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Influence of Biocontrol and Integrated Strategies and Treatment Timing on Plum Brown Rot Incidence and Fungicide Residues in Fruits

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Abstract: Brown rot caused by *Monilinia* spp. is the main disease of stone fruits. Our research aimed to identify an appropriate strategy to control plum brown rot and reduce fungicides residues in fruit through targeted application of the biocontrol agents (BCAs) *Papiliotrema terrestris* and *Bacillus subtilis*, alone or in combination with synthetic fungicides. The following treatments were evaluated: Biological (BIO1, BIO2), Integrated (INT1, INT2, INT3), and Combined (COMB), all compared with Chemical strategy. Five key treatments (seven for BIO2) were performed in the crucial phenological stages for the disease cycle: INT1, INT2, and INT3 approaches consisted, from the beginning of the season, of one, two, or three treatments, respectively, alternately with boscalid or cyprodinil followed by applications of BCA until the fruit harvest. After harvest, plums were subjected to an additional treatment with the two BCAs and the fruits were stored at 20 °C for 15 days. The results obtained by applying our BCA PT22AV, revealed, in the field and in postharvest, the highest level of disease protection with management strategies BIO2 (94.8–97.2% in field; 65–84% in postharvest) and INT3 (95.5–97% in field and 63% to 91% in postharvest). The level of fungicide residues in fruit was zero in BIO strategies and lower in INT strategies as compared to chemical strategy.

Keywords: *Papiliotrema terrestris* strain PT22AV; *Monilinia fructigena*; plum brown rot; biocontrol

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1. Introduction

Stone fruits are native to central Asia and apricots, cherries, nectarines, peaches, and plums are the main cultivated species. They are very susceptible to a widespread fungal disease known as brown rot that is responsible for high losses in both pre- and postharvest stages [1,2]. Plum brown rot (PBR) is caused by several fungal species of the genus *Monilinia* [3–7]: *Monilinia fructigena* (Aderhold and Ruhland) Honey mainly on pome fruits in Europe and on stone fruits in Brazil, China, and Europe; *Monilinia fructicola* (Winter) Honey on stone fruit in Australasia, Brazil, China, Europe, New Zealand, Japan, North, and South America; *Monilinia laxa* (Aderhold and Ruhland) Honey mainly on stone fruits in Europe, Brazil, Chile, China, Iran, South Africa, and USA; and the anamorph *Monilia polystroma* (van Leeuwen) in Japan, Poland, and Hungary [7]. Finally, two others less widespread *Monilinia* species, *M. mumecola* and *M. yunnanensis*, have also been reported to infect plants belonging to Rosaceae family (China and Japan) [5,8,9].

Host susceptibility coupled with the unavailability of genetic resistance, environmental factors, cultivation techniques, and onset in the pathogen populations of fungicide resistant strains are the main causes of the disease which, under favorable epidemiological conditions, can cause severe losses of plum fruits within a few days [2,5].

The plum brown rot (PBR) disease cycle comprises blossom blight and twig canker in early spring, fruit brown rot in summer, and overwintering inoculum of the fungal pathogen as mummified fruit [1].

In Europe, sexual reproduction of *Monilinia* spp. is not frequent, and primary infections in spring are caused by conidia originating from overwintering mummies on trees or on the ground [10,11]. The primary infections, which start from the flowers and then spread in the twigs causing blight canker, and the overwintering pathogen source provide the inoculum for latent infections on fruits [5,12].

Different studies reported that the incidence of PBR in pre- and postharvest is closely related to latent infections [5,12–14]. The time when latent infections occur is crucial for the disease severity that also depends on the host (species, cv), the environmental factors (temperature, humidity), and the type of cultivation [12,14]. The pathogen penetrates mainly through leaf stomata, lenticels, and fruit skin microcracks or wounds. Skin cracks and microcracks that often occur around the lenticels have a crucial role in the development of the disease. Different studies have demonstrated the effect of the cultivation practices (e.g., irrigation and thinning) and humidity of fruit surfaces on the density of pathogen propagules on fruits [1,15–18].

Latent infections and their time of establishment play a key role in the epidemiology and disease severity [13,19–21], so the timeliness of treatments and the number and effectiveness of fungicide applications are crucial to reduce the occurrence of *Monilinia* spp. infections. Therefore, to design the most appropriate disease control strategy, the host susceptibility, the seasonal trend, as well as the predisposition of the plum fruit to latent infections and the time at which they occur are the main factors to be taken into consideration.

Currently, the use of synthetic fungicides in the field is crucial for containing PBR close to the harvest, i.e., when the fruits are more susceptible to the disease, also for the presence of physiological cuticular cracks [5,22]. However, chemical control is increasingly limited due to environmental and toxicological risks as well as for the onset of fungicide-resistant pathogen strains. Moreover, very few fungicides are allowed in preharvest and often none in postharvest. The European regulations in force establish lower residue limits of the active ingredients [4].

For these reasons, sustainable alternative strategies are emerging, which also include the biocontrol based on the use of antagonistic microorganisms (bacteria, fungi, and yeasts) that is a promising tool to prevent pre- and postharvest fungal rots and to significantly decrease the use of synthetic fungicides [2,23–25]. Several studies have shown the effectiveness of many plant-beneficial microorganisms applied alone or combined with synthetic fungicides against different plant pathogens [2,26–34], and various biofungicides are commercially available [35,36]. Plant surfaces are efficiently colonized by bacteria, yeasts, yeast-like fungi, and filamentous fungi that play a central role in diverse ecological interactions [32,37,38]. Moreover, some of these epiphytic microorganisms are also known for their natural ability to hinder fungal plant pathogens development, and therefore they are ideal candidates as biocontrol agents (BCAs) because they can efficiently colonize plant surfaces, natural openings, as well as cracks and microcracks of the carpoplane, which are preferential ways of penetration and development of *Monilinia* spp. latent infections [2,26,34,38,39]. In our previous research, yeasts and yeast-like fungi were selected for their wide range of antagonistic activity against many fungal pathogens in pre- and postharvest [2,32,40–42] and were characterized for their mode of action also at a molecular genetic level [42–48], for the containment of some mycotoxigenic fungi as well as for the degradation capacity of some mycotoxins [33,49–53] and for the compatibility with some synthetic fungicides [2,34,42,53].

Under practical conditions, BCAs do not always reduce fungal decay when applied alone or when the number of treatments is insufficient and/or is not performed at critical stages for the disease development [2,24,54,55]. Therefore, for the control of an insidious disease such as PBR, an appropriate preventive strategy based on the use of BCAs in combination/alternation with synthetic fungicides is suggested considering the times of application [2,23,54,56]. Our previous research showed that the pre- and postharvest protective activity of the antagonist strains of yeasts or yeast-like

fungi *Aureobasidium pullulans*, *Rhodotorula kratochvilovae*, and *Papiliotrema terrestris*, is enhanced by combining/alternating them with a low dosage of fungicides and/or natural adjuvants [2,23,34,53,56].

The present study aims to compare three strategies (BIO, INT, and COMB) for the control of PBR caused by *Monilinia fructigena* that involves the use of the biocontrol agents *P. terrestris* PT22AV and *Bacillus subtilis* QST 713 applied alone (BIO), in alternance (INT), or in combination with synthetic fungicides (COMB), with treatments carried out at key phenological stages for the onset PBR latent infections.

2. Material and Methods

2.1. Biocontrol Agents

The BCA used in this research is the yeast *Papiliotrema terrestris* strain PT22AV (Patent Pending, AgroVentures Srl/LLC, Latina, Italy/Southbury, CT, USA, with ongoing registration applications for the European Union and United States of America) selected from a yeast collection isolated from the epiphytic microbial communities of various fruits and vegetables in central Italy. PT22AV has been characterized for its biocontrol activity in field and in postharvest trial programs for the commercial registration of a biofungicide formulate based on this strain as the active ingredient. The effectiveness of the BCA PT22AV in controlling PBR was compared with the commercial biofungicide Serenade Max® that is authorized against PBR and based on the biocontrol bacterium *Bacillus subtilis* strain QST 713 (Bayer Crop Science, Leverkusen, Germany).

The BCA PT22AV was supplied by Agroventures Srl/LLC in the form of a water-dispersible granules formulation at a concentration ranging from 0.5^{10} to 10^{10} CFU per g of product, and stored at the University of Molise, Department Agricultural, Environmental and Food Sciences, Section of Plant Pathology (Campobasso, Italy) before its use. For field efficacy trials, the yeast PT22AV was used by applying 1 kg of formulation per hectare and distributing it in a volume of 1000 L of water per hectare (10^7 CFU per mL of tank mix).

The biofungicide SerenadeMax® was stored in the appropriate commercial package at room temperature prior its utilization. Prior to its use, Serenade Max® was prepared according to the product label.

Both bioformulates were diluted in tap water (when applied alone) or in a low dose solution of cyprodinil or boscalid (in the combined control treatment COMB) as described below.

2.2. Fungicides

The fungicides used in this study were boscalid ((BOSC), chemical group pyridinecarboxamide, trade formulate Cantus®, 50%, *w*/*w* a.i., BASF, Milan, Italy); cyprodinil ((CYPR) chemical group anilinopyrimidines, trade formulate Chorus®, 50% a.i., *w*/*w*, Syngenta Crop Protection, Milan, Italy) and fenexamide ((FENEX), chemical group hydroxyanilides; trade formulate Teldor®, 50% a.i., *w*/*w*, Bayer Crop Science, Milan, Italy).

2.3. Sensitivity of the BCAs to Fungicides

The sensitivity of the BCAs *P. terrestris* strain PT22AV and *Bacillus subtilis* (Serenade Max®) to the main fungicides used for the control of plum brown rot was carried out as described elsewhere [2,34,53]. Briefly, the BCAs were tested *in vitro* for their sensitivity to commercial formulates of boscalid (BOSC), cyprodinil (CYPR), and fenexamide (FENEX). The assays were performed on BYA medium (Basal Yeast Agar: 18 g agar, 20 g dextrose, 10 g bacteriological peptone, 1 g yeast extract, 1 L of distilled water). Each fungicide was suspended in distilled water and mixed with the medium at 45 °C and, according to the full dose suggested by the manufacturers for application in on fruit trees, the following concentrations of fungicide active ingredients (a.i.) were tested: BOSC 450 μg mL^{-1} (100 g hL^{-1} of commercial product (i.e., about twice as the dose suggested by the manufacturer)), BOSC 200 μg mL^{-1} [40 g hL^{-1} of commercial product (full dose as suggested by the manufacturer)), 100 μg mL^{-1} (50% of the full suggested dose), and 50 μg mL^{-1} (25% of the full

suggested dose); CYPR 250 μg mL^{-1} (50 g hL^{-1} of commercial product (highest full dose as suggested by the manufacturer against *Monilinia* spp.)), 125 μg mL^{-1} (50% of the full suggested dose), and 62.5 μg mL^{-1} (25% of full dose); and FENEX 600 μg mL^{-1} ((120 g hL^{-1} of commercial product (full dose as suggested by the manufacturer)), 300 μg mL^{-1} (50% of the full suggested dose), and 150 μg mL^{-1} (25% of the full suggested dose). Each plate was poured with 100 μL of yeast suspension containing about 100 cells and incubated at 23 °C. After 7 days, the growing yeast colonies were counted in each plate and the minimum inhibitory concentration (MIC) was recorded. For each treatment, three replicates each consisting of five plates were used and the experiments were performed three times.

2.4. Microbial Epiphytic Population on the Surface of the Plum Fruits in the Field

The occurrence of yeasts was monitored on the surface of the plum fruits in the field from the end of flowering to preharvest according to the schedule reported in Figure 1. To this purpose, at the center of each replicate a total of 50 plum fruits, one per tree, were harvested. The fruits were washed in sterile distilled water, immersed in sterile distilled water, and kept in a rotary shaker at 150 rpm for 30 min and 21 °C. One hundred microliters of washing suspension, accurately diluted, was dispensed in Petri dishes containing NYDA. Five Petri dishes were prepared for each replicate. Plates were incubated at 23 °C and BCA colonies were counted and expressed as Log CFU/cm^2 of plum fruit surface. Based on colony color and morphology, phyllosphere yeasts were divided into pink yeasts, white yeasts, and yeast-like fungi. In these experiments only the white yeasts population, to which strain PT22AV belongs, were recorded.

2.5. Molecular Identification of the Plant Pathogen

Microbial isolation was performed from symptomatic fruits sampled in the field from untreated replicates. The sampled fruit were surface disinfected with a sodium hypochlorite water solution (2% *v*/*v*) for 2 min, rinsed with sterile distilled water, and small tissue pieces were placed in Petri dishes containing potato dextrose agar (PDA) with streptomycin sulphate (250 ppm) and incubated at 25 °C for 7 days. The isolation in pure culture of the fungal isolates was performed using the monoconidial isolation technique. From each representative fungal isolate, 500 mg of mycelium were collected to perform the total genomic DNA extraction using the DNeasy Yeast/Bact.Mini kit (Qiagen, GmbH, Hilden, Germany) following the manufacturer's instructions. The fungal gDNAs were used as a template to amplify the internal transcribed regions (ITS 1, 5.8S ribosomal RNA gene, and ITS 2. The ITS regions were amplified by PCR using the primers ITS1 and ITS4 [57,58]). PCR was carried out in a T Gradient ThermalCycler (Techne, mod. 512, Minneapolis, MN, USA) with a final reaction volume of 50 μL. The reaction mixture contained 25 μL of PCRMaster Mix (Promega, Madison, WI, USA), 4 μL of each primer (10 μM), 4 μL of template DNA and 13 μL of sterile water. The thermal cycling program was as follows: denaturation step at 94 °C for 150 s, followed by 15 s at 94 °C, 30 s at 55 °C, 90 s at 72 °C for 40 cycles and a final extension of 72 °C for 5 min. After amplification, 10 μL of the amplified product was loaded onto a 1% (*w*/*v*) agarose gel. The amplicons were sequenced by "Eurofins Genomics" (Ebersberg, Germany) and species-level identification was performed through BLASTn searches of sequence data from each PCR product in GenBank of National Center for Biotechnology Information (NCBI) at http://blast.ncbi.nlm.nih.gov (accessed on 13 June 2022).

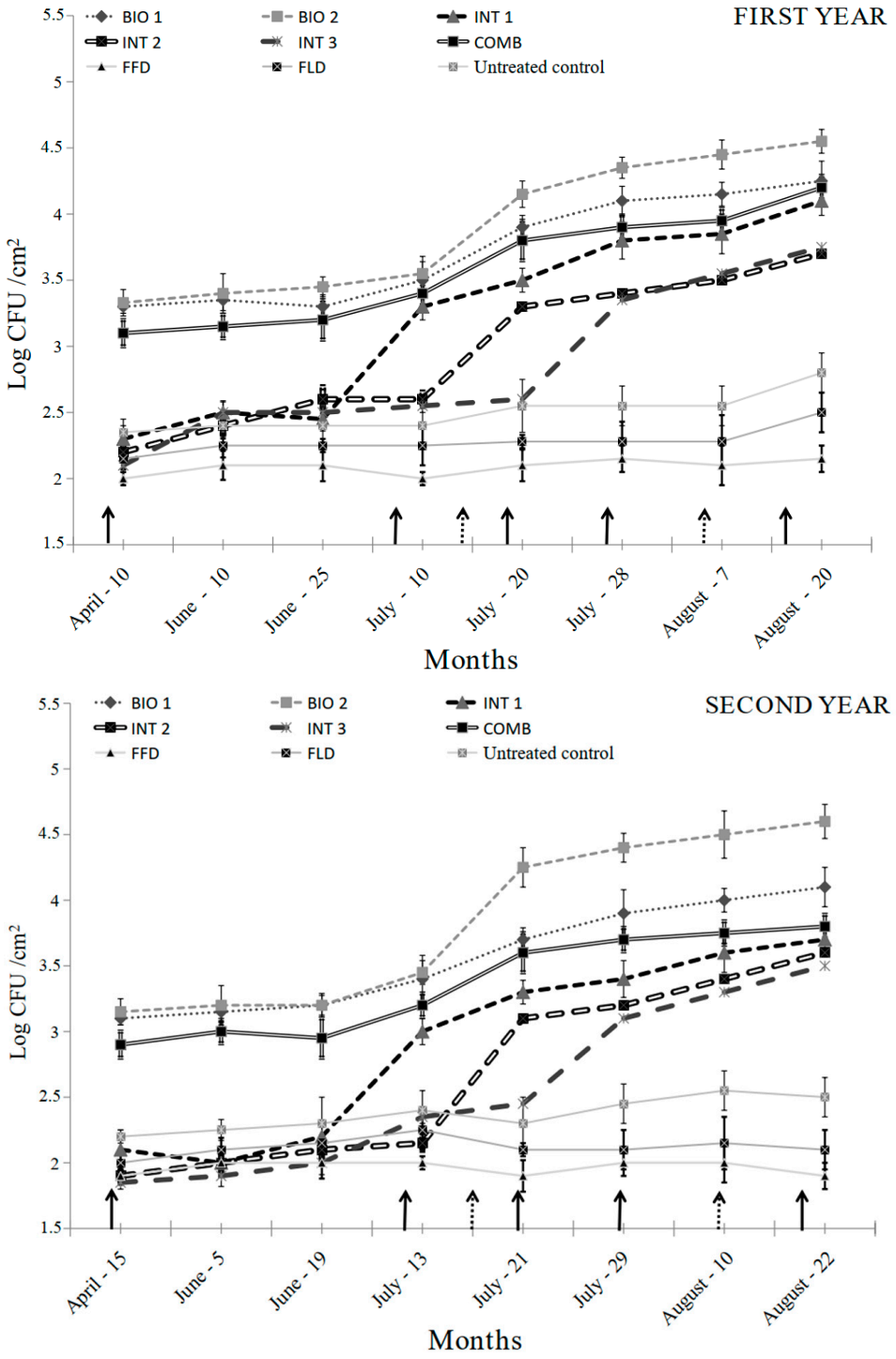

Figure 1. Dynamics of the total white yeasts population on plum fruits surface in field subjected to six different disease management strategies for two consecutive years: Biological, where the BCAs (the yeast *Papiliotrema terrestris* PT22AV or the commercial biofungicide Serenade Max® based on *Bacillus subtilis* strain QST 713), were applied alone in five (BIO1) or seven (BIO2) consecutive treatments; Integrated, i.e., INT1, INT2 and INT3, consisted of the application from the beginning of the growing season of one, two or three treatments respectively, with the chemical fungicides boscalid or cyprodinil applied alternately, and each strategy was followed by BCAs applications until fruit harvest; Combined (COMB), in which the biocontrol yeast PT22AV or Serenade Max® were applied in combination with a low dose (25% of the full label dose) of the synthetic fungicides Cantus® or Chorus® (alternately) in all treatments; Chemical, in which the chemical fungicides Cantus® and

Chorus® were alternately applied at low (25% of full dose) (FLD) or at full label dose (FFD) in all treatments. The arrows indicate the time of treatments. Bars represent the standard deviation from the mean.

2.6. Biological, Integrated, and Combined Control Experiments of Brown Rot of Plum Fruits

The field experiments were carried out in two consecutive years in Central Italy on the plum orchard of cv Stanley of the "Ciccaglione G. farm" (Latitude: 41°57′49.05″ N; Longitude: 14°53′57.52″ E). Distance between fruit trees was 5 m on the row and 6 m between rows. The orchards were irrigated and fertilized according to common farming practices in Central Italy. The field research was based on the application of the biocontrol agent *P. terrestris* PT22AV (BCAY) compared with the commercial biofungicide Serenade Max® (BCAB) (based on *Bacillus subtilis* strain QST 713 (Bayer Crop Science)) following six different strategies (Table 1): Biological 1 (BIO1), Biological 2 (BIO2), Integrated 1 (INT1), Integrated 2 (INT2), Integrated 3 (INT3), and Combined (COMB), all compared with chemical ones (CHEM) at full (FFD) or low (FLD, corresponding to 25% of FFD) dose of fungicides. All approaches were based on five (or seven only in in the case of BIO2 strategy) key treatments (Table 1) performed in the following crucial phenological stages for the disease cycle: 69–71 of BBCH-scale (end of flowering-ovary growing), 75 of BBCH-scale (fruit about half-final size), 77–78 of BBCH-scale (fruit 70–80% of final size), 81 of BBCH-scale (beginning of fruit coloring), and 87 of BBCH-scale (fruit ripe for picking). The BIO strategies involved the use of only the biological control agents (BCA), the yeast *P. terrestris* strain PT22AV or the commercial biofungicide Serenade Max®, applied alone, in all five (or seven in BIO2) treatments. The INT1, INT2, and INT3 strategies were based on the application of the chemical fungicides boscalid or cyprodinil (applied alternatively) at the first, first two or first three programmed times, respectively, and then followed by treatments with BCAs applied alone until harvest. The strategy COMB was performed, in all five treatments, with the use of the BCAs applied in combination with a low dose (25% of label dose) of chemical fungicide (boscalid or cyprodinil applied alternatively). Finally, in the CHEM strategy the chemical fungicides (boscalid or cyprodinil applied alternatively) were applied at label dose (FFD) or low dosage (FLD), in all five scheduled times. The application of water alone was the untreated control. The suspensions of the BCAs *P. terrestris* PT22AV and *Bacillus subtilis* strain QST 713 were accurately adjusted just before the treatment to a final concentration of 10^7 CFU and 2.05×10^8 CFU per ml of tank mix, respectively. The BCAs were suspended in tap water (when applied alone) or in a water solution of a low dosage (25% of label dose) of CYPR or BOSC when applied in combination (COMB strategy).

The field experiments in the farm were arranged in a completely randomized block design with four replicates (plots) per treatment each consisting of 75 plants. The treatments were carried out by spraying on fruit trees the equivalent of 1000 L Ha^{-1} of the liquid mixture with the equipment normally used in the farm. The experimental treatment schedule is reported in Table 1.

At the end of the second year the postharvest protective effects provided by the tested BCAs against *Monilia* spp. were also evaluated. Briefly, 100 plum fruits were sampled from each replicate, half of which were treated immediately after harvest with the BCA corresponding to the treatments, while the other half were treated with water. The BCAs were applied in postharvest stage using the same cell concentration as used in the field trials. After the treatments, each fruit was kept in individual plastic pots and stored at room temperature (25 ± 2 °C) for 15 days, and the incidence % of plum brown rot was assessed at the end of the incubation.

Table 1. Scheme of plum fruits brown rot management strategy used in two years of orchard research based on key treatments carried out at crucial phenological phases (BBCH) for the disease cycle.

Disease Control Strategy	Treatments and Application Timing (BBCH)				
			Preharvest (60–70 Days)	**Preharvest (30–35 Days)**	**Preharvest (7–15 Days)**
	69–71 of BBCH: End of Flowering-Ovary Growing	**75 of BBCH: Fruit About Half Final Size**	**77–78 of BBCH: Fruit 70–80% of Final Size**	**81 of BBCH: Beginning of Fruit Coloring**	**87 of BBCH: Fruit Ripe for Picking**
BIOLOGICAL 1 (BIO 1): 5 treatments with BCA [a]	BCA	BCA	BCA	BCA	BCA
BIOLOGICAL 2 (BIO 2): 7 treatments with BCA	BCA	BCA	BCA BCA	BCA BCA	BCA
INTEGRATED 1 (INT 1): One treatment with FFD [b] followed by four treatments with BCA	FFD (Cantus®) [c]	BCA	BCA	BCA	BCA
INTEGRATED 2 (INT2): Two treatments with FFD followed by three treatments with BCA	FFD (Cantus®)	FFD (Chorus®) [d]	BCA	BCA	BCA
INTEGRATED 3 (INT 3): Three treatments with FFD followed by two treatments with BCA	FFD (Cantus®)	FFD (Chorus®)	FFD (Cantus®)	BCA	BCA
COMBINED (COMB): Five treatments with BCA applied in combination with FLD [e]	FLD + BCA (Cantus® + BCA)	FLD + BCA (Chorus® + BCA)	FLD + BCA (Cantus® + BCA)	FLD + BCA (Chorus® + BCA)	FLD + BCA (Cantus® + BCA)
CHEMICAL (FLD): Five treatments with FLD	Cantus®	Chorus®	Cantus®	Chorus®	Cantus®
CHEMICAL (FFD): Five treatments with FFD	Cantus®	Chorus®	Cantus®	Chorus®	Cantus®
WATER (UTC)	Water	Water	Water	Water	Water

[a] The yeast biocontrol agent (BCA), isolate PTAV 22 of *Papiliotrema terrestris* or the biofungicide Serenade Max® (based on the bacteria *Bacillus subtilis* QST 713) were applied at 10^7 CFU/mL and 2.05×10^8 CFU/mL, respectively. [b] Fungicide applied at label Full Dosage (FFD): [c] Cantus® (a.i. boscalid). [d] Chorus® (a.i. cyprodinil). [e] Fungicide applied at Low Dosage (FLD = 25% of fungicide full dosage).

2.7. Analysis of Fungicide Residues in Plum Fruits

For the analysis of synthetic chemical fungicide residues, 50 plum fruits were collected from the center of each replicate (one from each plum tree at the center of the replicate) and for each disease control strategy just before harvest. The procedure was carried out according to the method reported in our previous research [2,53]. Briefly, for the analysis of fungicides 10 g of plum fruits were homogenized using an Ultra-Turrax T25 (IKA-WERKE, Staufen, Germany) and mixed with 10 g of fine diatomaceous earth at pH 10. The mixture was then placed onto a SPE polypropylene tube, and the sample eluted with 100 mL of a dichloromethane/ethyl acetate (80:20, v/v) solution. The extract was completely dried and then dissolved in 10 mL of a methanol/water (70:30, v/v) solution, centrifuged at 10,000 rpm for 3 min and used for LC/MS/MS analysis of fungicides. The standard working solutions of fungicides were prepared by appropriate dilutions with the same methanol/water (70:30, v/v) solution. Chromatographic separation was performed using an HPLC apparatus equipped with two micropumps Series 200 (Perkin Elmer, Waltham, MA, USA) and a Gemini 5 mm C18, 110 Å column (150 mm × 2 mm) (Phenomenex, Torrance, CA, USA). The adopted eluents were: (A) water 0.1% formic acid; (B) and acetonitrile. The gradient program was: 35–50% B (5 min), 50–95% B (7 min), 95% B (2 min), and 95–35% B (2 min), at a constant flow of 0.2 mL min^{-1}. The injection volume was 20 µL. MS/MS analyses of boscalid and cyprodinil were performed on an API 2000 triple quadrupole mass spectrometer (Applied Biosystems, Waltham, MA, USA) equipped with a Turbo Ion Spray source. Analyses were performed in the positive ion mode in MRM (multiple reaction monitoring). The calibration curve showed good linearity in the range 5–500 ng mL^{-1}. All chromatographic points of the calibration curve were run in triplicate, and the standard deviation was lower than 0.05. The limits of detection ((LOD) with a signal to noise ratio of 3) for CYPR and BOSC were 0.5 and 2.5 ng g^{-1}, respectively, and limits of quantification ((LOQ) with a signal to noise ratio of 6) were 2 ng g^{-1} for CYPR and 5 ng g^{-1} for BOSC.

2.8. Disease Assessment

The disease incidence was calculated on 50 plum trees at the center of each plot replicate; for each plum tree, 60 fruits (=20 fruits for each main branch of the plum tree) were examined for the presence of clear symptoms of PBR caused by *Monilinia* spp. The disease incidence was expressed as the percentage of diseased fruits. Protection efficiency (PE) was calculated according to the following formula, PE = [(A − B)/A]100 where A is the percentage of the diseased fruits in the untreated control, and B is the percentage of the diseased fruits in a specific treatment. Values ranged from 0 (no PE) to 100 (maximum PE).

2.9. Experimental Design and Statistical Analysis

In the field experiments, a completely randomized block design was arranged. For each disease control strategy, four replicates (plots) of 75 plum trees were used. Data on the effects of the treatments on brown rot disease incidence and fungicide residues were processed by the analysis of variance (ANOVA) according to a completely randomized block design using three replicates for each treatment. Data were submitted to factorial analysis of variance (one-way ANOVA) followed by Uncorrected Fisher's LSD comparison test ($p < 0.05$) and Tukey's multiple comparison test ($p < 0.05$). Differences were considered statistically significant when p-value was lower than 0.05. The percentages of infected wounds, assessed for all experiments, were converted into Bliss angular values (arcsine $\sqrt{\%}$) before statistical analysis.

3. Results

3.1. Compatibility of the BCA Papiliotrema terrestris PT22AV with Fungicides

To better define the partner fungicides to be used in combination with the yeast PT22AV in the INT and COMB strategies, the BCAs PT22AV and *Bacillus subtilis* were testedin vitro for their compatibility with the fungicides BOSC, CYPR, and FENEX, three

active compounds frequently used in the PBR control. Results of these assays showed that the biocontrol yeast was highly resistant to boscalid (MIC > 450 μgmL − 1) and cyprodinil (MIC > 200 μgmL − 1), whereas it was less resistant to fenexamide (MIC < 130 μgmL − 1). As expected, *B. subtilis* was resistant to all three fungicides applied at the maximum dosage. Based on these results, BOSC and CYPR were chosen for the orchard research.

3.2. Microbial Epiphytic Population on the Surface of the Plum Fruits in the Field

The dynamics of the total epiphytic white yeast population that was monitored on plum fruits in the two-year field experiments is shown in Figure 1. In both years, the values of the total population of epiphytic white yeasts recorded on fruits treated with the BCA PT22AV applied alone (BIO1 and BIO2), and after one, two, and three fungicide treatments (INT1, INT2, and INT3) and when applied in combination with the synthetic fungicides (COMB), were higher than those recorded on fruits that were not treated with the biocontrol yeast (FFD, FLD or water alone). For all treatments and in the two consecutive years, the trends of total white yeast populations recorded on plum fruits showed only slight variations. In both years, synthetic fungicides applied at low dose (FLD) or at the label full dose (FFD) reduced the total white yeast population compared with the untreated control and the other treatments. In particular, the total white yeast population (log CFU/cm^2) ranged from 1.99 × 10^3 to 1.79 × 10^4 log CFU/cm^2 (first year) and from 1.26 × 10^3 to 1.26 × 10^4 log CFU/cm^2 (second year) on plum fruits treated with the BIO1 strategy, and from 2.14 × 10^3 to 7.08 × 10^4 log CFU/cm^2 (first year) and from 1.41 × 10^3 to 3.98 × 10^4 log CFU/cm^2 (second year) on plum fruits treated with the BIO2 strategy. Furthermore, the total white yeast population ranged from 1.26 × 10^2 to 5.62 × 10^3 log CFU/cm^2 (first year) and from 0.79 × 10^2 to 5.01 × 10^3 log CFU/cm^2 (second year) on plum fruits treated with INT strategy, and from 1.26 × 10^3 to 1.58 × 10^4 log CFU/cm^2 (first year) and from 7.94 × 10^2 to 6.31 × 10^3 log CFU/cm^2 (second year) on plum fruits treated with the COMB strategy.

3.3. Pathogen Identification

From the PCR and sequence analysis performed on the ITS regions, was obtained an amplicon of 600 bp. The BLASTn searches with the obtained sequence in the NCBI database revealed a high similarity to the representative fungal sample of *Monilinia fructigena* (Table 2).

Table 2. Main results of alignments between the sequences of ITS region of isolated pathogen and other fungi sequences in GenBank.

PCR Products	Results of Blast						
	Description	Max Score	Total Score	Query Cover (%)	E Value	Ident. (%)	Accession
ITS	*Monilinia fructigena isolate DM1082*	922	922	100	0.0	100	MT644896.1
	Monilinia fructigena strain PHYTMN	922	922	100	0.0	100	MT522857.1
	Monilinia fructigena isolate MON23	922	922	100	0.0	100	MN049479.1
	Monilinia fructigena isolate MON7	922	922	100	0.0	100	MN049477.1
	Monilinia fructigena CBS:101500 strain	922	922	100	0.0	100	MH862738.1

3.4. Control Strategies of Brown Rot of Plum Fruits in Field Trials

The results of the different disease control strategies (Table 1) against plum brown rot in two years of research, are shown in Figure 2.

The disease incidence in plum fruits was more severe in the second year of research than in the first one. As expected, the disease incidence in the water-treated control (i.e., untreated control (UTC)) was higher than in all other tested strategies, and lower in the first (24.8%) than in the second year (48.7%). Conversely, the highest level of protection efficacy (PE) was recorded with synthetic fungicides applied alone at the full label dose

((FFD) 97.2–98.2% of disease reduction). As expected, the fungicides applied at a low dose ((FLD) 25% of the label full dose) yielded a significantly lower PE (16.1–33.8%).

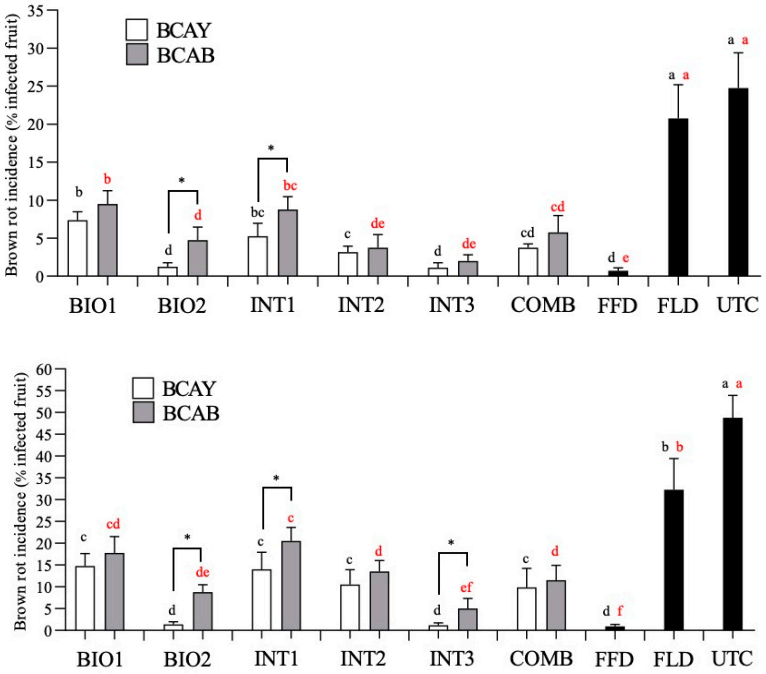

Figure 2. Brown rot incidence on plum fruits assessed in two consecutive years (year 1 and year 2) of field trials in which the plants in plum orchard were treated following six different disease management strategies: Biological, in which the BCAs (the yeast *Papiliotrema terrestris* PT22AV (BCAY) or the commercial biofungicide Serenade Max® based on *Bacillus subtilis* strain QST 713 (BCAB)), were applied alone in five (BIO1) or seven (BIO2) consecutive treatments; Integrated, i.e., INT1, INT2 and INT3, consisted of the application from the beginning of the growing season of one, two or three treatments respectively, with the chemical fungicides boscalid or cyprodinil applied alternately and each strategy was followed by BCAs applications until fruit harvest; Combined (COMB), in which the biocontrol yeast PT22AV or Serenade Max® were applied in combination with a low dose (25% of the full label dose) of the synthetic fungicides Cantus® or Chorus® (alternately) in all treatments; Chemical, in which the chemical fungicides Cantus® and Chorus® were alternately applied at low (25% of full dose) (FLD) or at full label dose (FFD) in all treatments. For each BCA (BCAY or BCAB) and for each year, means showing different letters or marked with an asterisk are significantly different according to Tukey's multiple comparison test ($p < 0.05$) and Uncorrected Fisher's LSD comparison test ($p < 0.05$), respectively.

In both years and for all the six disease management strategies (BIO1, BIO2, INT-1, INT-2, INT-3, and COMB), the application of biocontrol yeast PT22AV significantly reduced the incidence of PBR compared with untreated control, with disease incidence reductions ranging from 70.2% to 95.6% and from 69.7% to 97.5%, in the first and second year, respectively. PT22AV achieved the highest PE when applied alone for seven consecutive times in the BIO2 strategy (PE 94.8–97.2% in the first and second year, respectively); conversely, when the BCA was applied only five times (BIO1), the PE was significantly lower than the BIO2 strategy (PE 70.2% and 69.7% in the first and second year, respectively). In the integrated (INT) disease management strategies, INT-3 exhibited the highest PE (95.5% and 97% in the first and in the second year, respectively), whereas INT1 and INT2 were less effective than INT3 and BIO2 in both years. Nevertheless, the effectiveness of INT1 and INT2 can be considered satisfactory. Last, the COMB strategy that involved *P. terrestris* PT22AV combined with fungicides reduced the disease incidence by 84.7% to 79.7% in the first and in the second year, respectively.

The different strategies involving the commercial biofungicide Serenade® yielded similar results to those with biocontrol yeast, although in general *P. terrestris* PT22AV yielded a higher PE, and in some strategies (BIO2 and INT1) this difference was statistically significant (Figure 2).

3.5. Fungicide Residues in Plum Fruits

The residues of BOSC and CYPR, determined in the plum fruits subjected to the different treatments just after harvesting in the plum orchard, are shown in Figure 3. In plum fruits from plants treated with full and low dosage of fungicides alone the following two-years mean values were recorded: 452.5 and 92.5 µg/kg for BOSC; 60.0 and 10.5 µg/kg for CYPR (Figure 3). The level of residues detected in the fruits treated with fungicides applied at full dose was much higher than that detected in the fruits from plants subjected to Biological, Integrated, or Combined strategies. In the case of Integrated control strategies (INT) in which the fruits were treated one (INT1), two (INT2), or three consecutive times (INT3) with fungicides (BOSC or CYPR alternately) at full dose followed by the application of the biocontrol yeast PT22AV, the two years' mean values of both fungicides were 11.0, 19.5, and 50.0 µg/kg for BOSC, and 1.5, 2.7, and 2.9 µg/kg for CYPR.

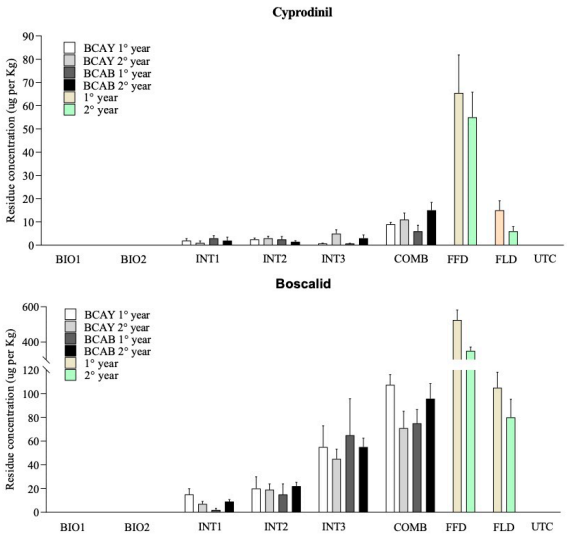

Figure 3. Residues of the fungicides boscalid and cyprodinil (µg/kg) in plum fruit after two years of field trials in plum orchard in which the plants were treated following six different disease management strategies: Biological, in which the BCAs (the yeast *Papiliotrema terrestris* PT22AV (BCAY) or the commercial biofungicide Serenade Max® based on *Bacillus subtilis* strain QST 713 (BCAB)), were applied alone in five (BIO1) or seven (BIO2) treatments; Integrated, INT1, INT2 and INT3, consisted of the application from the beginning of the growing season of one, two or three treatments respectively with the chemical fungicides boscalid or cyprodinil applied alternately and each strategy was followed by BCAs applications until fruit harvest; Combined (COMB), in which the biocontrol yeast PT22AV or Serenade Max® were applied in combination with a low dose (25% of the full label dose) of the synthetic fungicides Cantus® or Chorus® (applied alternately) in all treatments; Chemical, in which the chemical fungicides Cantus® and Chorus® were alternately applied at low (25% of full dose) (FLD) or at full label dose (FFD) in all treatments. Bars represent the standard deviation from the mean.

Regarding the combined strategy (COMB), of the mean values of residues of both fungicides of the two years were 91.3 µg/kg for BOSC and 10.0 µg/kg for CYPR. As expected, in the biological control strategy (BIO) performed with the BCA PT22AV applied alone and in untreated plum fruits, no BOSC and CYPR residues were detected.

3.6. Biocontrol Trials in Postharvest

Postharvest spraying of the two BCAs (PT22AV or Serenade Max®) on representative samples of fruit harvested from plum trees that underwent the different disease management strategies, reduced PBR incidence compared with untreated fruits (Figure 4). Overall, the highest degree of postharvest disease reduction was achieved by applying PT22AV and Serenade® on plums harvested from BIO2 and INT3 treatments. Specifically, the disease reduction in postharvest ranged from 65% to 84% and from 63% to 91% on plum fruits deriving from the field management strategies BIO2 and INT3, respectively, treated in the orchard with the BCA PT22AV; and from 43% to 81% and 59% to 83% on plum fruits deriving from field management strategies BIO2 and INT3, respectively, treated in the orchard with the commercial bioformulate Serenade®.

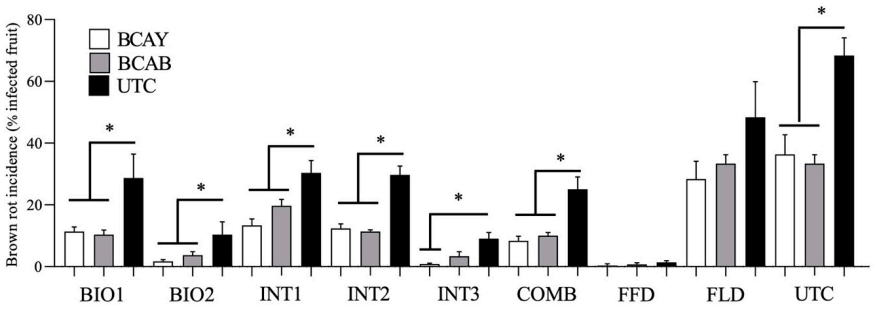

Figure 4. Postharvest brown rot incidence on plum fruits that were sprayed, immediately after harvest, with cell suspensions of either the BCA *Papiliotrema terrestris* PT22AV (BCAY) or the commercial biofungicide Serenade Max® based on *Bacillus subtilis* strain QST 713 (BCAB). The disease incidence was assessed after 15 days of storage at room temperature. For each treatment, the efficacy was compared with the respective postharvest untreated control (UTC). The columns marked with an asterisk are significantly different according to Uncorrected Fisher's LSD comparison test ($p < 0.05$).

All the strategies applied in the field produced significant reduction in brown rot incidence (Figure 5) even in the postharvest phase compared with the untreated field control. In particular, the rot average incidence in plums picked from the untreated field control was 68%, while for the most effective field strategies, i.e., fungicide at full dose (FFD), BCAY in BIO2 and INT strategies and BCAB in INT3 strategies the brown rot incidence was 1.3%, 10.3%, 9.0%, and 19.3%, respectively.

Figure 5. Plum fruit with the typical symptoms of brown rot.

4. Discussion

This study reports the results of two-year experiments aiming to evaluate new Biological and Integrated strategies to control plum brown rot (PBR) caused by *Monilinia fructigena*. Currently, the control of PBR is mainly based on an intensive use of synthetic fungicides. However, ecotoxicological issues related to the use of pesticides have led to increasingly restrictive regulations on their use and reduction in maximum tolerable chemical residues in fruit and into the environment [3,4,59]. Therefore, new appropriate management approaches are urgently necessary to obtain fruit with low or zero pesticide residues and safeguard the environment. On the other hand, these new approaches should also guarantee effective fruit protection from fungal diseases. In this regard, the timing schedule of fruit health management is crucial and must take into consideration the insidious life cycle of *Monilinia fructigena*. Indeed, application of biocontrol agents BCAs at appropriate stages of the fruit production chain has proved to be a valid alternative for the control of phyllosphere and carpoplane plant pathogens on many crops, both in the field and in the postharvest stage [2,34,55,60–62]. For microbe-based biocontrol to be effective, the timely colonization of the fruit surface by antagonistic epiphytic microorganisms plays a decisive role in outcompeting pathogens. Therefore, the application of these beneficial microorganisms must precede the settlement of the pathogen and its penetration, which occurs through natural or artificial openings [1].

Biological control relies on the use of bacteria, fungi, and yeasts whose high antagonistic activity is based on several mechanisms of action, such as competition for nutrients and space, secretion of cell-wall degrading enzymes and/or antifungal compounds, direct parasitism, and induction of resistance in host plant tissues [63]. Many synthetic fungicides operate through mechanisms of action targeting the products of one of few fungal genes [64]. Conversely, the multifaceted mode of action of BCAs can reduce the likelihood of the onset of resistant pathogen strains.

Although many effective BCAs have been formulated and commercialized [31,54], quite a few of them have provided swinging protection in practical conditions and do not ensure the same level of protection and/or reproducibility over time as compared to chemical fungicides [10,24,55,60]. PBR caused by *Monilinia fructigena* is an insidious disease and the most important economically for plum fruits worldwide because the pathogen is capable of infecting immature fruit, with infections decisive for the establishment of latent infections, as well as mature fruit, in the most sensitive phenological phase just before harvest. In this regard, the positive relationship between the number of conidia on the fruit surface and the incidence of latent infections in orchards was observed by many authors [12,13,19,20]; indeed, the presence of pathogen conidia on fruit surface in the presence of high humidity is the ideal combination for all the infectious phases (adhesion of the conidia to the cuticle, germination, formation of appressorium, and hyphae penetration) preceding the onset of latent infections [5,10,12]. Therefore, the key time to preventively protect the sites (e.g., natural and/or artificial openings) preferentially used by the pathogen to penetrate fruit tissues is when favorable humidity conditions and a high inoculum of the pathogen occur at a high infection risk phenological phase. Consequently, only a specific disease management model based both on the right strategy (Biological and/or Chemical) and on the right application timing, can protect from *Monilinia* infections.

In different studies, an integrated approach for plant health management based on the combined use of BCAs and synthetic fungicides proved to be effective in controlling different fungal diseases [2,10,34,53].

In the present research, we report the results of a two-year investigation carried out in Central Italy to compare the effectiveness of Biological (BIO), Integrated (INT), and Combined (COMB) approaches in controlling PBR, while minimizing the use of synthetic fungicides and of their residues on plum fruits. We tested plum health management strategies that were based on five key treatments (except for the BIO2 strategy, in which two additional treatments were performed only in the critical epidemiological phase) with the biocontrol agent *P. terrestris* PT22AV and the commercial biofungicide Serenade Max®

based on the bacterium *Bacillus subtilis* strain QST 713 (Bayer Crop Science). The two BCAs were applied alone (BIO1 and BIO2 strategies), or after the synthetic fungicide applications (INT1, INT2, and INT3 strategies), or in combination (COMB strategy) with low doses (25% of label dose) of the synthetic fungicides CYPR or BOSC.

All three strategies (BIO, INT, and COMB) significantly reduced PBR severity compared to the untreated control. Nevertheless, due to the insidious behavior of the pathogen *M. fructigena*, BIO1, COMB, INT1, and INT2 strategies led to a disease reduction that was lower than the highest level of protection achieved with full dose of synthetic fungicides (FFD). In both years, a satisfactory level of disease protection was obtained only with the BIO2 and INT3 strategies, in which the BCA PT22AV was applied alone for seven consecutive times (BIO2) or two times after three treatments with fungicides (INT3) at full dose (Figure 2).

Interestingly, in both years the two additional treatments with the BCA PT22AV (BIO2 strategy) at the key phenological phase with high risk for latent infections (77–85 BBCH) were crucial for a further significant reduction in the disease severity compared with BIO1 (Figure 2). Indeed, the surveys on the dynamics of the population of the antagonist applied, showed that the population of the BCA PT22AV in BIO2 was significantly higher than in BIO1 (Figure 1). As reported in previous studies, the 77–85 BBCH phase is the most important for latent infections because it is characterized by the coincidence of wetting of the surface of the plum fruit, natural wounds (lenticels and cracking), and the presence of the pathogen inoculum. Therefore, the other indirect but pivotal aim of the research was also to evaluate the efficacy of the BIO, INT, and COMB strategies in reducing latent infections. Indeed, the assessment of the population dynamics of white yeasts showed that these microorganisms were significantly more abundant in BIO2 than in BIO1 (Figure 1). The confirmation of this result is the level of reduction in the PBR achieved in the INT3 approach (strategy used as a chemical control until 81 BBCH), comparable with that in BIO2, in which, in the same key phenological period (75–81 of BBCH) for latent infections, the fruit is protected with an effective fungicide treatment at full dose. The differential and very useful results recorded between BIO1 and BIO2 depend on the degree of colonization of the plum carpoplane by the BCA applied (Figure 1), as also found in other studies [2,33,42]. In BIO2 strategy, compared with the other approaches (BIO1, INT1, INT2, and COMB), the high level of fruit surface colonization by the BCA results in a better protection of wounds through which the pathogen enters into the fruit tissues. This agrees with other research that biocontrol microorganisms, if adequately applied, can effectively colonize unwounded and wounded fruit surfaces, and can protect fruit wounds used by necrotrophic fungal pathogens as *M. fructigena* to penetrate and infect the host [1,16,17]. In particular, wound colonization by a BCAs is decisive for the control of brown rot of stone fruits, especially in critical phenological phases (75–81 of BBCH, i.e., from fruit about half final size to fruit ripe for consumption) in which these fruits are susceptible to frequent occurrence of cuticular cracking [1,5,16,17,65]. Several proteomics/transcriptomic studies have well demonstrated the defense activation in host tissues by different species of yeast BCA when they come into contact with the injured surface of the fruit [55,60,66–68]; therefore, in a necessary perspective of preventive management of this harmful disease, an application of BCAs even before wounds from cracking occur, would allow the BCA cells to colonize the wounds before the pathogen and the wound tissues strengthening from a histological view point towards *Monilia* spp.

Moreover, PBR incidence in the postharvest stage of the fruits that underwent the different experimental indicates that the treatments with good effectiveness levels in the field can effectively contain PBR also in postharvest. In addition, a further application of the two BCAs significantly improves the shelf life of plums. *P. terrestris* strain PT22AV applied after the harvest in the BIO2 and INT3 field strategies reduces the infection to levels that are comparable with those obtained by the fungicide applied at full dose in the orchard (Figure 5).

The Importance of consumer safety also raises health concerns related to the toxicity of fungicide residues that persist in fruits during the postharvest stages up to the final consumer. In the present study, in the integrated (INT1, INT2, INT3) and in the combined strategies (COMB), in which the fungicides were applied at the early phenological stages or low doses were applied in combination with the BCAs, the residues of fungicides in plum fruits were significantly lower than in fruit treated with fungicides at full dosage. As expected, no residues of either BOSC or CYPR were detected in BIO1 and BIO2. The residue levels of CYPR in the fruits were much lower than those recorded for BOSC, most likely because the last CYPR treatment was performed before the one with BOSC (30 days versus 7 days before harvest).

In summary, our work evidenced that new and most appropriate biocontrol and integrated strategies can be designed for phytosanitary management and for prevention of PBR. Such strategies are not only effective against fungal disease, but also drastically reduce or even eliminate fungicide residues in stone fruit. To achieve these objectives, the targeted application of biocontrol agents, such as in BIO2 strategy of this research, should consider the following key aspects: (i) the phenological phases of the fruit tree (i.e., those that are critical for the occurrence oof latent infections), (ii) the lifestyle of the necrotrophic fungal pathogen and (iii) the environmental conditions (e.g., humidity). Considering the behavior of the pathogen *M. fructigena* and the importance of the fruit wounds for latent infections in a specific phase, an effective BCA must be applied in a preventive way to "occupy" the gates of the pathogen penetration. Although chemical control contributes to prevent food losses and addresses the problem of food security, its negative side effects have generated a growing demand for healthier foods. To reduce or eliminate the use of synthetic fungicides in agriculture while maintaining an economically sustainable level of fruit protection from the pathogen, it is necessary to replace these products with targeted strategies on a case-by-case basis, considering together the (i) pathosystem, (ii) type of BCA and (iii) environmental conditions.

5. Conclusions

The main results of this research highlight the following:

- The high antagonist activity of the yeast *P. terrestris* PT22AV as a biological control agent (BCA) against *Monilinia fructigena*, applied alone (BIO2) and applied after (INT3) treatments with synthetic fungicides;
- The phenological phase 77–81 BBCH for plums is a vulnerable period for the entry of the pathogen;
- Decisive is the timing of the application of BCAs in the key phenological phase for brown rot as well as the entire control strategy, for the containment of this insidious pathogen;
- The plum fruits harvested from plants treated with the BIO strategy have zero residues of synthetic fungicide.

In conclusion, considering the results obtained in this research, it emerges that in organic farming it is appropriate to use a control strategy based on the application of BCAs, paying attention to the critical periods for the onset of latent infections, and carrying out a sufficient number of treatments (as in the BIO2 strategy). On the other hand, in integrated agriculture, this objective can be overcomed by using synthetic fungicides from flowering to the critical phenological period for the disease, and then continuing up to the pre-harvest phase with BCA-based treatments (as in the INT3 strategy) in order to reduce, even to zero, the residues of synthetic fungicides.

Author Contributions: D.P., G.L., G.I. and F.D.C. designed the experiments and methodology. D.P. and F.D.C. carried out the experiments. D.P., G.L., G.I., T.C., R.C. and F.D.C. analyzed the data. G.L., G.I., R.C. and F.D.C. acquired funds. D.P., G.I. and F.D.C. wrote the manuscript (MS). G.L., R.C. and F.D.C. revised and edit MS. F.D.C. supervised the MS. All authors have read and agreed to the published version of the manuscript.

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