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REGULAR ARTICLE

Differential susceptibility of Morettini pears to blue mold caused by *Penicillium expansum*

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ABSTRACT

Pear fruit (*Pyrus communis* L. cv Beurré Precoce Morettini) developing on the outside of canopy and exposed to sunlight acquires a bright redness on the exposed side, compared to the green peel fruit that develop within the canopy. At harvest, the main physicochemical parameters of the red and green side of the fruit, including firmness, brix and titratable acidity, were similar in pears harvested at the same day and stage of maturity. However, it was observed that fruits with a red side or that mostly green showed a different response to biotic stress, namely to pathogenic fungi attack. Actually, after 7 days under shelf-life conditions, red fruits demonstrated to be more resistant than green ones when challenged with a postharvest *Penicillium expansum* inoculation. Significant differences were observed in antioxidant activity and total phenolic compounds between both sides of the fruits. Several polyphenols related to mechanisms of induced resistance against fungal plant pathogens were identified in the extracts, such as catechin, quercetin, quinic acid or caffeoylquinic acid. These results suggest that the accumulation of phenolic compounds triggered by sun exposure may be one of the mechanisms responsible for the increased resistance of red-skinned Morettini pears.

Keywords: Antimicrobial properties; Antioxidant activity; Pathogenic fungi; Phenolic compounds; Fungal diseases

INTRODUCTION

'Beurré Precoce Morettini' pear is an Italian variety originated from the cross between 'Coscia x Williams' pears. The tree is vigorous in growth and fruits early and abundantly. Pears are harvested during the 3rd week of July and can be stored under refrigeration during a maximum period of 1- 2 months. Fruits are middle large, pyriform and have a very good quality. They have a juicy, crunchy and sweet white pulp with a sweet-spicy flavor. Fruit skin is thin, semi-hard and smooth, with a basic green-yellow coloration that can develop a bright redness on the side exposed to the sunlight.

This cultivar is susceptible to pests like psylla and scab, and extremely susceptible to bacterial diseases like fire blight (*Erwinia amylovora*) and blossom blast (*Pseudomonas syringae* pv. *syringae*). In the field, an interesting characteristic was observed; pears with a red side or that mostly green show a different response to biotic stress, namely to pathogenic fungi attack. The green side of the fruit seems to be more susceptible to fungal infections.

Host plant resistance against fungal pathogens involves multiple defense mechanisms that can be included into several categories such as physiological factors and passive or active defense responses (Lattanzio et al., 2006). Fruit physiological factors such as firmness, pH, sugar content and epicuticular waxes can affect the development of fungal pathogens (Alkan and Fortes, 2015).

Preformed phenolic compounds, like resorcinols or flavonoids may be particularly important in antifungal resistance. Moreover, active defense responses including induced antimicrobial compounds, defense-related proteins and reactive oxygen species may also be a key point in the fruit resistance against pathogens (Prusky and Keen, 1993; van Loon et al., 2006).

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This study aimed to explain the differences in the susceptibility/resistance to fungal infection observed in Morettini pears from the same orchard and at the same stage of maturity.

MATERIALS AND METHODS

Fruits

Beurré Precoce Morettini pears (*Pyrus communis* L.) grown in an orchard located in Alcobaça (west region of Portugal) were hand-harvested at the stage of commercial maturity and stored in a cold room at 1ºC and 90-95% RH until use.

For all the assays described throughout this work, pears were divided in 2 groups, in one group only the reddish part of the fruit was considered and in the other group only the greenish yellow part, hereafter called red and green, respectively.

Pathogen

The fungal pathogen *Penicillium expansum* was obtained from infected Rocha pears and maintained on potato dextrose agar (PDA). The pathogen was incubated at 25ºC for 7 days. Spore suspensions were prepared as described in Janisiewicz and Marchi, 1992.

Infection assays

Fruits were surface-disinfected with 0.5% sodium hypochlorite and artificially wounded twice (each wound 15 mm apart from the equator of the fruit and to a depth and a diameter of 4 mm), in the red or in the green part. The wounds were inoculated with 20µL of an aqueous suspension of *P. expansum* (10^4 and 10^3 conidia/mL). The infected fruits were placed in boxes and incubated at 20-22ºC (shelf-life conditions) during 7 days. The number of infected wounds and the diameter of the wounds were measured daily.

Skin colour

The colour of the fruit was determined with a Minolta chromameter (model CR-300, Data Processor 301, Minolta, USA). Three measurements were evaluated from each side of the fruits (red and green). The colour values were expressed by the CIELAB colorimetric system. The L* value was used as an indicator of brightness. The values a* and b* were used to determine hue angle (Hue^o = arc tan b*/a*). Hue values refers to a color wheel, with red-purple at an angle of 0º, yellow at 90º, bluish-green at 180º and blue at 270º (Mcguire, 1992).

Firmness

Firmness was measured by puncture, after skin removal, with a penetrometer (Penefel) fitted with an 8 mm diameter plunger. The firmness was expressed in kgf cm^{-2}.

Titratable acidity and total soluble solids

Pear samples were mashed and 20 mL of the pulp obtained was homogenized with 20 mL of distilled water. The homogenate was filtered and then titrated with 0.10 N NaOH to pH 8.2. The volume of NaOH added was used to calculate the titratable acidity (TA), applying a multiplication factor of 0.67. The results were expressed as g malic acid L^{-1} of juice.

The total soluble solids (TSS) content was determined by using a hand-held refractometer (Atago ATC-1E) at 20ºC. The results were expressed in °Brix (AOAC 2000).

Extract preparation

Extracts of pear were prepared following the method proposed by Swain and Hillis (1959). Briefly, 1 g of frozen sample (pulp and peel) was mixed with 5 mL of pure methanol (Merck), homogenized (Ultra-turrax apparatus) for 2 min and stored in darkness at 5ºC for 24h. Afterward, samples were centrifuged at 12000g at 10°C for 5 min. The initial supernatants were separated and the pellets were rinsed once with the extraction solution. The extracts were then concentrated under vacuum (30°C) until dryness. The residue was recuperated with purified water until a total volume of 1 mL. All experiments were carried out in triplicate.

These extracts were used for the determination of both antioxidant activity and phenolic content.

Total phenolic content and antioxidant activity

Phenolic compounds were determined according to Vieira et al. (2009) with minor modifications. The extract (0.25 mL) was mixed with deionised water (2 mL) and 0.25 mL of Folin-Ciocalteu reagent. After 5 min, 5 mL of a sodium carbonate solution (20%) plus 5 mL of distilled water were added and mixed well. One hour later, the absorbance was measured at 750 nm with an UV-VIS spectrophotometer (Shimadzu UV-160), using gallic acid (GA) as standard. The results were expressed as mg GAE 100 g^{-1} FW.

The antioxidant activity was determined by the DPPH method according to Shimada et al. (1992) with slight modifications. Extracts (0.1 mL) were mixed with 3.9 mL of a 0.05 mM methanolic solution of DPPH (2,2-diphenyl-1-picrylhydrazyl radical) and shaken vigorously. The reaction mixture was allowed to stand for 30 min at room temperature in the dark. The absorbance of the mixture was determined at 517 nm using a UV-VIS spectrophotometer (Shimadzu UV-160). Trolox and ascorbic acid were used as standards. The antioxidant activity of pear extracts was expressed as mg trolox 100 g^{-1} FW and mg AAE 100 g^{-1} FW.

HPLC-ESI-MS/MS analysis of phenolic compounds

The identity of the phenolic compounds was confirmed by tandem mass spectrometry analysis. Two extracts were analyzed on an HPLC Dionex Ultimate 3000 composed of a binary pump HPG3200, an autosampler WPS300 and a column oven TCC3000 coupled in-line to a LCQ Fleet ion trap mass spectrometer equipped with an ESI ion source (Thermo Scientific). Aliquots of 10 µL were injected into the column via a Rheodyne injector with a 25 µL loop, in the partial injection mode. Separations were carried out with a Zorbax Eclipse Plus C18 (150 x 2.1 mm, 5 µm, Agilent Tecnologies) at 30 ºC, using a mobile phase of 0.1% of acid formic in water (v/v, eluent A) and acetonitrile (eluent B), and a gradient elution program of 0-2 min isocratic 5% B, 2-20 min linear gradient to 40% B, 20-25 min linear gradient to 90% B, 25-27 min isocratic 90% B, and 27-30 min linear gradient to 5% B (10 min reequilibration time). A flow rate of 0.8 mLmin^{-1} was used, and the LC eluent was introduced into the ESI source in a post-column splitting ratio of 3:1.

The mass spectrometer was operated in the ESI positive and negative ion modes, with the following optimized parameters: ion spray voltage, ±4. 5kV; capillary voltage, 16/-18 V; tube lens offset, -70/58 V, sheath gas (N_2), 80 arbitrary units; auxiliary gas (N_2), 5 arbitrary units; capillary temperature, 270ºC. Spectra typically correspond to the average of 20–35 scans, and were recorded in the range between 100-1000 Da. Tandem mass spectra (MS^n, n=2-3) were obtained with an isolation window of 2 m/z units, 20-30% relative collision energy and with an activation energy of 30 msec. Data acquisition and processing were performed using the software Xcalibur 2.2.

MS^2 experiments were used to characterize both flavonoid aglycones and glycosides; whereas MS^3 analysis was used in the fragmentation of flavonoid glycosides to their aglycones. The nomenclature for flavonoid aglycone fragmentations proposed by Claeys and co-workers were adopted to denote the various fragment ions (Vukics and Guttman, 2010).

Statistical analysis

All the results were submitted to analysis of variance (ANOVA). The mean values were compared by using Tukey test. The statistical significance was assessed at $p < 0.05$.

RESULTS AND DISCUSSION

Morettini pears were harvested on the same day from the same orchard and divided in two groups according with the color of the skin. Fruits grown on the outside of the tree, more exposed to the sunlight, developed an attractive red blush during ripening (Hue° = 62). Meanwhile, pears developed in the tree interior showed a greenish yellow skin, with a hue value of about 112 (Fig. 1a and Table 1). Significant differences in lightness were observed between both sides (Table 1).

In general, the physicochemical parameters of the red and green side of the fruit were similar, without significant differences in firmness, brix and titratable acidity (Table 1).

Aiming at confirming the differences observed in the field between red and green side in terms of incidence of rots, a controlled infection assay was performed. The pathogenic fungus *P. expansum*, one of the main responsible for postharvest decays in apples and pears, was chosen for the assay (Conway et al., 2004; Sánchez et al, 2012). Artificially wounded pears were inoculated with a suspension of *P. expansum* and incubated at room temperature. After 72h, all the wounds were colonized by *P. expansum* (disease incidence = 100%); however the progress of the disease in the green side was clearly faster than in the red side of the fruit (Fig. 1b). Five days after inoculation, the size of the infected wounds remained significantly different ($p < 0.05$). The diameter of the lesions in the red part was about 8% and 11% smaller than in the green part, for pathogen concentrations of 10^4 and 10^3 conidia/mL, respectively (Fig. 2). After 7 days of incubation, the evolution of the lesions maintained the trend, with significant differences of about 5% and 9% (data not shown).

A similar behavior was previously observed in mango fruit. Fruits with a red coloration in their peel were more resistant than entirely green when challenged with a *Colletotrichum gloeosporioides* fungal inoculation. This increased resistance was linked to the accumulation of anthocyanin and flavonoids (Sivankalyani et al., 2015).

Fig 1. (a) Differences in skin coloration of Morettini pears related to sunlight exposure during ripening. (b) Aspect of the artificially wounded pears inoculated with 20 µL of an aqueous suspension of *P. expansum* (10^3 conidia/mL) after 72h of incubation at 20-22°C (shelf-life conditions).

Table 1: Physicochemical parameters of Morettini pears according to the fruit side analyzed: Lightness (L*), hue angle (Hue°), firmness, total soluble solids (TSS) and titratable acidity (TA)

Fruit side	Parameter				
	L*	Hue°	Firmness (kgf cm^{-2})	TSS (°Brix)	TA (g malic acid L^{-1})
Red	50.99±1.42^{a}	61.98±5.08^{a}	4.81±0.26^{a}	12.80±0.35^{a}	6.07±0.18^{a}
Green	68.08±0.13^{b}	112.54±0.36^{b}	4.96±0.23^{a}	12.60±0.13^{a}	6.28±0.18^{a}

Values represented as mean±SD (n=18 for L*, Hue angle and firmness, n=9 for TSS and n=3 for TA). Different letters within the same column indicate significant statistical differences ($p \leq 0.05$)

Table 2: Total phenolic content and antioxidant activity of methanolic extracts of Morettini pears

Fruit side	Total phenols (mg GAE 100 g^{-1} FW)	Antioxidant activity	
		(mg trolox 100 g^{-1} FW)	(mg AAE 100 g^{-1} FW)
Red	336.59±16.42^{a}	141.45±6.56^{a}	77.12±3.58^{a}
Green	214.97±16.02^{b}	118.01±5.47^{b}	60.28±2.80^{b}

All the values are means±SD (n=6). Values within a column followed by different letters are significantly different ($p \leq 0.05$). GAE: Gallic acid equivalent, FW: Fresh weight, AAE: Ascorbic acid equivalent

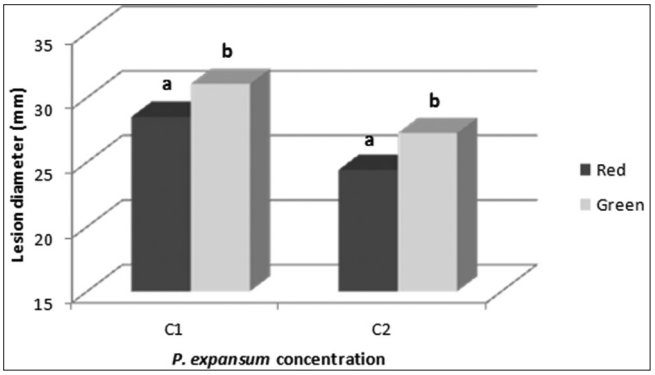


Fig 2. Diameter of the lesions caused by *P. expansum* inoculated into pear wounds, after 5 days of incubation at room temperature and 85% RH (shelf-life conditions). Data shown are the means for replicates of 18 wounds each. C1, 10^4 conidia/mL; C2, 10^3 conidia/mL. Different letters indicate significant differences ($p \leq 0.05$) between red and green side, for each pathogen concentration used.

It is well described that plants exposed to ambient sunlight protect themselves from the harmful ultraviolet-B radiation by adjusting their antioxidant systems, as well as by synthesizing phenolic compounds, which act as a protective screen inside the epidermis (Carletti et al., 2003).

In the other hand, there is already evidence that the accumulation of phenolic compounds in the tissues is one of the mechanisms developed by plants against fungal pathogens. Plants react to diverse phytopathogens by using constitutive or induced phenolic substances, affecting the susceptibility or resistance characteristics of the plant (Beckman, 2000).

Therefore, in Morettini pears the accumulation of phenolic compounds in the red side as response to sunlight exposure could be one of the causes for the higher resistance to fungal infection when comparing with the green side. Total soluble phenolic compound content in pear extracts was measured by the Folin-Ciocalteau method. Results showed a high concentration of phenolic compounds in both types of samples, however in the red side the accumulation was significantly higher (≥50%) than in the green part of the fruit (Table 2).

In general, the phenolic concentration measured in both samples was in the order of magnitudes reported for fruits considered at an intermediate level of phenolic content, like apple (211–347 mg GAE 100 g^{-1}), banana (52–231 mg GAE 100 g^{-1}), grape (145–196 mg GAE 100 g^{-1}), orange (31–337 mg GAE 100 g^{-1}), pineapple (47–174 mg GAE 100 g^{-1}), apricot (133–178 mg GAE 100 g^{-1}) and guava (170–345 mg GAE 100 g^{-1}). Specifically, for pears, the values reported in literature are around 40–220 mg GAE 100 g^{-1} (Brat et al., 2006; Wu et al., 2004).

When compared with other pear cultivars like D'Anjou, Packams, Abate, General Leclerc, Passe Crassane and Rocha, Morettini pears presented the highest concentration of phenolic compounds, mainly in red fruits (Salta et al., 2010; Galvis Sánchez et al., 2003).

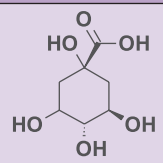
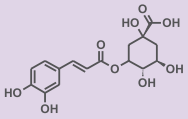
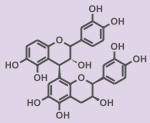
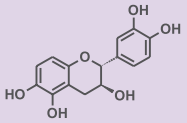
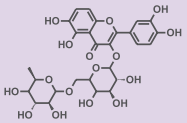
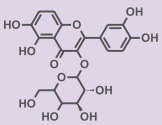
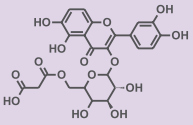
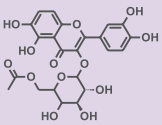
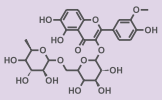
It is well established that polyphenols have strong antioxidant activities and there is a significant correlation between phenolic concentration and free radical scavenging activity (Triantis et al., 2005). The antioxidant activity of the extracts was determined by the DPPH radical scavenging method. The differences observed in the content of total antioxidant compounds agreed with the results previously observed for the phenolic contents. The antioxidant activity measured in the extracts of the red part was approximately 20-28% higher than in the green one (Table 2).

In a first approach to analyze the phenolic compounds present in the samples of Morettini pears, the HPLC-PDA analysis of the extracts showed identical phenolic profiles, yielding phenolic acids, catechins and flavonoids (data not shown). However, the proportion of each compound differed in the two types of analyzed samples (red or green part of the fruit). These results allow explaining the differences in the antioxidant activities found in the fruit extracts analyzed (Table 2). On the other hand, neither the red nor the green part of the fruit contained anthocyanins (data not shown).

Identification and peak assignment of individual polyphenols was carried out by HPLC-ESI-MS/MS based on comparison of their retention times, MS spectra and fragmentation patterns with those of standards (rutin and quercetin-3-O-glucoside) or published data. The 12 compounds identified or tentatively identified are listed in Table 3, along with retention times, structures and mass spectrometric characterization for each compound.

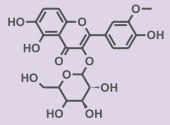
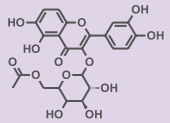
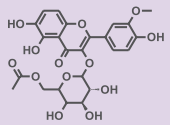
The presence of catechin, rutin and quercetin is well described in *Pyrus communis* L. (Galvis Sánchez et al., 2003) and these compounds have been confirmed in the analyzed extracts (peak 4- catechin, m/z 289; peak 5-rutin, m/z 609/611; and peak 6-quercetin-3-O-glucoside, m/z 463/465, Table 3). Moreover, the identification of several additional glycosides of quercetin and isorhamnetin is highlighted with two quercetin glycosides derivatives: quercetin-manonylhexoxide (peak 7, m/z 549/551) and quercetin-acetyl hexoside (peak 8, m/z 505), and four compounds that involved isorhamnetin glycosides and derivatives, namely isorhamnetin-O-rhamnose-glucoside (peak 9, m/z 625) isorhamnetin-3-O-glucoside (peak 10, m/z 477/479), isorhamnetin-manonyl hexoside (peak 11, m/z 563/565), and isorhamnetin-acetyl hexoside (peak

Table 3: Identification by HPLC-ESI-MS/MS of the phenolic compounds present in methanolic extracts of Morettini pears

Peak	Rt (min)	Structure	$[M+H]^+/[M-H]^-$ (*m/z*)	MS^2 (*m/z*, %, Ion attribution)	Proposed compound
1	0.8		-/191	MS^2[191] : 173 (10) $[M-H-18]^-$, 129 (15) $[M-H-62]^-$, 111 (100) $[M-H-62]^-$	Quinic acid
2	4.3		-/353	MS^2[353] :191 (90) $[M-H-152]^-$, 179 (100) $[M-H-146]^-$, 123 (80) $[M-H-202]^-$	Caffeoylquinic acid
3	5.3		-/577	MS^2 [577]: 425 (100) $[M-H-152]^-$, 407 (40) $[M-H-(152+18)]^-$, 289 (25) $[Cat-H]^-$	Procyanidin dimer
4	5.9		-/289	MS^2 [289]: 245 (100) $[M-H-44]^-$, 205 (40) $[M-H-84]^-$, 179 (20) $[M-H-110]^-$	Catechin or epicatechin
5	8.6		611/609	MS^2 [609]: 463 (2) $[M-H-162]^-$ (Y_1-), 343 (20) $[M-H-162-120]^-$ ($^{0,2}X_0$-), 301 (100) $[M-H-308]^-$(Y_0-), 300 (80) (Y_0-H$^•$), 179 (4) $^{1,2}A^-$.	Rutin
6	8.9		465/463	MS^2 [463]: 301 (100) $[M-H-162]^-$ (Y_0-), 300 (50) (Y_0-H$^•$), 179 (7) $^{1,2}A^-$,151 (5) $^{1,2}A^-$-CO	Quercetin-3-O-glucoside
7	9.4		551/549	MS^2 [549]: 505 (100) $[M-H-44]$ MS^3 [505]: 463 (45) $[M-H-42]^-$, 301 (100) $[M-H-(162+42)]^-$ (Y_0-), 300 (70) (Y_0-H$^•$), 179 (10) $^{1,2}A$-,151 (4) $^{1,2}A^-$-CO	Quercetin- manonylhexoxide
8	9.6		-/505	MS^2 [505]: 463 (50) $[M-H-42]^-$, 301 (100) $[M-H-(162+42)]^-$ (Y_0-), 300 (90) (Y_0-H$^•$), 179 (5) $^{1,2}A$-,151 (4) $^{1,2}A^-$-CO	Quercetin-acetyl hexoside
9	9.8		625/-	MS^2 [625]: 479 (50) $[M-H-146]^-$, 317 (100) $[M-H-(162+146)]^-$ (Y_0-).	Isorhamnetin-O-rhamnose-glucoside

(Contd)...

Table 3: ***(Continued)***

Peak	Rt (min)	Structure	[M+H]$^+$/[M-H]$^-$ (*m/z*)	MS^2 (*m/z*, %, Ion attribution)	Proposed compound
10	10.4		479/477	MS^2 [477]: 357 (10) [M-H-120]$^-$, 315 (40) [M-H-162]$^-$ (Y_0), 314 (100) (Y_0-H$^•$), 285 (20) (Y0-CH2O).	Isorhamnetin-3-O-glucoside
11	11.3		565/563	MS^2 [563]: 519 (100) [M-H-44]$^-$, MS^3 [519]: 357 (10) [M-H-(120+42)]$^-$, 315 (100) [M-H-(162+42)]$^-$ (Y_0-), 314 (7) (Y_0-H$^•$), 300 (15) (Y_0-CH3).	Isorhamnetin-manonyl hexoside
12	11.4		-/519	MS^2 [519]: 477 (7) [M-H-42]$^-$, 357 (10) [M-H-(120+42)]$^-$, 315 (100) [M-H-(162+42)]$^-$ (Y_0-), 314 (40) (Y_0-H$^•$), 300 (15) (Y_0-CH3), 285 (8) (Y_0-CH2O).	Isorhamnetin-acetyl hexoside

12, *m/z* 519) (Schieber et al. 2002, Barros et al. 2013) (Table 3). Other phenolic compounds identified in the pear extracts are phenolic acids, namely quinic acid (peak 1, *m/z* 191) and caffeoylquinic acid (peak 2, *m/z* 353); and one procyanidin dimer (peak 3, *m/z* 577) (Long-Ze and Harnyl, 2007).

Among the phenolic compounds identified, several of them have already been associated to disease resistance in fruits and vegetables (Lattanzio et al., 2006). For example, the first case confirmed was the increased resistance against smudge disease conferred by the accumulation of catechol and protocatechuic acid in onion scales (Walker and Stahmann, 1955). Phenolic compounds identified in olive plants like tyrosol, catechin, and oleuropein, have antifungal activity and protect plants against *Phytophtora* sp. (Del Río et al., 2003). Conidial germination of the fungus *Neurospora crassa* was completely inhibited by quercetin 3-methyl ether and its glucosides (Parvez et al., 2004). In citrus, phenolic compounds like quercetin, scopoletin and scoparone proved to be effective in reducing the severity and incidence of green mold disease caused by *Penicillium digitatum* (Sanzani et al., 2014).

Further studies are needed to investigate the accumulation patterns and the role of each one of the phenolic compounds identified in red and green Morettini pears.

CONCLUSION

The accumulation of phenolic compounds as consequence of a higher or longer exposition to sunlight is pointed out as one of the causes for the differential susceptibility or resistance of Morettini pears to fungal pathogens. The identification of phenolic compounds previously linked to fungal resistance supported these findings. Based on the results, it can be suggested that a late spring pruning, for example the removal of some branches from the interior of the tree 3-4 weeks before harvest to allow light penetration, could be a good agro-technical approach to increase the resistance to postharvest diseases in Morettini pear, increasing at the same time its nutritional value due to bioactive phenolic compounds accumulation.

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Authors' contributions

C.V.S. was involved in the overall planning and supervision of the work, writing and paper review; M.S. and P.V. were mostly involved in experimental procedures; R.M.S. was responsible for the adaptation and production of Morettini pears; M.C.O. and C.S. performed the chromatographic analysis and contributed to writing the paper.

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