rot in postharvest strawberries and plants while promoting plant growth.

Methods: The CF extract was obtained from the liquid culture of a strawberry pathogenic fungus, gauze-filtered and inactivated by autoclave. The *Fragaria x ananassa* cultivars Camino Real, Benicia, and Merced were used. For the postharvest assay, fruit natural decay was evaluated by simulating real storage conditions. For the phytopathological assays, first-generation plants inoculated with *B. cinerea* were evaluated 26 days post infection. The effect of CF on plant growth and biomass promotion was evaluated 60 days post treatment (DPT).

Results: The treatment with CF yielded healthier fruits of the three varieties, which presented minor severity of grey mould symptoms compared to the controls treated with water or potato dextrose broth (PDB) by 7 DPT. For instance, 40% of Camino Real, 80% of Benicia, and 20% of Merced fruits presented an incipient lesion (severity 1), whereas no fruits treated with water or PDB showed this minor level of infection. On the contrary, around 50% and 70% of the control fruits presented the highest level of severity. Moreover, CF pre-treatment of Benicia "mother plants" protected first-generation untreated plants. Likewise, the best biostimulant effect was observed for Benicia plants, which presented an increased number of leaves and runner production 60 DPT.

Conclusion: The fungal-derived extract CF constitutes a promising alternative to be used as a biological control agent of *B. cinerea* in strawberry. CF efficiently controlled the causal agent of grey mould in harvested strawberries and first-generation untreated plants. Yet, showed a minor effect as a plant growth promoter. Thus, the current study opens up the possibility to develop "phytovaccines" using extracts of almost any fungus, including pathogenic ones, as long as they are previously inactivated.

Keywords: biological control agents, *Botrytis cinerea*, grey mould, strawberry, postharvest, shelf-life extension, phytovaccines

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1 INTRODUCTION

Strawberries (*Fragaria* x *ananassa* Duch.) are high-value fruits but their marketing is limited due that their perishability determining a short postharvest life[1]. Tucumán is the second most important strawberry-productive region in Argentina, whilst Argentina is the third producer in South America[2]. Tucumán produces strawberries throughout the whole year. The bulk is produced during the spring, which runs from September to December, and yields the largest volumes with more accessible prices for mass consumption[3]. Because this period is characterized by fluctuating temperatures and unpredictable rainfall, postharvest fresh fruits are exposed to deterioration by postharvest fungi, especially *Botrytis cinerea.*

The fungus *B. cinerea* is regarded as the most common and important pathogen during the pre- and post-harvest of strawberries[4-6]. It is the causal agent of grey mould and affects fruit in the field, storage, transport and market, leading to significant economic losses[7,8]. Chemical fungicides are the mainstay for the control of grey mould disease in strawberries. However, most of them are associated with toxic effects on human and animal health, and take an environmental toll[9]. Similarly, the application of chemical fertilizers also contaminates the land ecosystems and the water bodies since only 20% is taken by plants and the remaining 80% runs off[10]. The increasing social awareness for preserving the environment and the interest in consuming healthier foods appeal to engage in the search for sustainable phytosanitary strategies. In this vein, biocontrol and biostimulation based on the use of biological control agents (BCAs) and biostimulant products constitute an attractive, viable and promising alternative to reduce, or even replace the use of chemical pesticides, fungicides and fertilizers in agriculture[11-13].

If appropriate postharvest practices are followed, losses can be minimized and fresh fruit adequately preserved for longer periods. In this vein, an efficient strategy to slow down ripening, respiration, senescence, water loss, and decay due to infection of quiescent *B. cinerea* fungi, is the precooling of freshly harvested fruits combined with a chemical or biological postharvest treatment, which helps for quality maintenance and prolonging shelf life[1,6,14,15].

Novel postharvest treatments of strawberries have been suggested to prevent *B. cinerea* infection during storage[6,16]. Examples of different kinds of BCAs that efficiently control *B. cinerea* in postharvest strawberries include the application of living microbes, such as bacteria of the *Bacillus* genera[7,8,17], the yeast *Aureobasidium pullulans*[18,19], the marine yeast *Sporidiobolus pararoseus*[20], the filamentous ascomycete *Ulocladium atrum*[21], the fungus *Beauveria bassiana*[18], the commercial product BOTRY-Zen, with *Ulocladium oudemansii* U3 strain as the active ingredient[22], and many others. Other techniques use different biological compounds to produce edible coatings that protect against *B. cinerea* and prevent water loss, such as silk fibroin[23], methylcellulose[24], chitosan[7,25,26], modified chitosan[27], and chitosan enriched with turmeric and green tea extracts[28]. Plant defence elicitors like the protein-like-subtilisin AsES were also reported to protect postharvest strawberries[29], as well as brassinosteroids[30] and volatile organic compounds derived from *Candida intermedia*[31] or from strawberry leaves[32]. Plant-based BCAs include the leaf ethanolic extract of *Prosopis juliflora*[33], essential oils[34], and volatile essential oils of *Origanum onites* and *Ziziphora clinopodioides*[35].

All the BCAs mentioned above protect strawberries from *B. cinerea* through a variety of mechanisms[1,36,37]. In this study, we propose a novel concept of BCA that would induce the effect in plants that vaccines have in humans as if it was a "phytovaccine". This BCA consists of a heat-inactivated culture of a strawberry fungal pathogen, namely culture filtrate (CF)[38]. Thus, this study aimed to evaluate the capacity of CF to biocontrol grey mould rot in postharvest strawberries and plants while promoting plant growth.

2 MATERIALS AND METHODS

2.1 CF Extract Obtention

The fungal-derived extract CF was prepared from the culture of the isolate M11 of *Colletotrichum acutatum*, according to the method described by Hael Conrad et al.[38], 2020. The fungus was gently provided by the fungi collection from the plant biotechnology laboratory at Universidad Nacional de Tucumán, Argentina. Briefly, a liquid static culture of M11 was grown in potato dextrose broth (PDB) at 28°C under continuous fluorescent light (300μmol/m^2 s). After ten days, it was gauze-filtrated and the liquid fermented culture constituted the extract CF, which was autoclaved (121°C, 1atm, 20min) to inactivate conidia and cell debris[38]. CF was diluted 1:10 in distilled water (CF 0.1x) previous to its use.

2.2 Postharvest Assay

2.2.1 Strawberries Treatment and Storage

In this study, healthy strawberries of the varieties 'Camino Real', 'Benicia' and 'Merced' (*Fragaria* x *ananassa* Duch.) were used. Freshly harvested fruits with uniform sizes and maturities, and with no mechanical injuries were selected and 5 fruits were randomly assigned to either the treatment or the control group. To simulate the real postharvest and storage conditions in which strawberries are commercialized in the local market, fruits were treated immediately after harvest, rapidly cooled down at 4°C and kept in plastic trays to keep high relative humidity (RH) (close to 100%). After 48h, closed trays were stored at 25±2°C and 85%±5% RH, under continuous white light (150μmol photons/m^2·s). The treatments consisted in spraying 800μL of one of the following: CF 0.1x; the commercial fungicide Switch® 0.8mg/mL (cyprodinil 37.5%w/w and fludioxonil 25%w/w, Syngenta, USA); and the controls distilled water or PDB (1:10 diluted in distilled water).

2.2.2 Grey Mould Incidence and Severity

Grey mould disease progress was monitored over time at 3, 6, 7 and 9 days post treatment (DPT). The incidence of the infection and severity of symptoms were recorded at 6 DPT. The incidence was recorded as the number of decayed fruits, whereas the severity was assessed according to an empirical scale defined by Romanazzi et al. adapted in this study for strawberry fruits: 0=healthy berry; 1=one brownish lesion, 2 to 3mm in diameter (beginning of infection); 2=one brownish water-soaked lesion, 10mm in diameter; 3=several brownish water-soaked lesions or 25% of the berry infected; 4=26 to 50% of the berry surface infected, sporulation starting to visualize; 5=>50% of the berry surface infected, sporulation present[25,39]. Three independent assays were performed for two consecutive years.

2.3 Biostimulation of Plant Growth

2.3.1 Plant Material

The strawberry varieties evaluated ('Camino Real', 'Benicia' and 'Merced') are short-day cultivars from the genetic improvement program at the University of California. Their main features are summarized in Table 1. Benicia is an early variety that provides scoop fruit, whilst Camino Real and Merced are late varieties. The plants were obtained from a nursery in El Maitén, Chubut, Argentina (GPS coordinates: 42° 3' 1.26" S latitude and 71° 10' 4.512" W longitude), as standard fresh plants (without leaves and with bare roots); they were washed with tap water to reduce the amount of primary inoculum that can potentially be attached and sowed in the field. For the biostimulation assays, plants were agamically propagated from the in-field plants. For the phytopathological assays, plants were agamically propagated from CF- and water - treated plants. In both cases, runners were fixed into pots with a mixture of sterile substrate humus:perlome (2:1), and after one month attached to the "mother plant", such plantlets were cut out, transferred into blown plastic pots of 13.5cm diameter and 11.2cm height, and kept in the greenhouse for one month until assays were carried out. Plants were regularly watered and did not receive any fertilization or pesticide treatments.

2.3.2 CF Pre-treatment

All the strawberry plants were pruned to keep the 3 newest fully expanded leaves 10 days before the assay. These plants were entirely sprayed with enough amount of CF 0.1x, water or Switch® (0.8mg/mL) until run-off, kept in the greenhouse, and watered three times a week. The assay was randomized with 6 plants per treatment, which were used for further evaluations of non-destructive (growth promotion) and destructive (biomass promotion) parameters.

2.3.3 Growth Promotion Parameters

The number of total leaves, leaf area, leaf greenness, and the total number of runners (counting for propagated plants) was evaluated 60 DPT. The total number of leaves and runners and the leaf greenness were determined *in planta* (non-destructive measures). The leaf greenness analyses were determined by measuring the relative chlorophyll content using a chlorophyll meter (Minolta SPAD-502, Spectrum Technologies, USA). The results were expressed as SPAD values, and measures were obtained from the central leaflet of 10 randomly selected leaves from each of the 6 replicates per treatment (*n*=60). The main leaf area was determined using the software Image J version 1.44 (NIH)[43].

2.3.4 Biomass Promotion Parameters

The root length, crown diameter, and dry weight (DW) were evaluated 60 DPT. After carefully washing over a sieve to eliminate the rest of the soil, plants were placed on the bench and root length was determined using a millimetre ruler. The crown diameter was evaluated with a calibre. To obtain the DW, both the aerial part (leaves and stems) and the roots (containing the crown) were placed in paper bags and oven-dried at 65°C±5°C for 1 week. The DW was determined with a digital scale with an accuracy of 1g (CS5000, OHAUS), and the total DW was calculated as the sum of the aerial part and roots. 6 plants per treatment were used.

Table 1. Main Features

Variety	Origin	Market	Plant	Fruit	Yield (g/plant)*	Disease Resistance**	Abiotic Tolerance	Obser-vations	Ref.
Camino Real	University of California, USA	Outstanding fruit for both the fresh market and for processing.	Small, very compact and erect plant, which allows high planting density per hectare and facilitates harvesting.	Big, firm, and typically rounded or symmetrical-conic fruit of very good quality and flavour, in a good yield.	1.813	Moderately susceptible to common leaf spots and somewhat sensitive to powdery mildew. Quite resistant to Verticillium wilt and Phytophthora crown rot, and relatively resistant to Anthracnose crown rot. When treated properly, it has tolerance to two-spotted spider mites. It is tolerant to strawberry viruses encountered in California.	Tolerant to rains and adverse weather conditions.	Low fraction of non-marketable fruit. No pollination problems (very low percentage of deformation).	[40]
Benicia	University of California, USA	Good aptitude for agroindustry (frozen fruit) and fresh market	Medium to high vigour plant, which allows high planting density per hectare.	Great firmness, dark red external colour and intense red in the pulp; typically medium to long conic fruit, which can be flattened or slightly obovate; outstanding flavour.	2.566	Moderately resistant to powdery mildew, but is moderately susceptible to Anthracnose crown rot, and very susceptible to Verticillium wilt. It is moderately susceptible to Phytophthora crown rot and common leaf spot. When treated properly, it has tolerance to two-spotted spider mites. It is tolerant to strawberry viruses encountered in California.	Tolerant to rain.	When treated with appropriate planting regimes, 'Benicia' has larger fruit and produces individual-plant yields greater than that of 'Camarosa' (U.S. Plant Pat. No. 8,708).	[41]
Merced	University of California, USA	Exceptional fruit for both fresh market and processing.	Vigorous plant and more compact than Benicia.	Big, firm, and typically medium to long conic fruit, which is rarely flattened or slightly obovate; external and internal colour lighter than that of 'Benicia'.	2.339	Moderately resistant to powdery mildew, but is moderately susceptible to Anthracnose crown rot, and susceptible to Verticillium wilt. It is resistant to Phytophthora crown rot and common leaf spot. When treated properly, it has tolerance to two-spotted spider mites. It is tolerant to strawberry viruses encountered in California.	N.s.	When treated with appropriate planting regimes, 'Merced' is similar to 'Camarosa' (U.S. Plant Pat. No. 8,708), but with greater productivity, higher quality fruit, less vigorous plant, and lighter coloured fruit.	[42]

Notes: N.s.: Not specified. *The informed yields derive from independent assays, as stated in each of the patents (cited references). **The patents do not specify any information about resistance to *Botrytis cinerea,* the causal agent of grey mould, the disease evaluated in the current study. The causal agent of the common leaf spot is *Ramularia tulasnei,* powdery mildew is *Sphaerotheca macularis,* Verticillium wilt is *Verticillium dahlia,* Phytophthora crown rot is *Phytophthora cactorum,* and Anthracnose crown rot is *Colletotrichum acutatum.* The two-spotted spider mites refer to the species *Tetranychus urticae.*

2.4 Priming the Plant Defences

2.4.1 Plant Material and Treatments

The first generation of propagated plants from CF- and water-treated plants were used for the phytopathological assay. These plants were pruned to keep the 3 newest fully expanded leaves 10 days before the assay. The assay was randomized with 6 plants per treatment.

2.4.2 *B. cinerea* Infection

The strain BMM of *B. cinerea* was kindly provided by Brigitte Mauch-Mani (University of Neuchâtel, Switzerland). It was grown on PDA at 22°C under a 16h light cycle (200μmol/m^2·s) for 2 weeks. The preparation of conidia suspension and the drop infection procedure was performed according to the protocol described by

Hael-Conrad et al[29]. Briefly, 6μL droplets of a *B. cinerea* suspension (5×10^4 conidia/mL) were applied to the centre of the adaxial side of each leaflet (*n*=9/plant). Plants were immediately transferred into closed and hermetic infection chambers at 20°C, 100% RH and darkness for 48h, to promote the infection. After this time, plants were transferred into disease evaluation chambers at 20°C, 70% RH and 16h light cycle (350μmol/m^2·s). Grey mould disease symptoms were evaluated at 26 days post infection (DPI) by measuring the lesion area (cm^2) with the software ImageJ version 1.44 (NIH)[43]. The experimental design was a completely randomized block with 6 plants per treatment.

2.5 Statistical Analysis

The statistical analysis of data was carried out using the software InfoStat version 2013[44]. Differences between means were evaluated by Student's T-Test ($P\leq0.05$) as stated in the figure legend.

3 RESULTS

3.1 Biocontrol Effect on Postharvest Strawberries

In general, the fungal-derived extract CF induced a protective effect on post-harvested strawberry fruits of the varieties Camino Real, Benicia and Merced until 7 DPT, which was similar to the effect exerted by the commercial fungicide Switch®. Moreover, such fruits maintained a notoriously better appearance along the timeline analyzed compared to the control fruits treated with water or PDB, which presented earlier brownish water-soaking lesions by 6 DPT, most of them started developing fungal mycelia by 7 DPT and were completely covered with grey mould by 9 DPT (Figures 1A, 2A and 3A).

After quantification of the incidence of grey mould infection 7 DPT, we observed that for Camino Real, 50% of water-treated fruits presented level 5 of the severity of symptoms, which means that more than half of the berry surface was infected with some grade of sporulation present, whereas 40% of PDB-treated fruits presented level 5 of infection. On the contrary, Switch®-treated fruits did not present this level of infection and only 10% of CF-treated fruits did (Figure 1B). The percentage of water- or PDB-treated fruits with level 4 was 20% and 30%, respectively, i.e., 26% to 50% of berries' surface was infected with incipient sporulation. Whereas no fruits treated with Switch® presented this severity of infection, and only 20% of CF-treated fruits did. In addition, 30% of water- and PDB-treated fruits presented a level of disease severity between 2 and 3, which corresponded to fruits that presented one or several brownish water-soaked lesions, but neither of such treated fruits was healthy (level 0) or presented an incipient brownish lesion (level 1). Whereas 30% of Switch®-and CF-treated fruits presented severity 2 or 3, and 70% or 40% of Switch®- and CF-treated fruits entered the group of healthy or incipient infected fruits (severity 1), respectively (Figure 1B).

Similar outcomes were achieved with fruits of the variety Benicia evaluated 7 DPT (Figure 2B). For instance, 40% of water- and PDB-treated fruits presented advanced infection of severity level 5, whereas Switch®- and CF-treated fruits did not present such a level of infection. The percentage of water- or PDB-treated fruits with level 4 was 20% and 30%, respectively; no Switch®-treated fruits presented this level of infection and only 10% of CF-treated fruits did. In addition, 20% of water- and PDB-treated fruits presented a

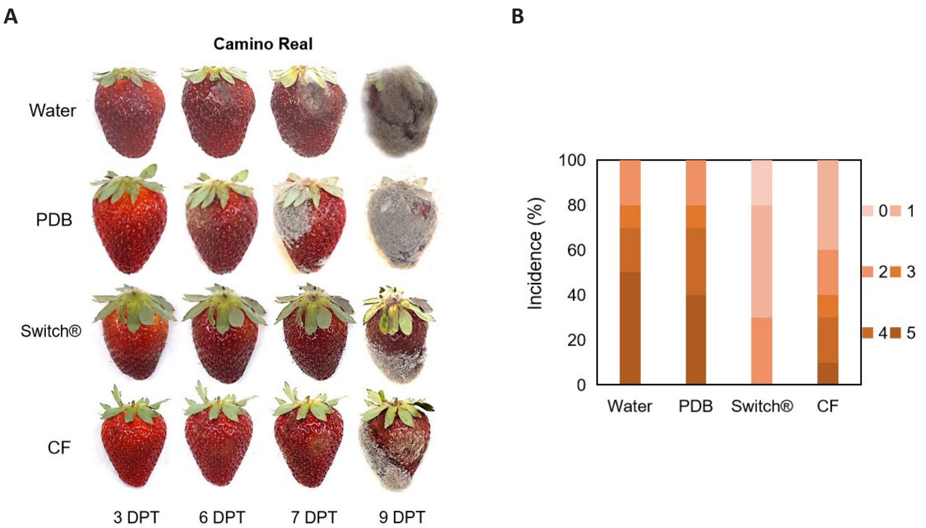

Figure 1. Biocontrol effect of the fungal-derived extract CF on postharvest strawberries of Camino Real. Fruits were sprayed with the fungal-derived extract CF, the fungicide switch®, or water and diluted PDB as the controls. A: The infection of spontaneous occurring grey mould was monitored for 9 days post treatment (DPT); B: Incidence of the disease and severity of symptoms were quantified 7 DPT. The incidence was calculated as the % of fruits infected in different degrees, and the severity was calculated on a scale from 0=healthy fruits to 5=>50% of the berry surface infected and sporulation present. One representative picture of each treatment is presented. Assays were repeated three times during each of the campaigns in 2016 and 2017, with similar results.

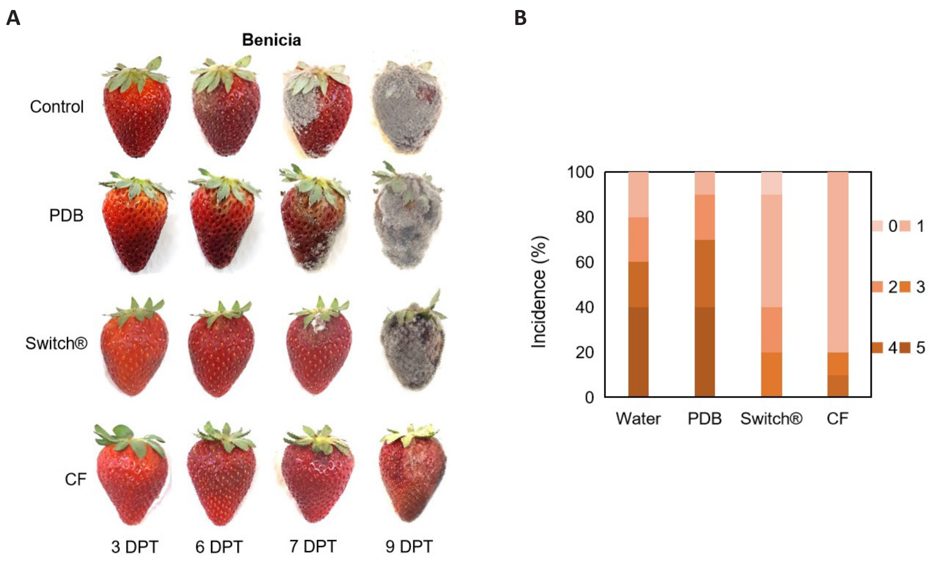

Figure 2. Biocontrol effect of the fungal-derived extract CF on postharvest strawberries of Benicia. Fruits were sprayed with the fungal-derived extract CF, the fungicide switch®, or water and diluted PDB as the controls. A: The infection of spontaneous occurring grey mould was monitored for 9 days post treatment (DPT); B: Incidence of the disease and severity of symptoms were quantified 7 DPT. The incidence was calculated as the % of fruits infected in different degrees, and the severity was calculated on a scale from 0=healthy fruits to 5=>50% of the berry surface infected and sporulation present. One representative picture of each treatment is presented. Assays were repeated three times during each of the campaigns in 2016 and 2017, with similar results.

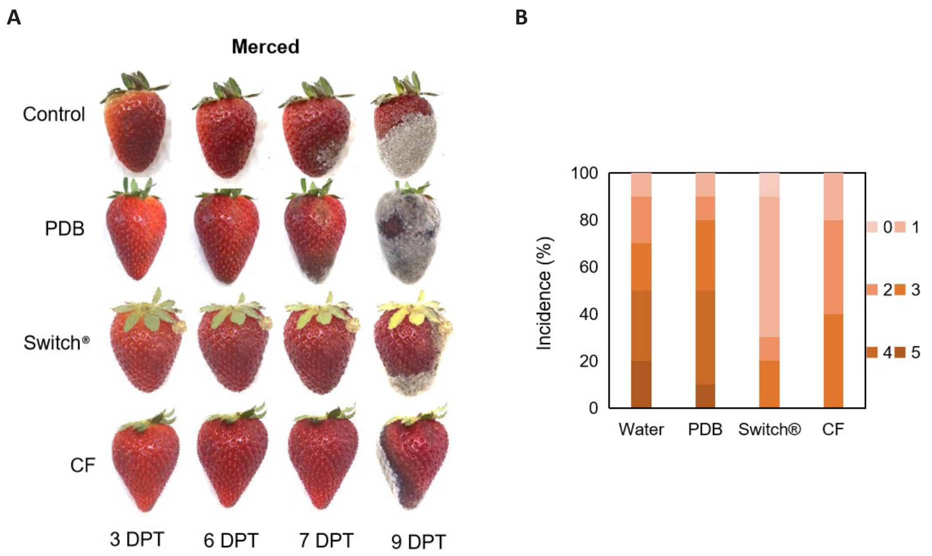

Figure 3. Biocontrol effect of the fungal-derived extract CF on postharvest strawberries of Merced. Fruits were sprayed with the fungal-derived extract CF, the fungicide switch®, or water and diluted PDB as the controls. A: The infection of spontaneous occurring grey mould was monitored for 9 days post treatment (DPT); B: Incidence of the disease and severity of symptoms were quantified 7 DPT. The incidence was calculated as the % of fruits infected in different degrees, and the severity was calculated on a scale from 0=healthy fruits to 5=>50% of the berry surface infected and sporulation present. One representative picture of each treatment is presented. Assays were repeated three times during each of the campaigns in 2016 and 2017, with similar results.

level of disease severity 2 (no fruits with severity 3), and the remaining fruits presented a level of severity between 1 and 2. Whereas 40% of Switch®-treated fruits presented a severity level between 2 and 3, 50% severity presented level 1 and 10% were healthy by 7 DPT. Surprisingly, 10% of CF-treated fruits presented a severity of 3, and the remaining ones (80%) presented an incipient lesion (level 1) (Figure 2B).

Merced was the variety that better performed since only 20% and 10% of water- and PDB-treated fruits presented advanced infection of level 5, and 30% and 40%, respectively presented level 4 (Figure 3B). Whereas

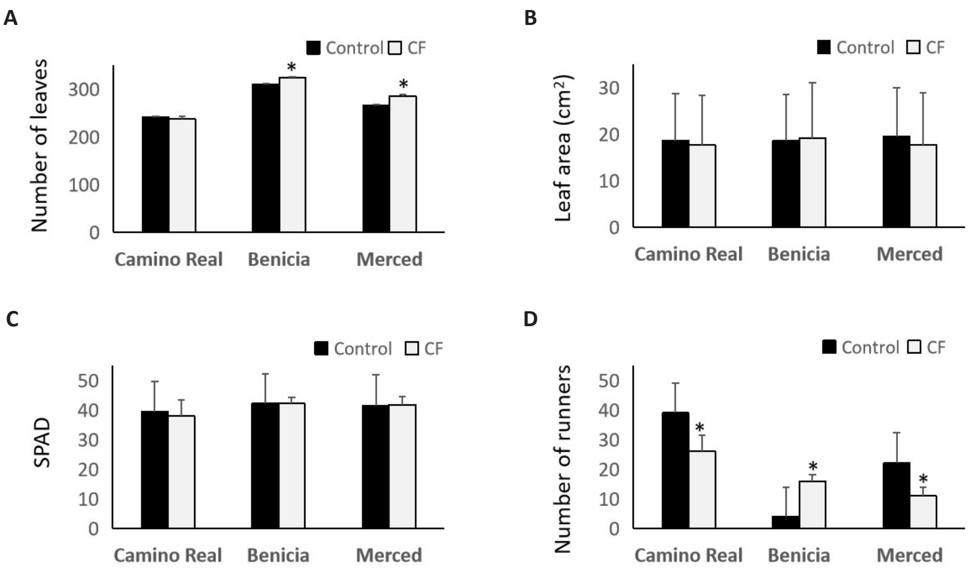

Figure 4. Biostimulant effect of the fungal-derived extract CF on strawberry growth. Plants of *Fragaria* x *ananassa* varieties Camino Real, Benicia and Merced were CF- or water-treated, and the following growth parameters were evaluated 60 days post treatment (DPT). A: Total number of leaves leaf area; B: Leaf area; C: SPAD (greenness index); D: The number of runners. Mean values ± SD were obtained from one independent assay with six replicates per treatment (n=6). Differences between the means of CF- and water-treated plants were evaluated by Student's T-Test ($P≤0.05$) and denoted with an asterisk.

Switch®- and CF-treated fruits did not present such levels of infection. In addition, 40% of water- and PDB-treated fruits, 30% of Switch®-treated fruits and 80% of CF-treated fruits presented a level of disease severity between 2 and 3. Whereas the healthier fruits with severity 1 were the minority among the water- and PDB-treated fruits (only 10%), represented the majority for Switch®-treated ones (60%) and only 20% for CF-treated fruits (Figure 3B). For the three varieties, healthy fruits (level 0) were only observed for the ones treated with the commercial fungicide Switch®.

3.2 Biostimulant Effect on Plant Growth

CF induced a slight increment in the number of leaves of Benicia and Merced cultivars compared to water-treated control plants 60 DPT (Figure 4A). In general, the fungal-derived extract did not function as a strong biostimulant since no significant differences regarding leaf area and greenness index (SPAD) were detected compared to the control (Figure 4B and C). A strong stimulating effect in cultivar Benicia was observed, which produced 75% more runners than the control (Figure 4D). However, for Camino Real and Merced, a negative effect was detected producing 33% and even 50% fewer runners than the control, respectively (Figure 4D).

CF further induced a slight increment in the root length of the three cultivars compared to the control, but such an increase was statistically significant only for Camino Real (Figure 5A). On the contrary, no effect was detected for the crown diameter (Figure 5B), aerial and root DW, and thus the total DW (Figure 5C).

3.3 Primed Protection in First-generation Plants

We tested whether the fungal-derived extract CF could induce the innate defences in plants that were not previously treated, which were obtained by agamic propagation of plants that were induced with CF. CF induced slight protection against *B. cinerea* in Camino Real and significant protection in Benicia (Figure 6), which presented smaller lesions compared to the water-treated control. On the contrary, control plants were completely infected and dead by 26 DPI. Among the three cultivars, Benicia acquired the highest protection (53% relative to the control).

4 DISCUSSION

4.1 CF Protective Effect: Biocontrol of Grey Mould Disease

B. cinerea causes grey mould rot that affects fruit in the field, storage, transport and market, and plants in the field, leading to significant economic losses. In the present study, we demonstrated that the fungal-derived extract was efficient to reduce and slow down the natural decay due to grey mould in three commercial varieties of strawberries, extending the shelf life to seven days. Interestingly, the effect of CF on strawberry fruits does not depend on the particular genotype. Likewise, the results are consistent with previous studies in which we demonstrated CF could induce local and systemic protection against *B. cinerea* in *Arabidopsis thaliana*[38] and *Fragaria* x *ananassa* cultivar "Pájaro"[45].

In the way to reduce the use of chemical fungicides to

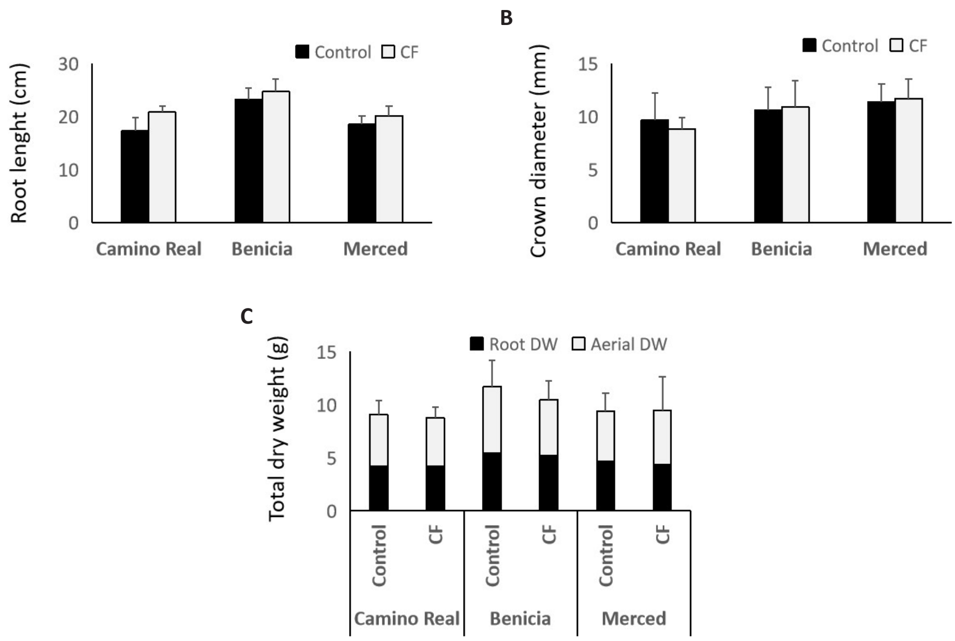

Figure 5. Biostimulant effect of the fungal-derived extract CF on the biomass. Plants of *Fragaria* x *ananassa* varieties Camino Real, Benicia and Merced were CF- or water-treated, and the following biomass parameters were evaluated 60 DPT. A: Root length (cm); B: Crown diameter (mm); C: Total DW (g), which is calculated as the sum of root DW and the aerial part DW. Mean values ± SD were obtained from one independent assay with 6 replicates per treatment (*n*=6). Differences between water- and CF-treated plants were analysed using Student's T-test (*P*<0.05).

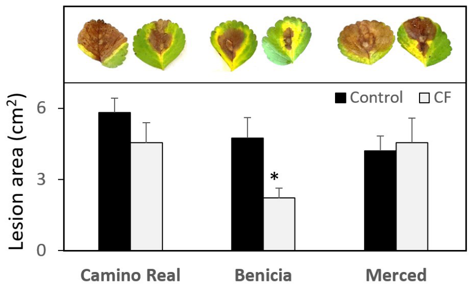

Figure 6. Protective effect of the fungal-derived extract CF in strawberry plants infected with *B. cinerea*. "Mother plants" of *Fragaria* x *ananassa* varieties Camino Real, Benicia and Merced were spray treated with CF or water (as the control) until run-off. The induced plants were maintained in the greenhouse until started producing runners, which were fixed on the soil to obtain "daughter plants" that were used for the phytopathological assays. Two-month-old plants were infected with *B. cinerea* and lesions size were measured 26 days post infection (DPI). Mean values ± SD were obtained from one independent assay with six replicates per treatment (*n*=6). A representative image of each treatment is presented. Asterisks indicate a statistically significant difference between CF- and water-treated plants, according to Student's T-test (*P*<0.05).

control grey mould disease in strawberries, we propose a novel postharvest treatment, which is friendly to the environment and human and animal health. To our best knowledge, it is the first time that a culture filtrate derived from an inactivated strawberry fungal pathogen is reported to efficiently control *B. cinerea* in postharvest strawberries. The extract was obtained from the culture of *Colletotrichum acutatum* M11, a pathogen causal of anthracnose in strawberries[30]. Unlike a pathogenic behaviour, CF behaved more as a "phytovaccine", comparable to some extent to what pathogen-inactivated vaccines are for humans. The protective effect was more notorious for Benicia and Merced cultivars because while CF-treated fruits were maintained healthy for 7 days, control fruits were completely rotted and covered with grey mycelium by that time. Interestingly, the biocontrol effect of CF was similar to the commercial fungicide Switch®, which is extensively used to control *B. cinerea*.

Many BCAs of different natures have been reported to control grey mould in postharvest strawberries during storage[3,4,15,17-34,43,44]. In general, these BCAs showed a significant reduction in the incidence and symptoms of grey mould in storage strawberries. Particular long-lasting protection was achieved using coatings of chitosan mixed with essential oils[34,35,47], which ranged between 9 days and 21 days post infection with *B. cinerea.* The protective effect in such studies was evaluated during refrigerated storage at low temperatures (4°C and 5°C), which is a strategy widely used as efficiently helps to reduce fungal infections and slow down the deterioration and the rotting process of fresh fruit[1]. However, these are not the real conditions in which fresh fruit is commercialized in the local market. Thus, in our assays, we simulated real postharvest cooling, storage and commercial conditions for the local market with satisfactory results. Under similar storage conditions than us, but through artificial *B. cinerea* inoculations, Wang et al.[48] extended by 2 DPI the shelf life of strawberries inoculated with the strain KLBC XJ of *Bacillus halotolerans,* whereas Yang et al.[28] extended it by 6 DPI when strawberries were coated with chitosan or by 14 DPI when coated with chitosan enriched with turmeric acid extracts. Likewise, Furio et al.[30] evaluated the natural decay of strawberries under identical storage conditions than we assayed and found between 20% and 30% of grey mould incidence in strawberries pre-treated with two kinds of brassinoesteroids, compared to the 70% of incidence registered in water-treated fruits by 5 DPT.

4.2 Biocontrol Mechanisms Triggered against *B. cinerea*

CF consists of a blend of fungal-derived molecular patterns, including fragments of the cell membrane, conidia and cell wall, oligosaccharides, carbohydrates, lipids and peptides, among many other compounds produced by the fungus during its active growth[38]. As it is inactivated by humid heat treatment (autoclave), any protective effect from thermo-labile compounds or functional proteins is discarded. Unlike the commercial fungicide Switch®, which inhibits conidia germination and thus shows an antifungal mode of action, CF does not affect the elongation of conidia germ tubes, the mycelium growth, or the hyphae morphology or membrane cells permeability when tested *in vitro*[38]. However, CF exerted some kind of damage on *B. cinerea* that was unable to infect detached strawberry leaves, despite it could normally grow *in vitro*[45]. Thus, this phenomenon could be happening in postharvest strawberry fruits pre-treated with CF, which may inhibit the virulence of quiescent *B. cinerea* fungi slowing down the appearance of grey mould symptoms and diminishing the incidence of the fungus. Likewise, it is also valid to hypothesize that CF may activate the innate defences of strawberry fruits, as it does in plants. For example, CF rapidly induces the upregulation of defence-related genes such as *PR1*, *ERF6* and *WRKY70* (between 48 and 96 HPT) and callose (120 HPT) in *F.* x *ananassa* cv. "Pájaro"[38]. In this vein, it was demonstrated that the volatile organic compound linalool protected strawberry fruits from grey mould rot by down-regulating *B. cinerea's* expression of key enzymes in the ergosterol biosynthesis pathway, which affected the membrane integrity, damaging mitochondrial membranes, and increasing the levels of reactive oxygen species[32].

The induced protection in first-generation plants was cultivar dependent, at least under the conditions evaluated. Plants of the cultivar Benicia were the only ones that achieved protection against *B. cinerea* after one generation. The "mother plants" received one treatment with CF. Perhaps to achieve protection over generations, Camino Real and Merced cultivars need to be pre-treated more times until they start to produce runners. Such kind of induced protection over generations was also demonstrated by Salazar et al.[49] in strawberry plants of the cultivar 'Pájaro' that were agamically propagated from a plant that had previously been treated with the avirulent pathogen *Acremonium strictum.*

This kind of induced protection is known as "priming", which is defined as "a physiological status of plants leading to faster and stronger activation of defence responses to subsequent biotic and abiotic stresses"[50]. Priming in plants is governed by chromatin modifications due to histones methylation and acetylation, which cause the rapid upregulation of transcription factors after stress exposure[51-55], leading to fast defence responses.

4.3 Biostimulation of Strawberry Plant Growth

There is a current trend to develop bioproducts with the dual function as biocontrol agents and plant biostimulants, with plant growth-promoting bacteria leading the research[56]. In this study, we demonstrated that the treatment with the fungal-derived extract CF yielded a protective effect in postharvest fruits and plants and also a desirable biostimulant effect on the vegetal growth of strawberry plants in the greenhouse. Unlike the biocontrol effect, the biostimulation effect was cultivar dependent. In general, CF led to plants with more leaves (Benicia and Merced cultivars), larger roots (cv. Camino Real), and more runners. Regarding the latter feature, it is important to highlight that whereas CF induced significant production of runners in Benicia, an opposite effect was observed for Camino Real and Merced. In a previous study, we observed that the fungal-derived extract CF slightly inhibited *A. thaliana* seedlings' growth *in vitro,* yielding plants with lower fresh weight compared to the control[38]. Similar to our findings, Furio et al.[30] demonstrated that brassinosteroids functioned both as biocontrol agents and biostimulants in strawberry plants. Overall, these results lead us to hypothesize that although strawberry crops can withstand *B. cinerea* under field conditions and have extended fruit storage, CF may also affect the efficient production of the crop. More investigations are required to validate this hypothesis.

5 CONCLUSION

The fungal-derived extract CF constitutes a promising alternative to be used as a biological control agent of *B. cinerea* in strawberry. CF efficiently controlled the causal agent of grey mould in harvested strawberries and first-generation untreated plants. Yet, showed a minor effect as a plant growth promoter. Thus, the current study opens up the possibility to develop "phytovaccines" using extracts of almost any fungus, including pathogenic ones, as long as they are previously inactivated.

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Conflicts of Interest

The authors declared no conflict of interest.

Author Contribution

All authors conceived and designed the experiments. Hael Conrad V performed the experiments, analysed the data, and wrote the manuscript. All authors edited and proofread the manuscript.

Abbreviation List

B. cinerea, Botrytis cinerea
BCAs, Biological control agents
CF, Culture filtrate
DPI, Days post infection.
DPT, Days post treatment;
DW, Dry weight
PDB, Potato dextrose broth
RH, Relative humidity
SPAD, Soil plant analysis development

References

[1] Kuchi VS, Sharavani CSR. Fruit physiology and postharvest management of strawberry. Asao T, Asaduzzaman M ed. In: Strawberry - Pre- and Post-Harvest Management Techniques for Higher Fruit Quality. IntechOpen: London, UK, 2019; 23-40. DOI: 10.5772/intechopen.84205

[2] Calzada J, Corina S, Rozadilla B. Tucumán and its agricultural and agro-industrial potential [in Spanish]. Accessed June 10, 2022. Available at https://www.bcr.com.ar/es/mercados/investigacion-y-desarrollo/informativo-semanal/noticias-informativo-semanal/tucuman-y-su

[3] Kirschbaum D. Strawberry: Crop conditions at the beginning of the harvest in Tucumán [in Spanish]. Accessed June 10, 2022. Available at https://inta.gob.ar/archivos/frutilla-condiciones-del-cultivo-en-el-inicio-de-la-cosecha-en-tucuman/view

[4] Romanazzi G, Feliziani E. Postharvest decay: Control strategies. Elsevier: Amsterdam, The Netherlands, 2014; 131-146. DOI: 10.1016/B978-0-12-411552-1.00004-1

[5] Dean R, Van Kan JAL, Pretorius ZA et al. The top 10 fungal pathogens in molecular plant pathology. *Mol Plant Pathol*, 2012; 13: 414-430. DOI: 10.1111/j.1364-3703.2011.00783.x

[6] Petrasch S, Knapp SJ, van Kan JAL et al. Grey mould of strawberry, a devastating disease caused by the ubiquitous necrotrophic fungal pathogen Botrytis cinerea. *Mol Plant Pathol*, 2019; 20: 877-892. DOI: 10.1111/mpp.12794

[7] Feliziani E, Romanazzi G. Postharvest decay of strawberry fruit: Etiology, epidemiology, and disease management. *J Berry Res*, 2016; 6: 47-63. DOI: 10.3233/JBR-150113

[8] Droby S, Lichter A. Post-harvest botrytis infection: Etiology, development and management. Springer: Dordrecht, Netherlands, 2007. DOI: 10.1007/978-1-4020-2626-3_19

[9] United Nations Development Programme (UNDP). Accessed 17 July 2022. Available at https://link.springer.com/chapter/10.1007/978-94-6265-515-7_36

[10] United Nations Environment Programme. Fertilizers: Challenges and solutions. Accessed 17 July 2022. Available at https://www.unep.org/news-and-stories/story/fertilizers-challenges-and-solutions

[11] Ajeng AA, Abdullah R, Malek MA et al. The Effects of biofertilizers on growth, soil fertility, and nutrients uptake of oil palm (Elaeis Guineensis) under greenhouse conditions. *Processes*, 2020; 8: 1681. DOI: 10.3390/pr8121681

[12] Bhardwaj D, Ansari MW, Sahoo RK et al. Biofertilizers function as key player in sustainable agriculture by improving soil fertility, plant tolerance and crop productivity. *Microb Cell Fact*, 2014; 13: 1-10. DOI: 10.1186/1475-2859-13-66

[13] Raja N. Biopesticides and biofertilizers: Ecofriendly sources for sustainable agriculture. *J Biofertilizers Biopestices*, 2013; 4: e112. DOI: 10.4172/2155-6202.1000e112

[14] Thompson J, Mitchell FG, Kasmire RF. Cooling horticultural commodities. *Postharvest Tech Hortic Crop*, 2002; 97-112.

[15] Nunes MCN, Brecht JK, Morais A et al. Physical and chemical quality characteristics of strawberries after storage are reduced by a short delay to cooling. *Postharvest Biol Tec*, 1995; 6: 17-28. DOI: 10.1016/0925-5214(94)00048-W

[16] Romero J, Albertos I, Díez-Méndez A et al. Control of postharvest diseases in berries through edible coatings and bacterial probiotics. *Sci Hortic-Amsterdam*, 2022; 304: 111326. DOI: 10.1016/j.scienta.2022.111326

[17] Chen X, Wang Y, Gao Y et al. Inhibitory abilities of bacillus isolates and their culture filtrates against the gray mould caused by botrytis cinerea on postharvest fruit. *Plant Pathology J*, 2019; 35: 425-436. DOI: 10.5423/PPJ.OA.03.2019.0064

[18] Sylla J, Alsanius BW, Krüger E. Control of Botrytis cinerea in strawberries by biological control agents applied as single or combined treatments. *Eur J Plant Pathol*, 2015; 143: 461-471. DOI: 10.1007/s10658-015-0698-4

[19] Adikaram NKB, Joyce DC, Terryc LA. Biocontrol activity and induced resistance as a possible mode of action of Aureobasidium pullulans against grey mould of strawberry fruit. *Austral Plant Path*, 2002; 31: 223-229. DOI: 10.1071/AP02017

[20] Shen H, Wei Y, Wang X et al. The marine yeast Sporidiobolus pararoseus ZMY-1 has antagonistic properties against Botrytis cinerea in vitro and in strawberry fruit. *Postharvest Biol Tec*, 2019; 150: 1-8. DOI: 10.1016/

j.postharvbio.2018.12.009
[21] Boff P, Köhl J, Gerlagh M et al. Biocontrol of grey mould by Ulocladium atrum applied at different flower and fruit stages of strawberry. *BioControl*, 2002; 47: 193-206. DOI: 10.1023/A:1014553221510
[22] Thomidis T, Pantazis S, Navrozidis E et al. Biological control of fruit rots on strawberry and grape by BOTRY-Zen. *New Zeal J Crop Hort*, 2015; 43: 68-72. DOI: 10.1080/01140671.2014.958502
[23] Marelli B, Brenckle MA, Kaplan DL et al. Silk fibroin as edible coating for perishable food preservation. *Sci Rep-Uk*, 2016; 6: 1-11. DOI: 10.1038/srep25263
[24] Nadim Z, Ahmadi E, Sarikhani H et al. Effect of methylcellulose-based edible coating on strawberry fruit's quality maintenance during storage. *J Food Process Pres*, 2015; 39: 80-90. DOI: 10.1111/jfpp.12227
[25] Romanazzi G, Gabler FM, Margosan D et al. Effect of chitosan dissolved in different acids on its ability to control postharvest gray mould of table grape. *Phytopathology*, 2009; 99: 1028-1036. DOI: 10.1094/PHYTO-99-9-1028
[26] Romanazzi G. Chitosan treatment for the control of postharvest decay of table grapes, strawberries and sweet cherries. *Fresh Produce,* 2010; 4: 111-115.
[27] Vu KD, Hollingsworth RG, Leroux E et al. Development of edible bioactive coating based on modified chitosan for increasing the shelf life of strawberries. *Food Res Int*, 2011; 44: 198-203. DOI: 10.1016/j.foodres.2010.10.037
[28] Yang C, Lu JH, Xu MT et al. Evaluation of chitosan coatings enriched with turmeric and green tea extracts on postharvest preservation of strawberries. *LWT*, 2022; 163: 113551. DOI: 10.1016/j.lwt.2022.113551
[29] Hael-Conrad V, Abou-Mansour E, Díaz-Ricci JC et al. The novel elicitor AsES triggers a defense response against Botrytis cinerea in Arabidopsis thaliana. *Plant Sci*, 2015; 241: 120-127. DOI: 10.1016/j.plantsci.2015.09.025
[30] Furio RN, Salazar SM, Martínez-Zamora GM et al. Brassinosteroids promote growth, fruit quality and protection against Botrytis on Fragaria x ananassa. *Eur J Plant Pathol*, 2019; 154: 801-810. DOI: 10.1007/s10658-019-01704-3
[31] Huang R, Li GQ, Zhang J et al. Control of postharvest botrytis fruit rot of strawberry by volatile organic compounds of candida intermedia. *Phytopathology*, 2011; 101: 859-869. DOI: 10.1094/PHYTO-09-10-0255
[32] Xu Y, Tong Z, Zhang X et al. Unveiling the Mechanisms for the plant volatile organic compound linalool to control gray mould on strawberry fruits. *J Agr Food Chem*, 2019; 67: 9265-9276. DOI: 10.1021/acs.jafc.9b03103
[33] Saleh I, Abu-Dieyeh M. Novel Prosopis juliflora leaf ethanolic extract coating for extending postharvest shelf-life of strawberries. *Food Control*, 2022; 133: 108641. DOI: 10.1016/j.foodcont.2021.108641
[34] Abd-Elkader DY, Salem MZM, Komeil DA et al. Post-harvest enhancing and botrytis cinerea control of strawberry fruits using low cost and eco-friendly natural oils. *Agronomy*, 2021; 11: 1246. DOI: 10.3390/agronomy11061246
[35] Kahramanoğlu İ, Panfilova O, Kesimci TG et al. Control of postharvest gray mould at strawberry fruits caused by botrytis cinerea and improving fruit storability through origanum onites L. and Ziziphora clinopodioides L. volatile essential oils. *Agronomy*, 2022; 12: 389. DOI: 10.3390/agronomy12020389
[36] Ghorbanpour M, Omidvari M, Abbaszadeh Dahaji P et al. Mechanisms underlying the protective effects of beneficial fungi against plant diseases. *Biological Control*, 2018; 117: 147-157. DOI: 10.1016/j.biocontrol.2017.11.006
[37] Köhl J, Kolnaar R, Ravensberg WJ. Mode of action of microbial biological control agents against plant diseases: Relevance beyond efficacy. *Front Plant Sci*, 2019; 10: 845. DOI: 10.3389/fpls.2019.00845
[38] Hael Conrad V, Tomas Grau RH, Moschen SN et al. Fungal-derived extracts induce resistance against Botrytis cinerea in Arabidopsis thaliana. *Eur J Plant Pathol*, 2020; 158: 45-58. DOI: 10.1007/s10658-020-02054-1
[39] Romanazzi G, Gabler FM, Smilanick JL. Preharvest chitosan and postharvest UV irradiation treatments suppress gray mould of table grapes. *Plant Disease*, 2007; 90: 445-450. DOI: 10.1094/PD-90-0445
[40] Shaw DV, Larson KD. Strawberry plant named 'Camino Real'. Accessed 17 July 2022. Available at https://patents.google.com/patent/USPP13079P2/en
[41] Shaw DV, Larson KD. Strawberry plant named 'Benicia'. Accessed 17 July 2022. Available at https://patents.google.com/patent/USPP22542P3/en
[42] Shaw DV, Larson KD. Strawberry plant named 'Merced. Accessed 15 October 2002. Available at https://patents.google.com/patent/US20140325716P1/en
[43] Schneider CA, Rasband WS, Eliceiri KW. NIH Image to Image J: 25 years of image analysis. *Nat Methods*, 2012; 9: 671-675. DOI: 10.1038/nmeth.2089
[44] Di Rienzo JA, Casanoves F, Balzarini MG et al. Grupo InfoStat, FCA, Universidad Nacional de Córdoba. Accessed 17 July, 2022. Available at http://www.infostat.com.ar
[45] Tomas-Grau RH, Hael-Conrad V, Requena-Serra FJ et al. Biological control of strawberry grey mould disease caused by Botrytis cinerea mediated by Colletotrichum acutatum extracts. *BioControl*, 2020; 65: 461-473. DOI: 10.1007/s10526-020-10003-4
[46] Tomas-Grau RH, Di Peto P, Chalfoun NR et al. Colletotrichum acutatum M11 can suppress the defence response in strawberry plants. *Planta*, 2019; 250: 1131-1145. DOI: 10.1007/s00425-019-03203-5
[47] Mohammadi A, Hashemi M, Hosseini S. The control of Botrytis fruit rot in strawberry using combined treatments of Chitosan with Zataria multiflora or Cinnamomum zeylanicum essential oil. *J Food Sci Tech*, 2015; 52: 7441-7448. DOI: 10.1007/s13197-015-1871-7
[48] Wang F, Xiao J, Zhang Y et al. Biocontrol ability and action mechanism of Bacillus halotolerans against Botrytis cinerea causing grey mould in postharvest strawberry fruit. *Postharvest Biol Tec*, 2021; 174: 111456. DOI: 10.1016/j.postharvbio.2020.111456
[49] Salazar SM, Grellet CF, Chalfoun NR et al. Avirulent

strain of Colletotrichum induces a systemic resistance in strawberry. *Eur J Plant Pathol*, 2013; 135: 877-888. DOI: 10.1007/s10658-012-0134-y

[50] Wiesel L, Newton AC, Elliott I et al. Molecular effects of resistance elicitors from biological origin and their potential for crop protection. *Front Plant Sci*, 2014; 5: 655. DOI: 10.3389/fpls.2014.00655

[51] Jaskiewicz M, Conrath U, Peterhänsel C. Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *EMBO Rep*, 2011; 12: 50-55. DOI: 10.1038/embor.2010.186

[52] Conrath U. Molecular aspects of defence priming. *Trends Plant Sci*, 2011; 16: 524-531. DOI: 10.1016/j.tplants.2011.06.004

[53] Conrath U, Beckers GJM, Flors V et al. Priming: Getting ready for battle. *Molecular Plant-Microbe Interactions: MPMI*, 2006; 19: 1062-1071. DOI: 10.1094/MPMI-19-1062

[54] Pastor V, Luna E, Mauch-Mani B et al. Primed plants do not forget. *Environ Exp Bot*, 2013; 94: 46-56. DOI: 10.1016/j.envexpbot.2012.02.013

[55] Mauch-Mani B, Baccelli I, Luna E et al. Defense priming: An adaptive part of induced resistance. *Annu Rev Plant Biol*, 2017; 68: 485-512. DOI: 10.1146/annurev-arplant-042916-041132

[56] Morales-Cedeño LR, del Carmen Orozco-Mosqueda M, Loeza-Lara PD et al. Plant growth-promoting bacterial endophytes as biocontrol agents of pre- and post-harvest diseases: Fundamentals, methods of application and future perspectives. *Microbiol Res*, 2021; 242: 126612. DOI: 10.1016/j.micres.2020.126612