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Effectiveness of Four Synthetic Fungicides in the Control of Post-Harvest Gray Mold of Strawberry and Analyses of Residues on Fruit

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Abstract: Fungicides are usually applied on strawberries to manage gray mold, induced by the fungal pathogen *Botrytis cinerea*. In this study, four reduced-risk fungicides (formulations of pyrimethanil, PYR, 175 mL/hL; boscalid, BOS, 80 g/hL; combination fludioxonil, FLU, +cyprodinil, CYP, 110 g/hL) were applied before harvest for the management of post-harvest diseases of strawberries. The resulting fungicide residues on the strawberry fruit were also quantified. Strawberry fruits were harvested at 0, 4, 8, and 12 days following treatment (dft) and kept at 20 ± 1 °C for 4 days or cold-stored for 7 days at 0.5 ± 1 °C, followed by a 4-day shelf life at 20 ± 1 °C. All fungicides significantly reduced gray mold, according to the McKinney Index. At 0 dft and 4 days of shelf life, the FLU + CYP completely prevented post-harvest strawberry gray mold, while PYR and BOS reduced the disease by 88% and 42%, respectively, in comparison to the untreated control. For the duration of experiment, fungicide residues were always below the maximum residue levels, and FLU was the most degraded, thanks to the enzymatic pool of the strawberries. Monitoring fungicide residues in strawberries is essential to provide the consumer information on the safety of this widely consumed fruit. The present study points out the safety of strawberry fruits for consumers, even if the treatment strategy implies the use of fungicide mixtures before the consumption, with fungicide levels always being below the MRL.

Keywords: *Botrytis cinerea*; *Fragaria* × *ananassa*; fungicide residues; post-harvest decay; maximum residue level

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1. Introduction

Strawberry (*Fragaria* × *ananassa* Duch) is a fruit with many health benefits, due to its phytonutrients and vitamins, which can promote a strong defense against degenerative diseases [1]. Strawberry is one of the most economically important fresh and processed fruits, cultivated all over the world, that is consumed for its pleasant aroma [2]. However, strawberry is a perishable fruit that easily undergoes spoilage by *Botrytis cinerea* after harvest, limiting its commercialization and consumption [3–9]. *B. cinerea* is a necrotrophic phytopathogen considered the second most important fungal pathogen worldwide [5,10]. To prevent post-harvest gray mold, chemical control remains the primary strategy [11]; in particular, fungicides are sprayed on the strawberry canopy throughout the season, from flowering to harvest [12,13]. The use of pesticides results in several advantages, such as high efficiency, simplicity of employment, inexpensiveness, better yields, and better quality [14,15], but synthetic pesticides could represent a risk to environmental fate and food safety [16,17]. Residues in food may cause side effects on the human body, e.g., allergies [18]; chronic diseases, such as asthma, diabetes, leukemia, and Parkinson's disease [19,20]; or cancer [21]. Moreover, retailers increasingly require fruit and vegetables with a limited amount of pesticide residues [22].

The EC regulation 396/2005 adopted in 2005 on pesticide residues marked the turning point, as it consolidated and replaced all the old national maximum residue limits (MRL) by their harmonization throughout Europe [23]. The regulation above and more specific attention to public opinion [24–26] have directed research towards pesticides, which can ensure rapid foodstuff degradation and good disease control. In the last years, some reduced-risk fungicides were introduced to effectively manage pre- and post-harvest diseases of fruits [27–29]; moreover, these fungicides ensure low environmental impacts and human health risks [30–32]. Several reduced-risk fungicides have extremely low mammalian toxicity [33] (LD_{50} values from 2000 to more than 5000 mg kg^{-1}) and are not known or suspected to be carcinogens [28]. The effectiveness of reduced-risk fungicides at controlling post-harvest diseases in fruit crops has been demonstrated in many reports [27–29,34], and practices such as mixtures and rotations can be implemented to prevent resistance from developing and to ensure the lasting efficacy of these reduced-risk fungicides [35–38]. The amounts of residues found in food must be safe for consumers and must be as low as possible, with respect to the maximum residue level (MRL), that is, the highest level of a pesticide residue legally tolerated in or on food or feed [39]. Strawberry is the crop where the highest pesticide residues have been found for many years in the USA. The "Dirty Dozen" is the list of the twelve crops for which pesticides are detected at higher levels in a report from the Environmental Working Group of USDA (United States Department of Agriculture) [40], and the strict control of pesticide residues in these fruits is recommended. Several studies highlighted the presence of pesticide residues in strawberry fruits destined for consumption [41–43], in some rare cases exceeding the European Commission's fixed MRL for all food and animal feeds. Also, in the strawberry-processing by-products, high pesticide residues were found. Sojka et al. [44] found high levels of pesticide residues in three strawberry by-products, 96% of which were fungicides, even higher than the pesticide concentration in fresh fruits, while El-Sheik et al. [45], spiking tomato and strawberry samples with 16 pyrethroid insecticides, found that the home-processing of the two fruit types (juice and jam) showed a complete residue reduction of 100% in strawberries. The MRL for all crops and all pesticides can be found in the MRL database on the Commission website (https://food.ec.europa.eu/plants/pesticides/eu-pesticides-database_en accessed on 1 September 2023). A previous investigation conducted in Poland from 2005 to 2014, which tested the presence of over 70 fungicides, found dithiocarbamates (27.4%) and captan (26.3%) to be among the most often detected [41]. The percentage of samples in which exceedances of the MRL of fungicides were observed fell within the range of 0.8–2.6%.

In recent years, tentative efforts have been made to reduce pesticide residue concentrations in strawberry fruits through removing techniques, such as washing, ozone, boiling, and ultrasonic cleaning. The last technique resulted in the more effective reduction in residues at over 91% [46].

The above considerations strengthen the research about pesticide residues in strawberries, mainly used against fungal disease at harvest and post-harvest managements. The use of reduced-risk fungicides can contribute both to low residues in fruits destined for consumption and cause low levels of environmental risk. In the present study, we compared the effectiveness of four reduced-risk-fungicides in three different treatments (pyrimethanil, PYR; boscalid, BOS; fludioxonil, FLU + cyprodinil, CYP) applied on the canopy for the control of the post-harvest gray mold of strawberries. The consequent fungicide residues were also detected on the strawberry fruit in different storage conditions (refrigerated or not refrigerated) to asses if residues were present at concentrations exceeding the MRL at any moment of conservation. Control of pesticide residues in fruits destined for consumption was managed through random sampling with statistical relevance, and it could be possible that a considerable number of fruit stocks could become out of control. This investigation was carried out to assess how crucial it is to control fungicide residues in strawberry fruits, which require frequent chemical treatment for fungal diseases, during post-harvest management to guarantee food quality and safety.

2. Materials and Methods

2.1. Field Experimental Trial

The trials were run in an experimental strawberry field, cv 'Alba', located in central-eastern Italy (Agugliano; 43°31′60″ N, 13°22′60″ E). Strawberry plants from plantlets were grown in the field using the plastic hill production system in twin rows 30 cm apart, with plantlets at intervals of 30 cm and with each twin row separated by 1 m from the next, as reported by Feliziani et al. [12]. Throughout the season, a drip system was used to irrigate the plants. Before the treatments, the strawberries were labeled according to the phenological stage. The strawberries were sprayed with a volume equivalent to 500 L/ha using a motorized backpack sprayer (GX 25, 25 cm^3, 0.81 kW; Honda, Tokyo, Japan) on 15 May 2016 with commercial formulations and then collected at different times until 27 May 2016. Treatments used cyprodinil (CYP) + fludioxonil (FLU) (Switch, 37.5 + 25, Syngenta S.p.a., Milan, Italy), boscalid (BOS) (Cantus, 50, BASF S.p.a., Cesano Maderno, Italy), and pyrimethanil (PYR) (Scala, 37.4, Bayer S.p.a., Leverkusen, Germany), with applications at doses of 110 g/hL, 80 g/hL, and 175 mL/hL, respectively. The choice to apply the mixture of FLU and CYP was motivated by the proven efficacy in controlling *B. cinerea* [47,48]. The commercial formulation Cantus was chosen because it was the only one containing BOS, an ingredient authorized in Europe for strawberries, although not registered alone for the use on strawberries. The water treatment was used as a control. No gray mold symptoms were observed on the fruits in the field. The strawberry fruits were harvested at zero (T0), 4 (T1), 8 (T2), and 12 (T3) days following treatment (dft). The experimental field was designed with a randomized block design with four replicates, and each replicate consisted of six strawberry plants. At harvest, only ripe fruits in each plot that had the labels on the stems (previously placed during the flowering) and that were red over ≥2/3 of their surface were picked to be sure they received all the treatments from flowering to maturity. From the fruits thus collected, only those with uniform color and shape and absence of defects were selected. The fruits harvested from each plot were randomly split into groups of six fruits and placed into large, covered boxes. A layer of wet paper was placed at the bottom of the large boxes to keep humidity close to saturation (98%) during storage. The strawberry fruits were divided into two sets, one kept at room temperature (RT) at 20 ± 1 °C for 4 days and the other maintained at cold storage (CS) at 0.5 ± 1 °C for 7 days, and then exposed to 4 days of shelf life at 20 ± 1 °C and 98% relative humidity. Fungicide residues were measured at each sampling time, both for fruit kept at room temperature and for cold-stored samples, with three replicates per sample for each of the six strawberry fruits.

2.2. Decay Evaluation

During storage at room temperature or shelf life, the number of strawberry fruits with gray mold symptoms coming from natural infections was recorded, and then the disease incidence was calculated. Disease severity was also recorded, according to an empirical scale with six degrees (Figure 1): 0, healthy fruit; 1, 1–20% fruit symptomatic surface; 2, 21–40% fruit symptomatic surface; 3, 41–60% fruit symptomatic surface; 4, 61–80% fruit symptomatic surface; and 5, ≥80% fruit symptomatic surface and showing sporulation [12]. The empirical scale allowed the calculation of the McKinney's index, expressed as the weighted average of the disease, as a percentage of the maximum possible level [49]. This parameter included information on both disease incidence and disease severity.

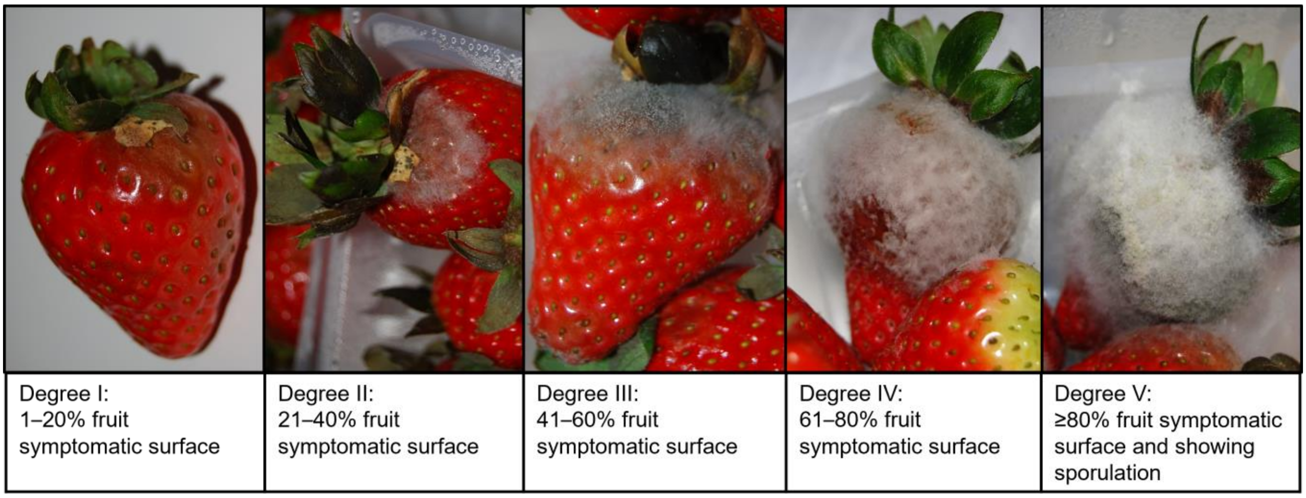


Figure 1. Strawberries with the 5 degrees of gray mold disease severity. Disease severity was recorded on the strawberries according to an empirical scale with six degrees: 0, healthy fruit; 1, 1–20% fruit symptomatic surface; 2, 21–40% fruit symptomatic surface; 3, 41–60% fruit symptomatic surface; 4, 61–80% fruit symptomatic surface; and 5, $\geq$80% fruit symptomatic surface and showing sporulation.

2.3. Fungicide Extraction and Analyses

Analytical standards (>99% purity) of all pesticides, used as external standards and to build calibration curves (0.1, 0.25, 0.5, 1, 2.5, and 5 mg L^{-1}), were purchased by Dr. Ehrenstorfer GmbH, Augsburg, Germany. All other reagents and solvents used were of analytical grade (Sigma-Aldrich, St. Louis, MO, USA). Fungicides from strawberry samples were extracted using a kit supplied by Phenomenex (KSO-8910 Phenomenex RoQ-QuEChERS, Bologna, Italy), combined with the purification method [50]. Briefly, starting from 10 fruits, 10 g of blended and homogenized strawberries (three replicates from three fruit lots) was added with 10 mL of acetonitrile, 4 g of $MgSO_4$, and 1 g of NaCl, shaken for 10 min, and centrifuged at 4000 rpm for 5 min. Aliquots (6 mL) were added to d-SPE (solid phase), shaken for 5 min, centrifuged at 4000 rpm for 5 min, and subsequently analyzed by HPLC. The HPLC analyses were performed using a Perkin Elmer 200 Series chromatograph equipped with a UV detector at 205 nm and Supelcosil C18 column (25 cm $\times$ 4.6 mm, i.d.) (Perkin Elmer Italia, Milano, Italy). The mobile phase was acetonitrile/water, 70/30, and the flow was 0.8 mL min^{-1}. Under these conditions, the retention times of the four fungicides were 9.2 min for FLU, 12.4 min for CYP, 5.1 min for PYR, and 4.8 min for BOS.

2.4. Statistical Analysis

The data were subjected to analysis of variance according to a randomized block design. All the data were first tested for normality according to the Shapiro–Wilk test. If the normal distribution of the data was not confirmed, even after arcsin square root transformation, non-parametric tests were applied using the Kruskal–Wallis test, followed by the Mann–Whitney test to determine differences among the strategies. The parametric data were tested for homogeneity of variance according to the Levene test ($p \leq 0.05$). If the homogeneity was confirmed, one-way ANOVA analysis was performed, and averages were separated according to the Fisher LSD test or Tukey's honestly significant difference (HSD) tests at $p \leq 0.05$ (Stat-soft, Tulsa, OK, USA). When the homogeneity of variance was not confirmed, Welch's ANOVA was performed, and means were separated using the Games–Howell post hoc test ($p \leq 0.05$). The linearity of the analytical method was evaluated by calculating a five-point linear plot with three replicates based on linear regression and the correlation coefficient (R^2).

3. Results

3.1. Decay Evaluation

The pre-harvest treatments with conventional fungicides reduced the development of gray mold on strawberries, as compared to the control in all tested conditions. The effectiveness of tested fungicides on disease control was usually high, with disease incidence, severity, and McKinney index significantly reduced by all fungicide applications. During shelf life, fruits were visually inspected daily and scored for gray mold symptoms. As representative results, we report the data of the McKinney index in strawberry fruits, cv Alba, sprayed and harvested soon after (0 dft), cold-stored for 7 days at 0.5 ± 1 °C, and then exposed to 4 days of shelf life, where gray mold was significantly reduced by all fungicide treatments, as compared to the control (Figure 2).

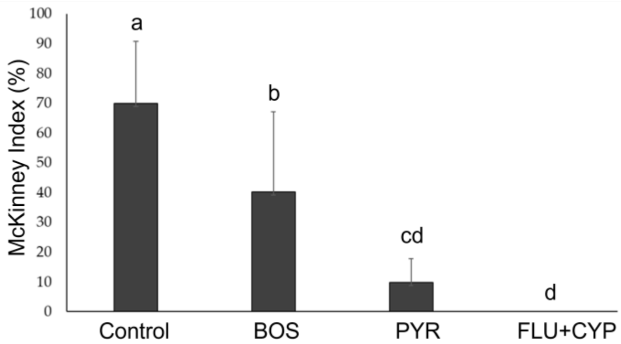
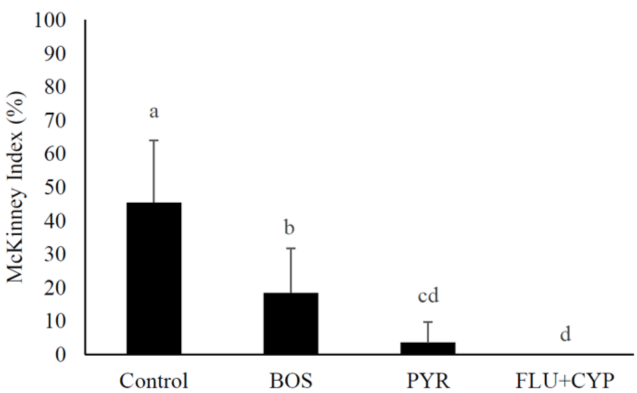
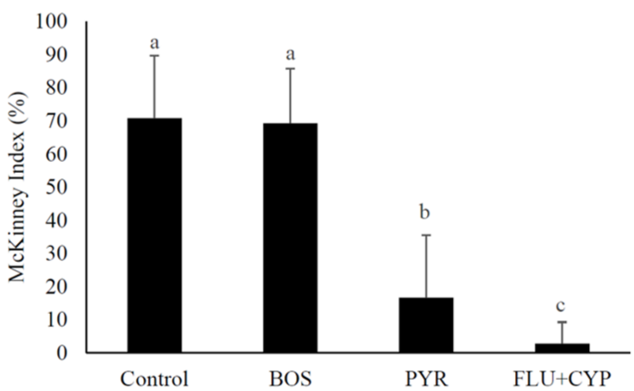


Figure 2. McKinney index of gray mold of 'Alba' strawberry fruit treated with fungicides, then collected soon after (0 dft), stored for 7 days at 0.5 ± 1 °C, and then kept for 4 days shelf life. BOS, boscalid; PYR, pyrimethanil; FLU + CYP, fludioxonil + cyprodinil. According to the Fisher LSD test, treatments with different lowercase letters were significantly different at $p \leq 0.05$. Bars above columns represent the standard deviation.

The treatment with CYP + FLU completely prevented post-harvest gray mold, while PYR and BOS provided 88% and 42% disease reduction in the McKinney index, as compared to the control, respectively. Similar results were recorded on the same fruits exposed to 3 and 5 days of shelf life (Figure 3a,b); on fruits harvested at 4 dft, cold-stored for one week at 0.5 ± 1 °C, then exposed to 3 days of shelf life (Figure 3c); and on fruits harvested at 8 dft, cold-stored for one week at 0.5 ± 1 °C, then exposed to 4 days of shelf life (Figure 3d). Overall, the highest gray mold reduction was consistently provided by the application of the combination of CYP + FLU, followed by PYR and by BOS; in all assessments (except for BOS at 0 dft exposed to 5 d of shelf life), they significantly reduced the infections, as compared to the control.

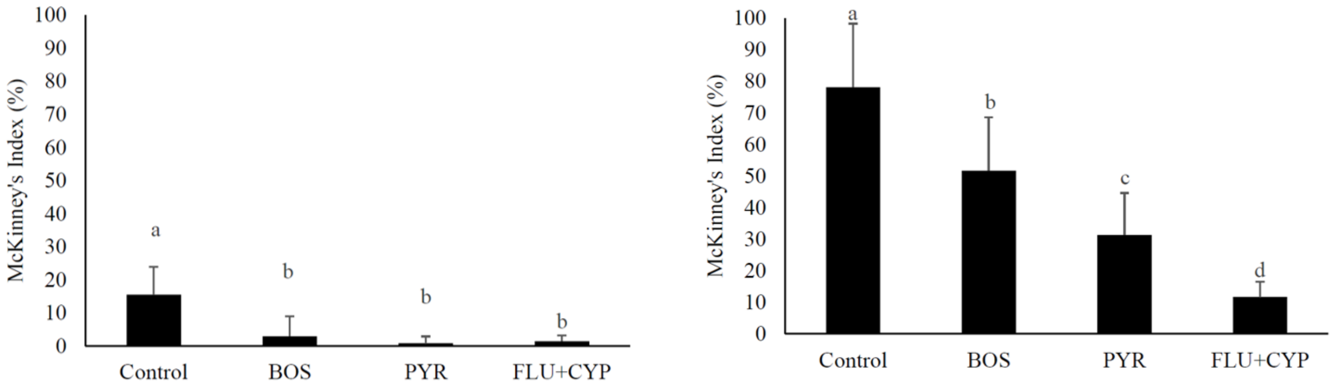


(**a**)



(**b**)

Figure 3. *Cont.*

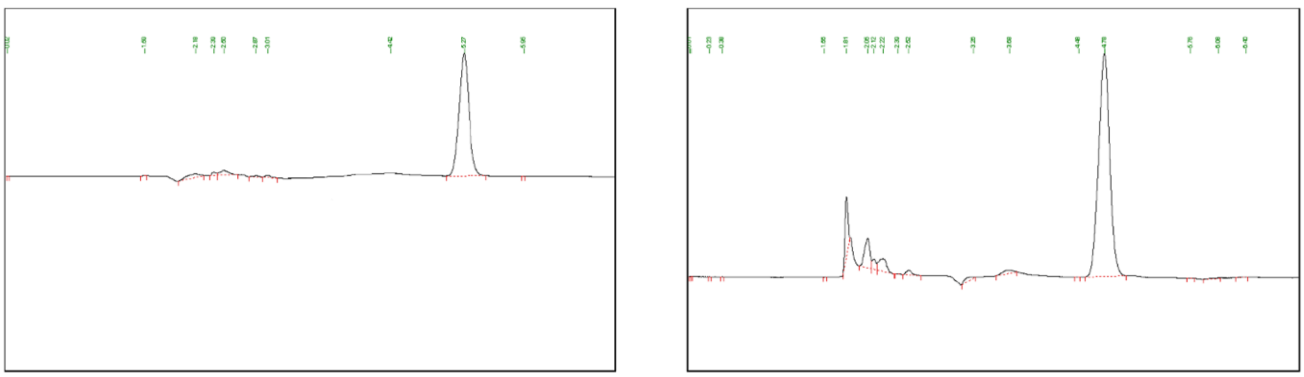


(**c**) (**d**)

Figure 3. McKinney index of gray mold of 'Alba' strawberry fruits treated with fungicides, then collected at different dfts and exposed to different days of shelf life: collected soon after (0 dft), stored for 7 days at 0.5 ± 1 °C, and then exposed to 3 (**a**) and 5 (**b**) days of shelf life; collected at 4 dft, stored for 7 days at 0.5 ± 1 °C, and then exposed to 3 days of shelf life (**c**); and collected at 8 dft, stored for 7 days at 0.5 ±1 °C, and then exposed to 4 days of shelf life (**d**). BOS, boscalid; PYR, pyrimethanil; FLU + CYP, fludioxonil + cyprodinil. According to Fisher LSD test, treatments with different lowercase letters were significantly different at $p \leq 0.05$. Bars above the columns represent the standard deviation.

3.2. Validation of the Analytical Method

All fungicides showed linearity in the concentration range of 0.05–5.0 mg kg^{-1}, with correlation coefficients higher than 0.99. In fruit samples, mean recoveries spiked at three fortification levels, ranging from 90.1 to 102.9%. The limit of detection (LOD) was measured at a signal/noise ratio of 3/1, ranging between 10 µg kg^{-1} and 30 µg kg^{-1} for all four fungicides. Examples of chromatograms for the calculations of LOD for PYR and BOS are reported (Figure 4a,b).



(**a**) (**b**)

Figure 4. Chromatograms for the calculation of LOD (limit of detection) for pyrimethanil (**a**) and boscalid (**b**).

The LOQs (limits of quantification) measured at a signal/noise ratio of 5/1 ranged between 0.04 and 0.1 mg kg^{-1} for all four fungicides. When observing the results of LOQs, the validation parameters were suitable, and fungicides were satisfactorily quantified using these methods.

3.3. Fungicide Residues in Fruits

The presence of fungicide residues in strawberry fruits during the 12 days of the experiment is reported in Figure 5.

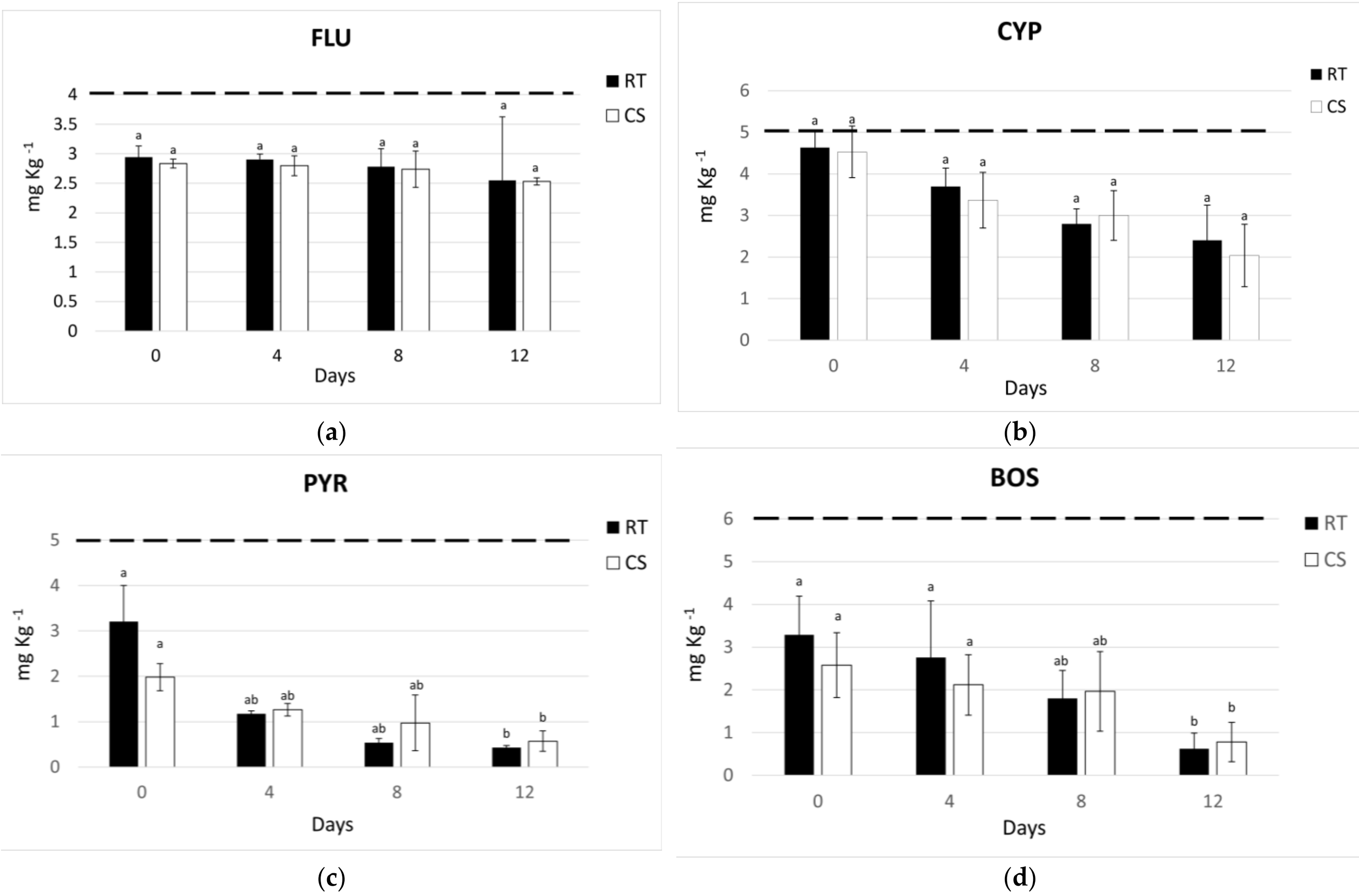


Figure 5. Fungicide residues that meet MRLs of strawberries in Europe. Dotter lines indicate MRL values. RT, room temperature; CS, cold storage; (**a**) FLU, fludioxonil; (**b**) CYP, cyprodinil; (**c**) PYR, pyrimethanil; (**d**) BOS, boscalid. Lower case letters refer to statistical differences within each fungicide. According to the HSD test, treatments with different lowercase letters were significantly different at $p \leq 0.05$. Bars above columns represent the standard deviation.

Looking at the residue levels found at a different time from fruit harvesting, FLU was the most persistent fungicide in fruits. Specifically, 86.7% of the initial concentration was still present in fruits immediately exposed to shelf life at 12 days after treatment, while 89.4% was measured in fruits kept for 7 days at 0.5 °C. The same data for the other fungicides were 51.9% and 44.8% for CYP, 13.4% and 28.8% for PYR, and 18.8% and 30.2% for BOS, respectively, for RT and CS.

4. Discussion

Despite the intensive efforts made to develop new strategies to control *B. cinerea*, such as horticultural management and a new genetic approach to induce resistance in strawberries, the use of pre- and post-harvest fungicides remain the best strategy to control this pathogen [5], provided that their residues remain below the MRL during the entire post-harvest period until consumption.

The effectiveness of these fungicides against *B. cinerea* was previously tested on strawberries or on other plant hosts [51–53]. However, resistances to multiple fungicides in *B. cinerea* have been reported in several strawberry production areas [11,54,55], resulting in reduced effectiveness toward the disease. In particular, previous studies related to resistance to fungicides of *B. cinerea* isolates from strawberries showed a lower level of resistant populations to both FLU and CYP + FLU, compared to BOS [56,57]. These previous investigations underline the importance of detecting the residues in the fruits to verify the contamination and human health risks.

The fungicides used have extremely low mammalian toxicity (LD_{50} values from 2000 to more than 5000 mg kg^{-1}) and are not known or suspected to be carcinogens. This is undoubtedly due to their selective mode of action, that is, BOS is a pyridinecarboxamide that inhibits succinate ubiquinone reductase (complex II) in the mitochondrial electron transport chain; PYR is an anylopyrimidine that inhibits the biosynthesis of methionine, affecting cystathionine β-lyase; FLU is a phenylpyrrole that inhibits the transport-associated phosphorylation of glucose, as well as prevents glycerol synthesis; and CYP is an anilinopyrimidine that inhibits the biosynthesis of methionine and other thionic amino acids. For this reason, the fungicides used in this experiment fall in the category of reduced-risk fungicides and prove to be the most valuable for the protection of fruits from post-harvest gray mold. Concerning fate and behavior, adsorption characteristics seem to be safe, considering that the $logP_{ow}$ values were always higher than 2.84, indicating a good adsorption potential for the four fungicides; K_{foc} data, reported as a range for different soils, had results higher than 500 mL g^{-1}, except for the PYR range, where 500 was the maximum value, corresponding to a mean capacity of adsorption on soil colloids and a minimum risk for environmental contamination. DT_{50} values, reported as a range of values measured for different soils with different physico-chemical characteristics, classify FLU and BOS in the "persistent" category, showing values ever higher than 100 days. The study of the persistence in strawberry fruits represents, in this case, valid information to ascertain if these two fungicides could cause problems for consumers' health, since their persistence in fruits could be similar to that in soils and exceed MRL. If we compare the results of the residues of FLU and CYP, as they are in the same formulation, as in the study of Fennol et al. [58], the first fungicide is more persistent than the second. With the exception of FLU and CYP, the other two fungicides show greater persistence in refrigeration tests (AFC), but this trend cannot be considered significant; it has been found in many other studies that have hypothesized how low temperatures slow down the degradation process of pesticides [58–63]. Looking at soil DT50 values, it would be expected that CYP should be the fungicide that disappears quickly from fruits, and FLU and BOS should be the fungicides that disappear later from fruits. The assumption was proved to be right for FLU, which resulted in it being the more persistent fungicide in fruits, as it is in soils; it was not confirmed for BOS, which showed a fast disappearance from fruits, similar to that in soils. PYR confirmed its low persistence in fruits, similar to that in soils, and CYP was more persistent in fruits than in soils. With pesticide degradation being a biological process, due to enzymatic pools present in the degradation substrate [59], it could be concluded that the different degradations in fruits, with respect to soils, could be due to enzymatic activities present in the fruits after harvesting, causing a faster degradation for BOS and a slower degradation for CYP. It is noted that the residues of the four fungicides measured in all strawberry fruits during the entire experimental period never exceeded the MRL values. The worst degradation condition in the enzymatic pool of the harvested fruits was found for FLU, while the other fungicides followed a more or less rapid degradation. Fungicide residues below MLR on the fruit are not dangerous for the consumer, and they protect the fruit during shelf life [64].

5. Conclusions

In conclusion, even if attempts to replace synthetic fungicides with natural fungicides and/or with some vegetable or microbiological extracts have been made in the last years [7,64–66], reduced-risk fungicides remain the most effective means to control the post-harvest gray mold of strawberries. FLU + CYP completely controlled *B. cinerea*, followed by PYR and BOS, in all conditions tested. PYR was the more degraded fungicide during the experiment, followed by BOS, while FLU and CYP degraded slowly but always remained under the MRL. The present study pointed out the safety of the strawberry fruits for consumers, even if the treatment strategy implied using fungicide mixtures before the consumption, with fungicide levels always below the MRL.

Author Contributions: Conceptualization, C.V. and G.R.; methodology, E.F. and L.L.; software, L.L.; validation, C.V., E.F. and L.L.; investigation, E.F., L.L., A.D.B. and E.M.; resources, C.V. and G.R.; data curation, L.L., E.M. and A.D.B.; writing—original draft preparation, C.V. and G.R.; writing—review and editing, C.V., G.R., L.L., E.M. and A.D.B.; visualization, L.L., E.M. and A.D.B.; supervision, G.R. and C.V.; project administration, C.V.; funding acquisition, G.R. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data presented in this manuscript is contained within the article.

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