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RESEARCH ARTICLE

Higher Levels of Protein and Phenolics in 'Braeburn' Apples Correlate with Fruit Tolerance to Grey Mould

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Abstract

Botrytis cinerea is a pathogenic fungus that causes grey mould responsible for significant economic losses in over 1400 plant species, including cultivated apples. The effect of storage at room temperature, the impact of wounding and the combined effect of wounding and phytopathogen infection by *B. cinerea* on development of grey mould was studied using cv. 'Braeburn' apples. We measured lesion size (disease development) and antioxidant metabolism in the sun-exposed and shaded sides of 'Braeburn' apples in both the peel and flesh tissues on day 0 (start of experiment) and 1, 3, 6, 8 and 10 days after inoculation with *B. cinerea.* Data obtained were analysed by principal component analysis and interaction tests to evaluate the importance of different parameters for disease development. Lesions developed faster on the shaded than on the sun-exposed side ($p<0.001$) and faster in the flesh than in the peel tissue ($p=0.002$).

High levels of protein ($p<0.001$) and phenolic compounds ($p=0.023$) predicted tolerance in fruits whereas high levels of protein, phenolics, vitamin C and oxygen radical absorbance capacity (all at $p<0.001$) suppressed the development of disease. We found that only ascorbate peroxidase (APX) activity ($p=0.021$) and flavonoid peroxidase (POX) activity ($p<0.001$) were influenced by the infection with *B. cinerea,* whereas superoxide dismutase (SOD) activity and catalase (CAT) activity remained mostly unaltered throughout this process. In conclusion the results of this study show an important role for protein and phenolics in apple fruit defence against grey mould and a positive effect of sunlight on the quality of apples.

Keywords: *Malus X domestica*; Protein; Phenolics; Apple; Sun-Exposed Side; Grey Mould; Fruit Tolerance

Abbreviations: ROS: Reactive oxygen species; SOD: Superoxide dismutase; POX: Flavonoid peroxidase; APX: Ascorbate peroxidase; CAT: Catalase; AsA: Vitamin C (L-ascorbic acid); ORAC: Oxygen Radical Absorbance Capacity

Introduction

Botrytis cinerea is a necrotrophic fungus responsible for both pre- and postharvest diseases, collectively known as grey mould, in over 1400 plant species, including cultivated apples (reviewed by [1-3]). The annual global economic losses due to *B. cinerea* are estimated at US$10-100 billion [3]. The interaction between *B. cinerea* and the host plant it infects has given rise to intense research worldwide [1,2]. *B. cinerea* often causes latent infection of immature apple fruits that are still attached to the tree, with grey mould becoming apparent as postharvest decay [2,4,5]. During harvest and cold storage, *B. cinerea* may infect mature apples and spread among stored fruits [6].

The influence of preharvest conditions on the levels of phytochemicals in fruit during postharvest storage and how this affects disease resistance are central issues in the prevention of grey mould. It is generally accepted that preharvest exposure of apple to stress, for instance from intense sunlight or high temperatures, increases the level of antioxidants in fruit [7,8]. Davey, *et al.* [9] and Bui, *et al.* [10] found that apple tissues that have been exposed to high intensity sunlight accumulated higher levels of antioxidants and had an improved ability to resist disease. Sunlight also stimulates the production of vitamin C, phenolics and antioxidants in apple peel, which makes the fruit less susceptible to infection by pathogens [9,11].

In addition, light has been reported to prevent the infection of grey mould in plants [12-14]. When a plant recognizes an attacking pathogen, one of the first reactions induced is an 'oxidative burst', during which rapid production of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) takes place. These highly reactive molecules, also known as Reactive Oxygen Species (ROS), prevent the spread of the pathogen to other parts of the plant by restricting fungal movement and reproduction [15]. ROS play a central role in redox-dependent signalling, which regulates several processes of importance in plant-pathogen interactions, e.g. cell death [16]. During host infection, *B. cinerea* contributes to programmed cell death as part of its infection strategy [17]. The fungus, together with the host plant, produces ROS at the infection site [18].

However, both fungus and plant produce antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), flavonoid peroxidase (POX) and ascorbate peroxidase (APX), during disease development [19] that counter the effect of ROS. Antioxidant enzymes remove ROS by catalysing antioxidant reactions. In the plant cells, SOD is in the first line of defence enzymes converting the superoxide radical (O_2^-.) into H_2O_2 [20]. An excessive amount of H_2O_2 is toxic to cells and this compound is decomposed by CAT, POX and APX, all of which can convert H_2O_2 to water [21]. CAT metabolises H_2O_2 via iron-heme groups that are attached to the enzyme. Furthermore, POX detoxifies H_2O_2 by using flavonoids as a substrate whereas APX uses vitamin C as a reducing agent [22].

In recent years, several papers have described the composition of phytochemicals in apple fruits. The levels of these vary, depending on the cultivar [23], growing season [24], stage of fruit maturity [25], time of harvest, postharvest storage conditions [23], and processing procedures [26]. Although grey mould is economically important, it has received little attention and we have thus only limited knowledge about detailed infection mechanisms important for the disease [27]. Postharvest storage diseases have been linked to preharvest environmental conditions [9,11]. Although the effect of sunlight on the physiology of apple fruit is quite well known [8,12,28], the effect of sunlight on defence mechanisms against grey mould in apple fruit has received little attention.

Davey, *et al.* [9] found a strong correlation between vitamin C content and postharvest infection with *B. cinerea*, whereas Tuyet, *et al.* [29] reported a complex and tissue specific antioxidant metabolism response upon infection. In contrast, our own recent study on 'Braeburn' and 'Golden Delicious' apple fruit, indicated that differences in antioxidant metabolism could not explain cultivar differences in the resistance to grey mould. However, sun-exposed, resistant peel tissue in 'Braeburn' apples contained about 4-fold higher levels of ascorbic acid suggesting that higher antioxidant content brought about by higher exposure to sun light correlates with improved resistance to pathogens, at least in 'Braeburn' apples. Here we extend previous studies suggesting that preharvest exposure to abiotic stress, such as high light intensity, can modulate postharvest susceptibility of apples to *B. cinerea* infection after harvest. Specifically, by including measurements of more antioxidants and through the use of principal component analysis the relative importance of different enzymes and antioxidant compounds in countering postharvest infection by grey mould is evaluated and compared in detail.

Materials and Methods

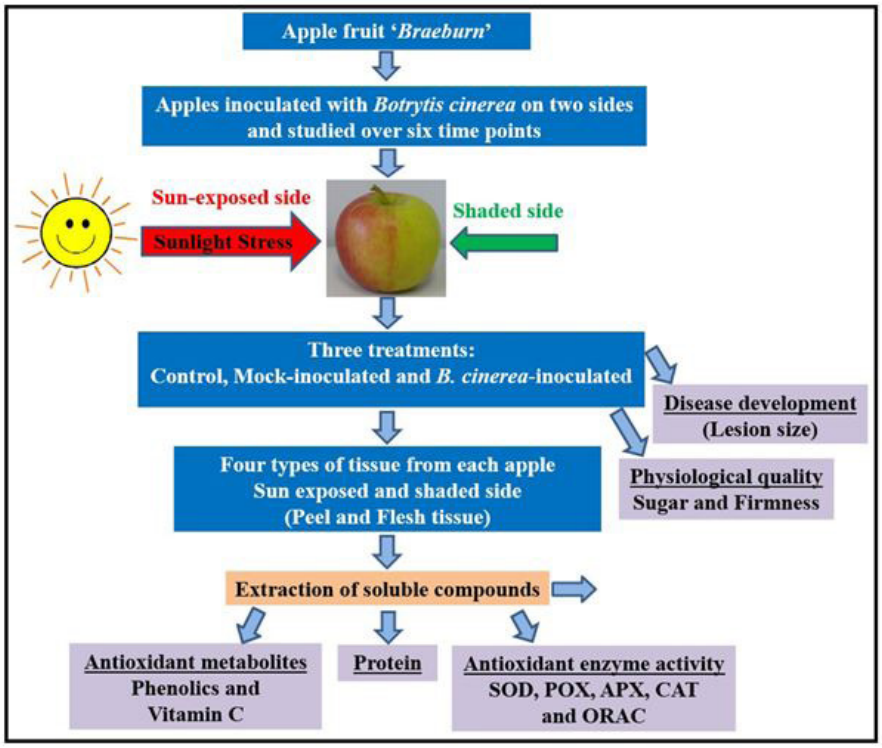

Figure 1: Flow chart and overview of sampling and analysis procedures. We extracted and analyzed protein for the determination of antioxidant metabolites and antioxidant enzyme activities. We also determined apple quality by analyzing sugar content (Brix) and firmness

An overview to sampling and analytical procedures is given in Figure 1. In this study, we collected 100 healthy 'Braeburn' apple fruits, of which 10 were used as control at the start of the experiment (day 0) and the 90 remaining fruit were sampled on days 1, 3, 6, 8 and 10 after inoculation with *B. cinerea*. For each time point 18 fruit were used; 6 as controls (neither wounded nor inoculated), 6 as mock-inoculated and 6 inoculated with *B. cinerea*. We measured 120 samples for lesion size (disease development) and analyzed 4000 samples for antioxidant metabolism. Four hundred individual tissues were analyzed for 10 different antioxidant properties: protein, phenolics, vitamin C, ORAC, SOD, POX, APX, CAT activity, sugar content (Brix) and firmness.

Fruit

The apple 'Braeburn' was selected because of the clear difference in colour between sun exposed (red) and shaded (green) sides, and its high susceptibility to *B. cinerea*. 'Braeburn' apple fruit was harvested between Oct. 22 and 29, 2010 at Fruitteelt, Experimental Garden for Pome and Stone fruit, Sint-Truiden, Belgium. That time was the optimal harvest period for long-term storage of cv. 'Braeburn' apples as recommended by Flanders Centre of Postharvest Technology (VCBT), Belgium. After picking, 100 healthy apple fruits were immediately transported and stored at VCBT under controlled atmospheric conditions, specifically at 0.5 °C, 1-2 % O_2, 2-2.5 % CO_2, and 95 % relative humidity. The apples were stored until July 2011, when inoculation was carried out at the Laboratory of Fruit Breeding and Biotechnology, Catholic University of Leuven (K. U. Leuven), Belgium.

Pathogen

Botrytis cinerea strain B05.10, a standard reference strain, was obtained from the Centre of Microbial and Plant Genetics (CMPG), K. U. Leuven. Cultivation and harvesting of *B. cinerea* spores was carried out as described by Davey, *et al.* [9]. Spores were stored frozen in a 25 % glycerol suspension at -80 °C. Two weeks prior to inoculation of apple fruits, the spore suspension was transferred to potato dextrose agar plates, which were incubated at room temperature. Spores were harvested from the agar surfaces by suspending them in 2 mL 0.05 % Tween 20 in sterile distilled water, followed by sonication for 5 min in a water bath. The spore density was determined using a Fuchs-Rosenthal counting chamber and the suspension was diluted to a final concentration of 1.5 x 10^5 spores per mL.

Fruit Wounding and Inoculation

As *B. cinerea* infects fruit tissue through wounds, we compared the changes in antioxidant levels using three treatments (below). Apples were inoculated with *B. cinerea* by making 0.3 cm wide and 0.6 cm deep wounds on opposite sides of 30 individual fruits using a sterile 3 mm wide and flat steel tool. Each apple was wounded midway between the calyx and the stem on both the sun-exposed ('red') and the shaded ('green') sides of the fruit. Inoculation took place immediately after wounding. There were three treatments, namely, a control (neither wounded nor inoculated), mock-inoculated (wounded and inoculated with 20 µL sterile water), and *B. cinerea*-inoculated (wounded and inoculated with 20 µL of a suspension containing of 1.5 x 10^5 spores/mL). Of the 20 µL of *B. cinerea* spore suspension used on each side of the fruit. After inoculation, the fruits were sealed in plastic boxes covered with plastic-foil and stored in a constant-climate room (around 20 °C) at 100 % relative humidity for the first 24 h, and then at 80% relative humidity for the remaining time of the experiment.

Fruit Sampling

Apple tissue was excised from around the point of inoculation using a 0.5 cm diameter cork-borer. The uppermost 0.3 cm from the surface was considered peel tissue (hypanthium – fruit cortex), and the part taken out from 0.3 cm to 2.0 cm of the fruit plug was considered flesh tissue. Samples were ground to a fine powder in liquid nitrogen and stored at -80 °C until analyzed.

Quality Analysis of Apples

Evaluation of the Development of Disease: The disease symptoms were quantified as the diameter of the lesion around the inoculation points of the apples on both the sun-exposed and shaded sides at 1, 3, 6, 8, and 10 days after inoculation. Lesion size was measured (in cm) using a plastic ruler.

Physiological Measurements: The firmness and the sugar content (Brix) were analyzed on individual fruits with sampling at two points on the circumference on the sun-exposed and the shaded sides of the fruit. The firmness (in kg/cm^2) was determined by using a penetrometer (Bishop, fruit pressure tester, model FT327, Italy). Sugar content was determined using a portable digital refractometer, model PR-32 (Atago Co. Ltd.), using juice obtained from the two holes in the apple made by the penetrometer [9], and calculated as g/kg fresh weight (g/kg fw).

Extraction of Protein

In a first step we extracted and separated protein from the powdered tissue. In a second step, we analyzed the extract for protein content, antioxidant metabolites and antioxidant enzyme activity (Figure 1). Extraction and separation were carried out according to Ahn, *et al.* [30] with minor modification. For the extraction, 0.1 g of tissue sample was homogenized with 1.0 mL of the extraction buffer (50 mM potassium phosphate buffer, pH 7.8), 1 mM ethylene-diamine-tetra-acetate (EDTA), 1 % polyvinyl-polyproline (PVPP), 0.3 % Triton X - 100, 10 % glycerol, 0.1 mM dithiothreitol (DTT), and 50 µM vitamin C. The samples were then centrifuged for 15 min at 14000 rpm at 10°C (centrifuge Hettich 220R, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany).

The supernatant (1.0 mL) was transferred to a sterile Eppendorf micro centrifuge tube and stored at - 80°C. In a next step the high-molecular components were separated from the low-molecular ones, using a Sephadex column and washing three times with column buffer. Column buffer consisted of 50 mM potassium phosphate buffer (pH 7.8), 1 mM EDTA, 1 % PVPP, and 10 % glycerol. The supernatant (1.0 mL) followed by 1.5 mL of column buffer was added and allowed to pass through the column to separate high molecular weight compounds. From the eluate, 1.5 mL aliquots were collected and used for determination of protein, antioxidant metabolites and antioxidant enzyme activities.

Analyses

Protein Content

Protein Content (g/kg fw): Protein content was determined using Bio-Rad Protein Assay, based on the method of Bradford described in Bui, *et al.* [10]. This method uses bovine serum albumin (BSA) as a standard and added acidic dye to protein solution to compare to a standard curve with a relative concentration of protein. We used 20 mL of ¼ diluted Bio-Rad solution (protein assay dye reagent concentrates - catalog number 500-0006, Bio-Rad, USA) and 80 mL of methanol. The 96-well plate contained 20 µL of supernatants and 180 µL of reaction mixture in each well. We used 20 µL of 5 different concentrations (0 mg/mL, 0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL and 0.8 mg/mL) of BSA standard substrate added in triplicate for each concentration. The plate was agitated briefly and incubated in the microplate spectrophotometer at room temperature for at least 5 minutes before absorbance was recorded at 595 nm, with triplicates for each treatment. The protein content was expressed as gram per kilogram fresh weight (g/kg fw).

Antioxidant Metabolites

Phenolic Compounds (mg GAE/kg fw): Total contents of phenolics was estimated using a photometric method with the Folin-Ciocalteu reagent as described in Davey, *et al.* [31]. Approximately 5 mg powder of apple tissue was extracted with 500µl of a solvent consisting of methanol and water (80:20, % v/v) containing 0.1% 1mM EDTA as antioxidant. For each sample, triplicate analyses were carried out using absorption at 280 nm. We used a standard curve based on gallic acid at 6 different concentrations (0, 25, 50, 100, 150, 200, and 250 µg/mL) and the concentration of phenolics was expressed as milligrams of gallic acid equivalents (GAE) per kilogram fresh weight (mg GAE/kg fw).

Vitamin C (g/kg fw): Vitamin C was determined by high-performance liquid chromatography (HPLC) analysis, essentially as described by Franck, *et al.* [32]. Approximately 0.2 g of tissue sample was homogenized with 1.0 mL of extraction buffer (3% metaphosphoric acid and 1 mmol L-1 EDTA). The samples were then centrifuged for 15 min at 14000 rpm at 10°C (centrifuge Hettich 220R, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). The supernatant (1.0 mL) was filtered through a PVPP filter with 0.45µm pore size (Millipore, Brussels, Belgium) and analyzed immediately with HPLC. Vitamin C content was analyzed as adsorption at 242 nm and expressed as gram per kilogram fresh weight.

Antioxidant Enzyme Activities

The Oxygen Radical Absorbance Capacity (ORAC) (µmol TE/g fw)

The ORAC assay is based on the measurement of the antioxidant scavenging activity against peroxyl radicals induced by 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH) [33]. All reagents were prepared using a 75 mM phosphate buffer (pH 7.4). In the final assay mixture (0.4 mL total volume), 6.3*10-8 M fluorescein (FL) was used as a target of free radical attack and AAPH (1.28*10-2 M) was used as a peroxyl radical generator. Samples of apple tissue were compared to 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox) standards, made in 7% Random Methylated β Cyclodextrin (RMCD) solvent (50:50% acetone-water mixture). Seven percent RMCD solvent was used as blank, and Trolox (12.5, 25, 50, and 100 µM) was used as control standard. The analyzer was programmed to record the fluorescence of FL every minute after the addition of AAPH. All measurements were expressed relative to the initial reading.

Final results were calculated using the differences in areas under the FL decay curves between the blank and the sample. The results were expressed as µmol Trolox equivalent (TE) per gram fresh weight (µmol TE/g fw). For the activities of selected antioxidant enzyme activities (SOD, POX, APX, and CAT) we used the supernatants in triplicate from extract (see extraction of protein), assayed as modified from Ahn, *et al.* [30]. All antioxidant enzyme activities were measured using 96-well microtiter quartz plates, containing 20 µL of enzyme extract and 180 µL of reaction mixture in each well in triplicate, and incubated at 18-20 °C. Readings were recorded every 30 seconds using a Multiscan Spectrum-Microplate Spectrophotometer (Thermo Lab systems, Helsinki, Finland) and expressed in units per gram of fresh weight for each enzyme (units/g fw).

SOD Activity (units/g fw)

SOD activity was determined by measuring the ability of SOD to inhibit the photochemical reduction of nitrobluetetrazolium (NBT) at 450 nm [30]. The assay mixture contained 50 mM PIPES buffer pH 7.5, supplemented with 0.4 mM o-dianisidine, 0.5 mM diethylene triamine pentaacetic acid (DTPA) and 26 µM riboflavin. SOD activity was measured at 450 nm at time = 0 min. To start the reaction the plate was placed 12-20 cm below two 15 W fluorescent lamps and the light switched on. The reaction was

stopped by switching off the light after which the plate was covered with aluminum foil until absorbance was measured at 450 nm. Eight different concentrations of SOD, each added in triplicate served as a standard. The SOD substrate stock solution had 2 mg protein/mL in water, equivalent to 3277 units/mg protein - Sigma-Aldrich, Saint Louis, Mo. USA, and was diluted to the eight concentrations with 1, 10, 20, 30, 40, 50, 80, and 100 units/mL.

POX Activity (units/g fw)

POX activity was determined as the rate of guaiacol oxidation in the presence of H_2O_2 (extinction coefficient, 26.6 mM^{-1} cm^{-1}) at 470 nm [30]. The reaction mixture, containing 50 mM potassium phosphate buffer at pH 7.0, 0.01 M EDTA, 0.02 M pyrogallol, and 1.47 mM H_2O_2, was added to the wells of a 96-well microtiter plate. The plate,which had 20 µL of enzyme extract and 180 µL of reaction mixture in each well, was agitated briefly and incubated in the microplate spectrophotometer for 4 minutes before absorbance was recorded.

APX Activity (units/g fw)

APX activity was determined by monitoring the decomposition of H_2O_2, based on the method described in [30] with a slight modification. The reaction mixture, containing 50 mM potassium phosphate buffer of pH 7.0, 0.1 mM EDTA, 0.88 mM vitamin C, and 0.1 mM H_2O_2, was added to the wells of a 96-well plate. The plate containing 20 µL of enzyme extract and 180 µL of reaction mixture in each well was agitated slightly and incubated in the microplate spectrophotometer for 30 seconds. Absorbance was measured at 290 nm (extinction coefficient of 2.8 mM^{-1} cm^{-1}).

CAT Activity (units/g fw)

CAT activity was determined using the method of Du and Bramlage described in Bui, *et al.* [10], with slight modifications. The reaction mixture contained 30 mM H_2O_2 in 50 mM potassium phosphate buffer of pH 7.0. The 96-well plate, which contained 20 µL of enzyme extract and 180 µL of reaction mixture in each well, was agitated briefly and incubated in the microplate spectrophotometer for 30 seconds before absorbance was recorded at 240 nm, with triplicates for each treatment. We also used 8 different concentrations of the CAT standard substrate (25 mg protein/mL, 423000 units/mg protein - Sigma, C-100), namely 20 µL of 0, 20, 50, 80, 100, 500, 1.000 and 5.000 units/mL added in triplicate for each concentration.

Statistical Analysis

Statistical analyses were performed in R 3.4.3 [34]. An over-all analysis of the data was made using a Principal Component Analysis (PCA). The input to the ordination was measured activities of enzymes and concentrations of metabolites for all samples. On this ordination, lesion size and tolerance (susceptible = 0, tolerant = 1) were tested using the procedure envfit in the vegan package [35]. Side of the apple and tissue (peel/flesh) were not part of the ordination but were identified by symbols. We analyzed the effect of the experiment using enzyme activity and metabolite concentrations as response variables, using linear mixed models with apple identity as a random factor since 4 different kinds of tissues/samples (peel sun exposed, peel shaded, flesh sun-exposed and flesh shaded) were taken per apple.

In separate analyses of tissue: side combination used to construct the supplementary table; ordinary linear models were used since each apple was represented only one time in those models. Mixed models used the lmer procedure in lme4 [36] and the models were evaluated using the package lmer Test [37]. A categorical variable is called a factor. The explanatory variable to construct figures and flowcharts was a single factor with factor levels constructed from all sampling days and treatment levels. Predictions from models were made using the package effects [38] and differences between factor levels interactions were tested using package phia [39]. All analyses of variance are of the ANOVA type 2 [40], which means that each term is evaluated when the effects of all other terms are taken into account.

Terminology

We have used the term 'tolerance' for an apple as the property of not developing disease symptoms despite having been inoculated with *B. cinerea*. We use the term 'susceptibility' of an apple as the lesion size after being inoculated with *B. cinerea.*

Results and Discussion

Recent work shows that the interaction of the pathogen *B. cinerea* with plant tissue is complex [19,41,42]. During infection, oxidative bursts are generated both by *B. cinerea* and the host as ways to facilitate infection and to defend itself against pathogenic attack, respectively [18]. Two clear phases are distinguished: an early stage characterized by local necrosis, and a later one, characterized by the development of a spreading lesion [5]. Accordingly, a current model predicts that *B. cinerea* uses different types of virulence/effector molecules at each stage. A complex signaling network regulates the secretion of a large set of proteins and phytotoxic secondary metabolites, which are necessary for the progression of the *B. cinerea* infection from the early to the late stages [41].

Influence of Sunlight on Apple Antioxidant Metabolism and their Tolerance

Antioxidant Metabolism

Sunlight during cultivation improved the quality of apples and this was reflected both in a higher level of antioxidant compounds and a higher tolerance to pathogen attack (Tables 1 & 2) (Figures 2 & 3). At the start of our experiment, the sun-exposed side of apples had significantly higher levels of phenolics, vitamin C and enzyme activities for POX and CAT, than the shaded side (Table 1). The peel tissue from the sun-exposed side of the apples had much higher ($p<0.001$) levels of both vitamin C and phenolics as compared to the shaded side. The shaded side a of the fruits had higher activities of CAT and POX than the sun-exposed side at $p=0.002$ and $p=0.025$, respectively. The peel tissue had about 2-3 times higher ($p<0.001$) levels of ORAC and phenolics than the flesh tissue. In addition, in the peel tissue, concentrations of vitamin C were approximately three times higher ($p<0.001$) than in the flesh on both sides (Table 1).

	Mean values (Lower 95 percent confidence limit/ Upper 95 percent confident limit				Pairwise t- test (p-values)			
Components	Peel sun exposed	Peel shaded	Flesh sun exposed	Flesh shaded	Peel tissue (sun exposed vs. shaded)	Flesh tissue (sun exposed vs. shaded)	Sun exposed side (Peel vs. Flesh)	Shaded side (Peel vs. Flesh)
Protein (g/kg fw1)	0.32 (0.30/0.34)	0.33 (0.31/0.35)	0.41 (0.39/0.43)	0.41 (0.39/0.43)	n.s.[2]	n.s.	<0.001	<0.001
Phenolics (mg GAE/ kg fw)	1.81 (1.59/2.02)	1.23 (1.02/1.45)	0.59 (0.37/0.80)	0.6 (0.38/0.82)	<0.001	n.s.	<0.001	<0.001
Vitamin C (g/kg fw)	0.53 (0.47/0.59)	0.33 (0.27/0.39)	0.21 (0.15/0.27)	0.16 (0.10/0.22)	<0.001	n.s.	<0.001	<0.001
ORAC (µmol TE/g fw)	23.33 (20.85/25.81)	22.79 (20.03/25.55)	13.09 (10.61/15.57)	11.44 (8.96/13.91)	n.s.	n.s.	<0.001	<0.001
SOD (units/g fw)	0.95 (0.62/1.27)	0.66 (0.34/0.99)	0.36 (0.04/0.69)	0.18 (-0.15/0.51)	n.s.	n.s.	=0.014	=0.025
POX (units/g fw)	0.44 (0.35/0.53)	0.54 (0.45/0.63)	0.24 (0.15/0.33)	0.24 (0.16/0.33)	=0.025	n.s.	<0.001	<0.001
APX (units/g fw)	0.49 (0.36/0.62)	0.43 (0.30/0.56)	0.07 (-0.06/0.20)	0.11 (-0.02/0.56)	n.s.	n.s.	<0.001	<0.001
CAT (units/g fw)	0.06 (0.05/0.08)	0.09 (0.08/0.11)	0.003 (-0.01/0.02)	0.006 (-0.01/0.02)	=0.002	n.s.	<0.001	<0.001
Firmness (kg)	9.46 (8.80/10.12)	9.02 (8.36/9.68)	9.46 (8.80/10.12)	9.02 (8.36/9.68)	n.s.	n.s.	n.s.	n.s.
Sugar content (g/kg fw)	14.01 (13.62/14.40)	13.83 (13.44/14.22)	14.01 (13.62/14.40)	13.83 (13.44/14.22)	n.s.	n.s.	n.s.	n.s.

Table 1: Concentrations of the main essential components, namely protein, phenolics, vitamin C, and ORAC as well as antioxidant enzyme activities (SOD, POX, APX and CAT) plus values for firmness and sugar (Brix). Healthy apples (n=10) were investigated at the start of the experiment. We compared four kinds of tissue, namely peel and flesh tissue from sun-exposed and shaded sides. Significance values result from pairwise t-test between sun-exposed and shaded sides, as well as between peel and flesh tissue fw stands for 'fresh weight', n.s. stands for 'not significant'

Response	Explanatory variable	Number of observations	Test statistic (Chi-square)	p-value	Direction of effect
	Day	96	1.07	n.s.[2]	
Tolerance (tissue inoculated with *B. cinerea* but did not have disease symptom-no lesions)	Tissue	96	0.31	n.s.	
	Side	96	9.44	=0.002	Sun exposed > Shaded
	Protein	96	11.86	<0.001	Positive
	Phenolic	96	5.19	=0.023	Positive
	Vitamin C	76	3.41	n.s.	
	ORAC	90	3.71	n.s.	
	SOD	96	2.62	n.s.	
	POX	96	0.14	n.s.	
	APX	96	0.69	n.s.	
	CAT	96	0.09	n.s.	
	Firmness	96	0.35	n.s.	
	Sugar	96	1.28	n.s.	

Response	Explanatory variable	Number of observations	Test statistic (Chi-square)	p-value	Direction of effect
	Day	96	22.81	<0.001	Positive
Susceptibility (tissue inoculated with *B. cinerea* and had disease symptom-Lesion-size Disease development)	Tissue	96	10.63	=0.002	Peel < Flesh
	Side	96	47.12	< 0.001	Sun exposed < Shaded
	Protein	96	26.35	< 0.001	Negative
	Phenolic	96	46.78	< 0.001	Negative
	Vitamin C	76	37.64	< 0.001	Negative
	ORAC	90	72.08	<0.001	Negative
	SOD	96	0.65	n.s.	
	POX	96	17.43	< 0.001	Negative
	APX	96	5.53	=0.021	Negative
	CAT	96	1.24	n.s.	
	Firmness	96	0.17	n.s.	
	Sugar	96	1.45	n.s.	

Table 2: One-way analysis of variance (ANOVA) based on generalized linear models explaining tolerance against infection (as opposed to susceptibility) assuming a binomial error distribution and including individual apples as a random factor in the *B. cinerea*-inoculated apples for days 3, 6, 8 and 10 n.s. stands for 'not significant'

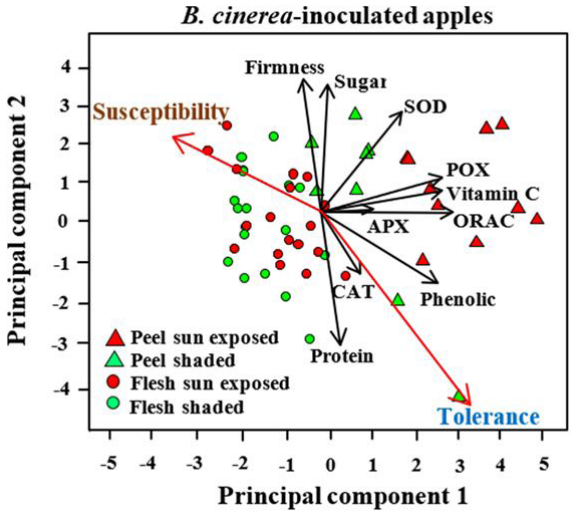

Figure 2: A Principal Component Analysis (PCA) based on the antioxidant (AOX) properties as well as sugar (Brix) and firmness. Samples from four kinds of tissue, namely sun-exposed and shaded peel, as well as flesh from the sun-exposed and the shaded sides are marked with different symbols. Values were taken with start on day 3 after inoculation. Regression of Susceptibility on the ordination gave R^2=0.46, p=0.001 and of Tolerance R^2=0.14, p=0.023

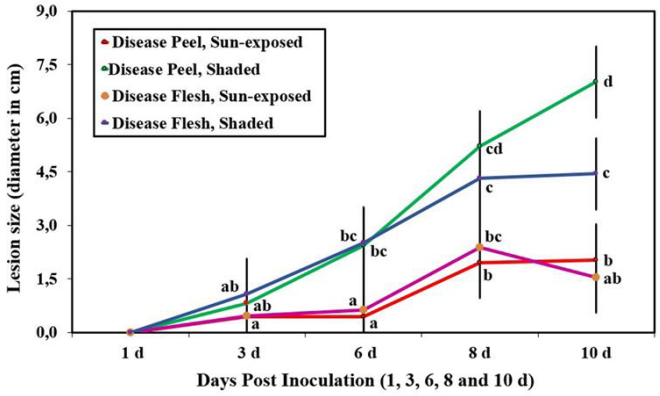

Figure 3: The development of disease (lesion size) from day 1 until day 10 after inoculation with *B. cinerea*. Measurements were made for each of all four kinds of tissue, namely peel and flesh from the sun-exposed and the shaded sides in inoculated fruit. Results are obtained from linear models using all combinations of tissue types, side and day as explanatory factors. The analysis is constructed so that all data points in the diagrams can be compared to each other and that non-overlapping confidence limit bars indicate significant differences while the exact probabilities can be found in ESM-1

Apple Tolerance

Disease symptoms (grey mould) appeared only in the *B. cinerea*-inoculated apples; there were no disease symptoms in the control and mock treatments. The symptoms appeared in both the peel and flesh tissues and on both sides of the inoculated apples. Lesion size increased significantly with time ($p<0.001$) (Table 2) (Figure 3) and developed significantly less on the sun-exposed (red) side of the apples as compared to the shaded (green) one. Lesions developed significantly slower in the peel tissue than in the flesh ($p=0.002$). The peel tissue on the sun-exposed side was the least susceptible part of the inoculated apples, while the flesh tissue on the shaded side was more susceptible. Lesion size was highest in peel tissue on the shaded side, somewhat lower in flesh on the shaded side and lowest on sun-exposed side for both the peel and flesh tissues on day 10 after inoculation (Figure 3; ESM-1: Lesion size).

The effect of sunlight is shown in individual apples with the sun-exposed side of the fruit being more resistant to infection than the shaded side (Table 2: Tolerance). The sun-exposed side of apples has previously been shown to accumulate higher levels of antioxidants and exhibit an improved capacity to resist pathogens [8-11,29]. Inoculated peel tissue developed smaller lesions than the flesh, demonstrating that disease developed faster in the flesh than in the peel. Our results are in further agreement with previous reports, which indicated that the peel had higher contents of antioxidants and an improved capacity to scavenge ROS, than the flesh [9,10,43-45]. The sun-exposed peel tissue had higher levels of antioxidants (protein, phenolics, ORAC, vitamin C and POX activity), and was less susceptible to *B. cinerea* infection, as previously reported [7-10].

Responses in apple antioxidant metabolites to the development of grey mould

Tolerance to grey mould was predicted by high levels of protein ($p<0.001$) and phenolics ($p=0.023$) (Table 2: Tolerance; Figure 2). Similarly, susceptibility was predicted by low concentrations of protein, phenolics, vitamin C, and ORAC (Table 2: Susceptibility; Figure 2). This means that the development of disease (lesion size) was negatively associated with high levels of protein and phenolics ($p<0.001$). Lesion size was also negatively associated with higher levels of vitamin C and ORAC ($p<0.001$). The sun-exposed side of the apples showed higher tolerance than the shaded side ($p=0.002$) (Table 2: Tolerance, Side-Red>Green). The lesions developed much more slowly ($p<0.001$) in the sun-exposed than in the shaded side of apples (Table 2: Susceptibility, Side-Red<Green; Figures 2 & 3). The general trends over time show that inoculation with *B. cinerea* led to strongly decreased levels of protein, phenolics, vitamin C, and ORAC during infection (Tables 2 & 3; Figure 4; ESM-1).

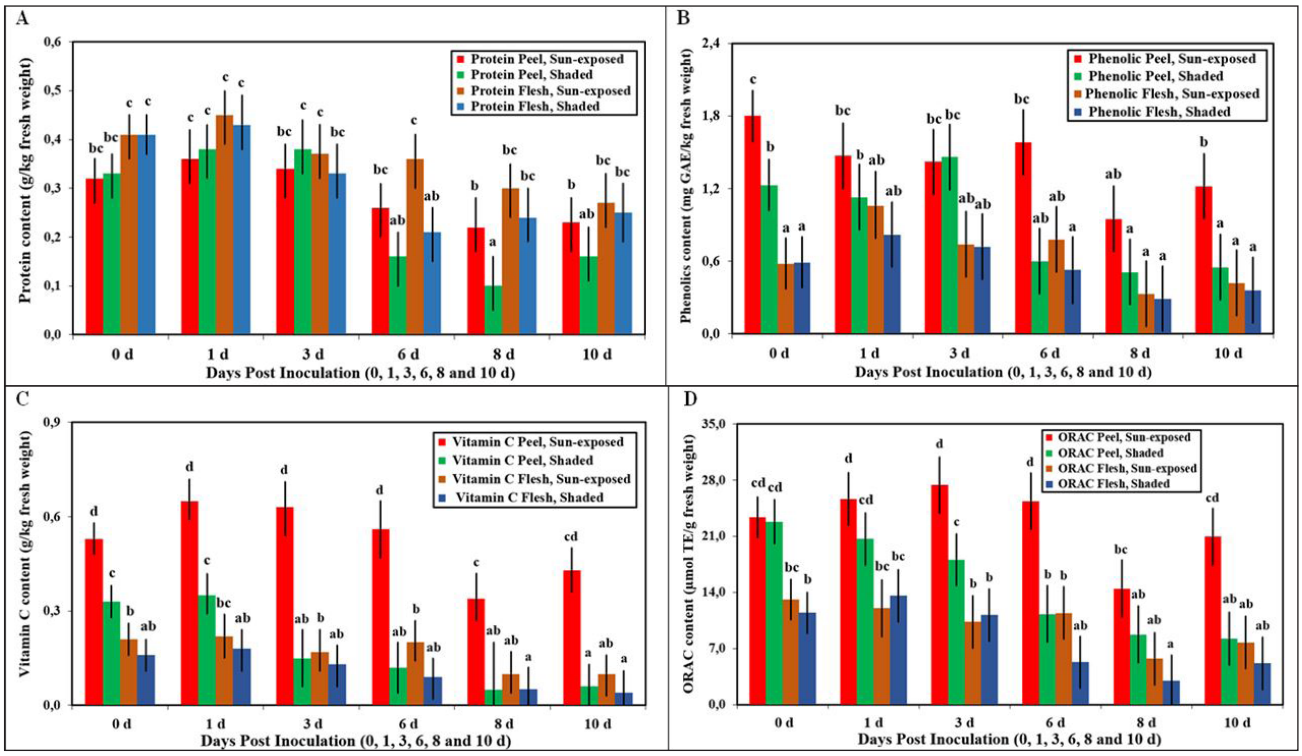

Figure 4: The levels of protein, phenolics, vitamin C and ORAC in 'Braeburn' apples from day 0 until day 10 after infection with *B. cinerea*. Measurements were made for each of all four kinds of tissue, namely peel and flesh from the sun-exposed and the shaded sides in inoculated fruit. Results are obtained from linear models using all combinations of tissue types, side and day as explanatory factors. The analysis is constructed so that all data points in the diagrams can be compared to each other and that non-overlapping confidence limit bars indicate significant differences while the exact probabilities can be found in ESM-1

Protein Content

High protein concentration was the strongest predictor of tolerance ($p<0.001$) and was negatively associated with lesion size ($p<0.001$) (Table 2: Tolerance, Protein; Figure 2). Protein levels were significantly different ($p<0.001$) among the three treatments (Table 3: Protein, Treatment). Using the prediction and interaction tests we found that protein concentration was significantly

lower in apples inoculated with *B. cinerea* than in both the control (p=0.002) and mock (p=0.004) treatments. Protein levels were significantly different in the two-way interaction for Treatment*Side (p<0.001) and for Treatment*Tissue (p=0.016) (Table 3). In general, the levels of protein did not change in the early stages of infection (days 1 and 3), whereas they became lower in the later stages of infection (days 6, 8 and 10) (Figure 4A; ESM-1: Protein; Figure 5; ESM-2: Protein). Using linear models for combinations of type of tissue, side and day as explanatory factors (Figure 4; ESM-1: Protein), we found that protein concentration was higher in the sun-exposed peel than in that on the shaded side on day 8 and that disease developed less in the sun-exposed peel than in both the peel and flesh tissues on the shaded side (Figure 4A).

Response variable/ Explanatory variables	Lesion size (diameter in cm)	Protein (g/kg fw)	Phenolics (mg GAE/ kg fw)	Vitamin C (g/kg fw)	ORAC (µmol TE/ g fw)	SOD (units/ g fw)	POX (units/ g fw)	APX (units/ g fw)	CAT (units/ g fw)	Firmness (kg/cm²)	Sugar content (g sugar/ kg fw)
Treatment	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**n.s.2**	**=0.007**	**n.s.**	**=0.022**	**n.s.**	**n.s.**
Day	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	n.s.	=0.025	n.s.	n.s.
Side	<0.001	n.s.	<0.001	<0.001	<0.001	n.s.	=0.012	=0.032	n.s.	0.003	<0.001
Tissue	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	n.s.	n.s.
Treatment *Day	<0.001	<0.001	<0.001	<0.001	<0.001	n.s.	n.s.	<0.001	n.s.	0.036	n.s.
Treatment*Side	<0.001	<0.001	n.s.	=0.004	<0.001	n.s.	n.s.	n.s.	n.s.	0.016	n.s.
Treatment*Tissue	<0.001	=0.016	=0.006	<0.001	=0.002	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Day*Side	<0.001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	=0.035
Day*Tissue	<0.001	n.s.	n.s.	<0.001	n.s.	<0.001	<0.001	=0.022	n.s.	n.s.	n.s.
Tissue *Side	=0.001	=0.015	<0.001	<0.001	<0.001	n.s.	=0.011	=0.008	n.s.	n.s.	n.s.
Treatment*Day*Side	<0.001	n.s.	n.s.	n.s.	=0.006	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Treatment*Day*Tissue	<0.001	0.001	n.s.	=0.001	=0.011	0.018	n.s.	=0.001	n.s.	n.s.	n.s.
Treatment* Side*Tissue	<0.001	n.s.	n.s.	n.s.	=0.001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Day*Tissue *Side	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	=0.026	n.s.	n.s.	n.s.

Table 3: Results from ANOVA type 2 analyses of linear mixed models using lesion size, antioxidant compounds and enzyme activity in apple fruit following three treatments over time course of the experiment. Response variables were protein, phenolics, vitamin C, ORAC, activity of SOD, POX, APX, and CAT, firmness and sugar content (Brix). Explanatory variables were those in the experimental setup with Day (0, 1, 3, 6, 8, and 10), Side (*sun- exposed* and shaded), Tissue (peel and flesh) and the three treatments (namely Control, *Mock- inoculated* and *B. cinerea*-inoculated)
n.s. stands for 'not significant'

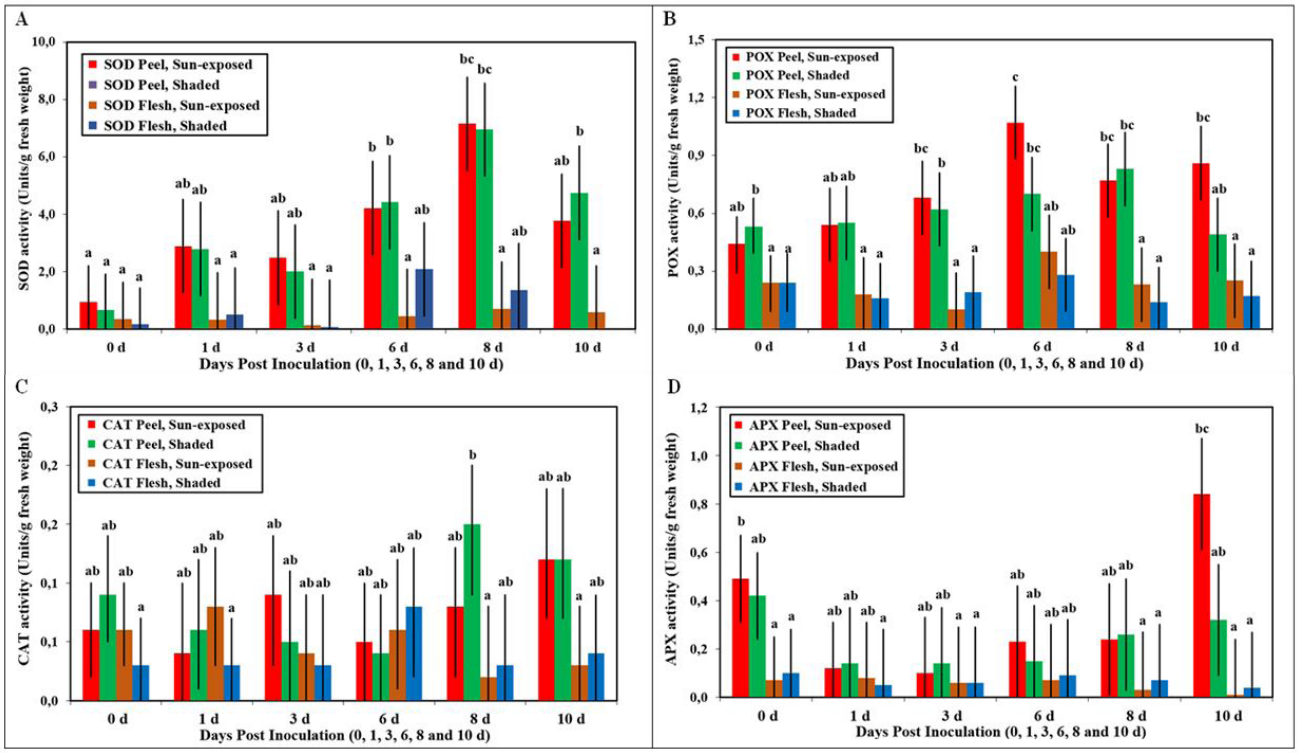

Figure 5: Antioxidant enzyme activities (SOD, POX, APX and CAT) in 'Braeburn' apples from day 0 until day 10 after infection with *Botrytis cinerea*. Measurements were made for each of all four kinds of tissue, namely peel and flesh from the sun-exposed and the shaded sides in inoculated fruit. Results come from linear models using all types of tissue, side, day and their combinations as explanatory factors. The analysis is constructed so that all data points in the diagrams can be compared to each other and that non-overlapping confidence limit bars indicate significant differences while the exact probabilities can be found in ESM-1

Thus, the higher protein content in the peel on the sun-exposed side reflects apples being more tolerant to infection by *B. cinerea*. The level of protein in all four kinds of tissue was lower in the *B. cinerea*-inoculated apples than in the control on day 10 after inoculation (Figure 6; ESM-2: Protein). Several proteins have previously been noted to be consumed by the fungus in the late phase of the infection [46]. Proteins have specific roles in the physiology and quality of apple fruits during harvest and postharvest handling as well as during maturation and in different stages of ripening [47]. The protein content is related to stress response and defense as well as to the energy metabolism of apples [47, 48].

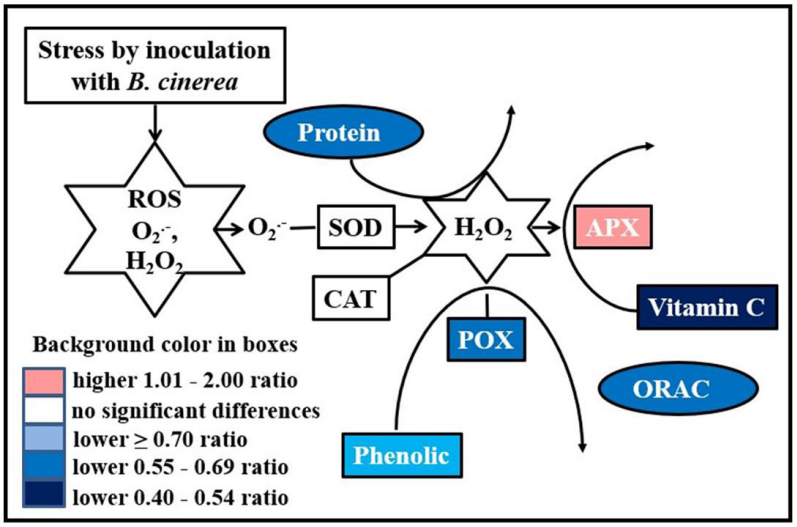

Figure 6: A summary of antioxidant responses in 'Braeburn' apples following infection with *Botrytis cinerea* on day 10 after inoculation. Measurements for all four kinds of tissue were pooled, namely peel and flesh from the sun-exposed and the shaded sides and no separation of tolerant and susceptible fruits. Colors represent relative changes in concentrations of metabolites and enzyme activities of infected apples compared to non-inoculated control apples. White colour indicates no significant change in antioxidant properties in *B. cinerea*-inoculated tissue and control tissue. Pink colour indicates significantly higher values in the inoculated apples at 1.01 to 2.00 ratio. Light blue colour indicates a lower value in the inoculated apples with a ratio ≥ 0.70, medium blue colour denotes decreased ratio (between 0.55 to 0.69) and dark blue colour denotes an even stronger decrease (ratio from 0.40 to 0.54) in *B. cinerea*-inoculated tissue and control tissue

Phenolics

The concentration of phenolics was a strong predictor of tolerance ($p=0.023$) in apples as the level of phenolics was negatively associated with lesion size ($p<0.001$) (Table 2) (Figure 2). Levels of phenolics' were significantly different among the three treatments ($p<0.001$) (Table 3: Phenolics, Treatment). Interaction tests showed that the levels of phenolics were lower in the *B. cinerea*-inoculated apples than in both control ($p=0.005$), and mock-treated apples ($p=0.019$). Levels of phenolics were significantly different in the two-way interaction for Treatment*Day ($p<0.001$), for Treatment*Tissue ($p=0.006$) and for Tissue*Side ($p<0.001$) (Table 3: Phenolic). In general, the levels of phenolics did not change in the early stages of infection (days 1 and 3), and levels of phenolics became lower in the later stages of infection (days 6, 8 and 10) (Figure 4B; ESM-1: Phenolics; Figure 6; ESM-2: Phenolics).

Figure 4(B) shows that the phenolics' content was higher in sun-exposed peel tissues as compared with that in flesh tissues on both sides of the apples over the duration of the experiment in *B. cinerea*-inoculated apples. The level of phenolics decreased as the lesion size increased strongly on the shaded side of fruit in the inoculated apples. Thus, the higher phenolics' content in the peel on the *sun- exposed* side could contribute to an improved oxidative defense and might also mirror apples being more tolerant to infection by *B. cinerea* [49]. Phenolics may be used in defense mechanisms against pathogens through the integration of phenolic esters into cell walls as suggested by Dixon [50]. Phenolic compounds are secondary metabolites with an important role in fruit quality and contribute to taste, color and nutritional properties [51]. Phenolics in fruits are important for postharvest preservation and human health benefits linked to the phenolic-associated antioxidant activity [50].

Vitamin C

Vitamin C content was highest in sun-exposed peel tissues as compared to the other three tissue types over the course of the experiment (Figure 4C; ESM-1: Vitamin C). Lesions developed more slowly on the sun-exposed side of both the peel and the flesh tissue than on the shaded side over the time of the experiment (Figure 3; ESM-1: Lesion size, Sun-exposed). Thus, in *B. cinerea*-inoculated apples, the peel on the sun-exposed side had the highest levels of vitamin C and slower disease development than the other three kinds of tissue. The level of vitamin C decreased strongly from day 6 on as the lesion size increased strongly (disease developed) in *B. cinerea*-inoculated apples (Figure 4C & 6; ESM-2: Vitamin C). We suggest that apple tissue used vitamin C in the defense against *B. cinerea*. Similar results for vitamin C were found by Davey, *et al.* [9] and by Bui, *et al.* [10]. Muckenschnabel, *et al.* [52] reported severe depletion of plant vitamin C during pathogen invasion of plant tissue before high ROS levels were detected. The water-soluble antioxidant vitamin C is the most abundant and powerful antioxidant and may prevent or minimize the damage caused by ROS in plant [53,54]. Fruit cultivars rich in vitamin C have the potential to improve not only fruit nutritional value but also storage properties [9].

The Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay [32] measures the antioxidant capacity of pure compounds or mixtures such as biological fluids. ORAC measurements thus simplify the complex antioxidant mechanisms in vivo to a single reaction *in vitro* measuring the ability of the compounds studied to protect a fluorophore from produced radicals [55]. ORAC levels were higher in peel tissues than in flesh for both the sun-exposed and the shaded side at the start of the experiment (Table 1; Figure 4D; ESM-1: ORAC). Peel on the sun-exposed side had the highest levels of ORAC and slowest development of disease compared to the other three kinds of tissue in *B. cinerea*-inoculated apples. In general, ORAC levels decreased strongly in *B. cinerea*-inoculated apples over the course of the experiment, with the exception of the sun-exposed peel tissue (Figure 4D & 6; ESM-2: ORAC). The timing coincided with the development of the necrosis. This suggests that the drop in ORAC likely is a result of the infection. We suggest that apple tissue also may use other antioxidants captured by the ORAC assay in the defense against *B. cinerea.*

Apple Antioxidant Responses to the Development of Grey Mould

In general, inoculation with *B. cinerea* seemed not to influence the activity of SOD and CAT, while the inoculation with *B. cinerea* had significant influences on the activity of POX ($p<0.001$) and APX ($p<0.021$) (Table 2: Susceptibility). Susceptibility was predicted by low activities of POX and APX in the host tissue (Table 2: Susceptibility). This means that the development of disease (lesion size) was negatively associated with higher levels of POX and APX activity. The primary lesion of *B. cinerea* is formed by fungal hyphae on the host surface. It triggers an oxidative burst leading to the production of H_2O_2 at the plasma membrane of the plant cells [18,19]. Through rapid formation of peroxide, lesions formed by *B. cinerea* deplete antioxidants and lead to a massive perturbation in the redox status in and around the infected tissue [15,19,21,22].

Disease developed slowly over time and peaked on day 8 in the peel tissue on both the sun-exposed and shaded sides (Figure 3; ESM-1: Lesion size, Peel tissue). In the peel tissue, antioxidant enzyme activities changed significantly on day 10 for both the sun-exposed and the shaded sides. Thus, in the inoculated apples POX activity had decreased and that of APX increased as compared with the control (Figure 5B, D & 6; ESM-1 and ESM-2: POX and APX). In the sun-exposed flesh tissue, the lesion size developed slowly until day 6, then strongly increased on days 8 and 10. In the shaded flesh tissue, lesion size developed more rapidly overtime (Figure 3; ESM-1: Lesion size, Flesh shaded). In the flesh tissue, there was no change in antioxidant enzyme activity over time neither for the sun-exposed nor for the shaded sides of the inoculated apples with the exception of a small change in CAT activity in the flesh tissue (Figure 5; ESM-1: Flesh tissue).

SOD Activity

Inoculation with *B. cinerea* led to large changes in SOD activity over time in the peel tissue for both the sun-exposed and the shaded sides (Figure 5A; ESM-1: SOD). The level of SOD varied in the early stages of infection (increased on day 1, decreased on day 3) and increased in the later stages (days 6 and 8) but then decreased on day 10 as compared to the control apples on day 0 (Figure 5A; ESM-1: SOD, Peel sun-exposed and shaded). On day 10, SOD activity for all four kinds of tissue in the inoculated apples returned to control values, viz. there was no difference in SOD activity between the inoculated and control apples (Figure 5A & 6; ESM-2: SOD activity day 10). This may reflect that the host synthesizes SOD in an attempt to detoxify ROS synthesized by the fungus during the early stage of infection [4]. In the early stages of infection, SOD mediates the first step in the formation of H_2O_2 by catalyzing the dismutation of the superoxide anion as an important part of plant detoxification of ROS, which may be involved in pathogenicity [19,21].

It is possible that the increased activity in the later stages of infection could be of *B. cinerea* origin [19]. However, an up regulation of SOD activity by the host in an attempt to deal with oxidative stress is a well-known response of tissues to freezing and pathogen infection [8,11,25,55]. The higher initial SOD activity in peel tissue could help explain its slower development of disease. Our results are in agreement with this notion, because SOD activity in apple fruits increased only at the early stages of the infection with *B. cinerea* (days 1, 6 and 8) then did not change at the late stages of the infection (day 10). It seems that SOD activity in apples was not influenced by the infection with *B. cinerea*, but it may be an important antioxidant enzyme activity in the defense reaction of apple tissue to fungal infection.

POX Activity

POX activity in the inoculated sun-exposed peel increased slightly on all samplings (days 1 through 6) then decreased on days 8 and 10, whereas POX activity in the inoculated shaded peel decreased slightly on day 10 (Figure 5B; ESM-1: POX). Only on day 10, POX activity for all four kinds of tissue in the inoculated apples decreased significantly in relation to control values (significant difference in POX activity between the inoculated and control apples only on day 10) (Figures 5B & 6; ESM-2: POX). Presumably, the phenolics were degraded as a substrate by POX to detoxify H_2O_2 during the infection [8,12,49].

The decrease in POX activity is associated with a lower level of phenolics in *B. cinerea*-inoculated apples in the later stages of the infection. The constitutively higher POX activities could be associated with the higher flavonoid (e.g. anthocyanin) content in sun-exposed tissues. Once again, a higher capacity for H_2O_2 detoxification (POX) seems to correlate with improved disease resistance. The higher capacity to deal with oxidative stress could explain the higher tolerance of peel tissue to *B. cinerea* infection. This suggests that the POX pathway may be down regulated at the later stages of the infection by *B. cinerea* because this enzyme is regulated by the availability of its substrate [10].

APX Activity

APX activity in the sun-exposed peel tissue decreased on days 1 and 3 and then increased on day 10 in *B. cinerea*-inoculated apples as compared to control apples on day 0, while in the shaded peel tissue the activity did not change over time (Figure 5D; ESM-1: APX). Inoculation with *B. cinerea* increased APX activity for all four kinds of tissue only on day 10 as compared to control values (Figures 5D & 6; ESM-2: APX). This may reflect an attempt by the host to detoxify the ROS synthesized during infection [4]. The increase in APX activity was accompanied by decreased levels of vitamin C on day 10.

This suggests that the fruit has reacted to the stress caused by disease development and that APX may be associated with susceptibility or a belated attempt to deal with oxidative stress. When grey mould develops lesions at a later stage, vitamin C is no longer recycled and starts being consumed as a consequence of APX activity [10]. Bui, *et al.* [10] and Tuyet, *et al.* [29], previously reported that inoculation with *B. cinerea* strongly increased APX activity in 'Braeburn' apples. The increase in APX activity may reflect the host's response to detoxify H_2O_2, although APX may also have been produced by the pathogen for a similar purpose. APX is considered to play a major role in scavenging ROS and in protecting both plant and pathogen cells from hydrogen peroxide [55].

CAT Activity

CAT activity in the peel tissue did not change over time, neither for the *sun- exposed* nor the shaded side (Figure 5C; ESM-1: CAT, Peel). CAT activity in the sun-exposed flesh tissue increased slightly on day 3 and in the shaded flesh tissue on day 6 (Figure 5C; ESM-1: CAT, Flesh). In flesh tissue, CAT activity was higher on the shaded than on the sun-exposed side, but CAT activity decreased over time after infection (Figure 5C; ESM-1: CAT, Flesh). On day 10, CAT activity for all four kinds of tissue in the inoculated apples returned to control values as there was no change in the values of CAT activity between the inoculated apples and the control apples (Figure 5C & 6; ESM-2: CAT activity day 10).

Similarly, Bui, *et al.* [10] previously reported that CAT activity did not change in 'Braeburn' apples after inoculation with *B. cinerea* and during the development of the disease. CAT activity could be of both plant and *B. cinerea* origin. Plant CAT activity may improve plant health, especially during infection by *B. cinerea* [55]. There are seven CAT genes in the *B. cinerea* genome, however, none of them seem to be important to infect and develop in the tissues of different plant species [4,18,19,51,55]. There are also other important detoxification systems some of which were mentioned above, that can lead CAT activity not to change during infection and disease development [4,10,15].

Conclusion

The tolerance mechanism of apples to grey mould infection is complex and is influenced by many different factors involved in ROS detoxification in the diseased tissue. Through advanced statistics and principal component analysis we could show that high levels of protein and phenolics were positively associated with the tolerance of apple fruits to grey mould. To our knowledge, such information has not been published before. The overall antioxidant capacity decreased over time during infection with grey mould, except for APX activity, which increased. The results also indicate that sun-exposure produces fruit with higher levels of antioxidants, which improves its tolerance to infection with grey mould. Ideally, this study should be repeated on a larger scale using entirely natural causes of infection. It would also be of value to follow physiological parameters during infection with increased time resolution, especially at the early stages when oxidative bursts are considered to be most prevalent.

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Supplementary Data

References

1. Fillinger S, Elad Y (2016) Botrytis - the Fungus, the Pathogen and its Management in Agricultural Systems. Springer, Switzerland.

2. Elad Y, Pertot I, Prado AMC, Stewart A (2016) Plant Host of Botrytis spp. In: Fillinger S and Elad Y (eds) Botrytis - the Fungus, the Pathogen and its Management in Agricultural Systems. Springer, Switzerland, pp. 413-86.

3. Romanazzi G, Feliziani E (2014) Botrytis cinerea (Gray Mold). In: Bautista-Banos S (1st Edn) Postharvest Decay Control Strategies. Elsevier, UK, pp.131-46.

4. Siegmund U, Viefhues A (2016) Reactive oxygen species in the Botrytis - host interaction. In: Fillinger S and Elad Y (eds) Botrytis - the Fungus, the Pathogen and its Management in Agricultural Systems. Springer, Switzerland, pp. 269-89.

5. van Kan JAL (2006) Review: Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends Plant Sci. 11: 247-53.

6. Sholberg PL, Conway WS (2004) Postharvest Pathology. In: Gross KC, Wang CY, SaltveitM (eds) Agriculture Handbook Number 66: The Commercial Storage of Fruits, Vegetables, and Florist and Nursery Stocks.

7. Solovchenko A, Schmitz-Eiberger M (2003) Significance of skin flavonoids for UV-B-protection in apple fruits. J Exp Bot 54: 1977-84.

8. Zupan A, Mikulic Petkovsek M, Slatnar A, Stampar F, Veberic R (2014) Individual phenolic response and peroxidase activity in peel of differently sun-exposed apples in the period favorable for sunburn occurrence. J Plant Physiol 171: 1706-12.

9. Davey MW, Auwerkerken A, Keulemans J (2007) Relationship of apple vitamin C and antioxidant contents to harvest date and postharvest pathogen infection. J Sci Food Agric 87: 802-13.

10. Bui TAT, Wright SAI, Falk AB, Vanwalleghem T, Van Hemelrijck W, et al. (2019) Botrytis cinerea differentially induces post-harvest antioxidant responses in 'Braeburn' and 'Golden Delicious' apple fruit. J Sci Food Agric 99: 5662-70.

11. Ballaré CL (2014) Light Regulation of Plant Defense. Annu Rev Plant Biol 65: 335-63.

12. Jakopic J, Stampar F, Veberic R (2009) The influence of exposure to light on the phenolic content of 'Fuji' apple. Sci Hortic 123: 234-39.

13. Canessa P, Schumacher J, Hevia MA, Tudzynski P, Larrondo LF (2013) Assessing the Effects of Light on Differentiation and Virulence of the Plant Pathogen *Botrytis cinerea*: Characterization of the White Collar Complex. PLoS One 8: e84223.

14. Schumacher J (2017) How light affects the file of Botrytis. Fungal Genet Biol 106: 26-41.

15. Torres MA, Jones JDG, Dangl J (2006) Reactive oxygen species signaling in response to pathogens. Plant Physiol 141: 373-78.

16. Dickman, MB, Fluhr, R (2013) Centrality of Host Cell Death in Plant-Microbe Interactions. Annu Rev Phytopathol 51: 543-70.

17. Shlezinger N, Minz A, Gur Y, Hatam I, Dagdas YF, et al. (2011) Anti-Apoptotic Machinery Protects the Necrotrophic Fungus Botrytis cinerea from Host-Induced Apoptotic-Like Cell Death during Plant Infection. PLoS Pathog 7: e1002185.

18. Tudzynski P, Kokkelink L (2009) Botrytis cinerea: molecular aspects of a necrotrophic life style. In: Deising HB (ed) The Mycota 5. Springer, Germany, pp. 29-50.

19. Heller J, Tudzynski P (2011) Reactive oxygen species in phytopathogenic fungi: signaling, development and disease. Annu Rev Phytopathol 49: 369-90.

20. Alscher RG, Erturk N, Heath LS (2002) Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J Exp Bot 53: 1331-41.

21. Larrigaudière C, Vilaplana R, Soria Y, Recasens I (2004) Oxidative behaviour of Blanquilla pears treated with 1-methylcyclopropene during cold storage. J Sci Food Agric 84: 1871-7.

22. Foyer CH, Noctor G (2011) Review: Ascorbate and glutathione: The heart of the redox hub. Plant Physiol 155: 2-18.

23. Kevers C, Pincemail J, Tabart J, Defraigne JO, Dommes J (2011) Influence of cultivar, harvest time, storage conditions, and peeling on the antioxidant capacity and phenolic and ascorbic acid contents of apples and pears. J Agric Food Chem 59: 6165-71.

24. Lata B (2007) Relationship between apple peel and the whole fruit antioxidant content: Year and cultivar variation. J Agric Food Chem 55: 663-71.

25. Awad MA, De Jager A, van der Plas, LHW, van der Krol AR (2001) Flavonoid and chlorogenic acid changes in skin of 'Elstar' and 'Jonagold' apples during development and ripening. Sci Hortic 90: 69-83.

26. Rahman MM, Das R, Hoque MM, Zaman W (2015) Effect of freeze drying on antioxidant activity and phenolic contents of mango (Managifera indica). Int Food Res J 22: 613-17.

27. Williams B, Kabbage M, Kim H, Britt R, Dickman MB (2011) Tipping the Balance: Sclerotinia sclerotiorum Secreted Oxalic Acid Suppresses Host Defenses by Manipulating the Host Redox Environment. PloS Pathog 7: e1002107.

28. Grappadelli CL (2003) Light Relations. In: Ferree DC, and Warrington IJ (eds) Apple: Botany, Production and Uses. CABI, UK, pp. 195-216.

31. Davey DW, Dekempeneer E, Keulemans J (2003) Rocket-powered high-performance liquid chromatographic analysis of plant ascorbate and glutathione. Anal Biochem 316: 74-81.

32. Franck C, Baetens M, Lammertyn J, Verboven P, Davey MW, et al. (2003) Ascorbic acid concentration in cv. Conference pears during fruit development and postharvest storage. J Sci Food Agric 51: 4757-63.

33. Ou BX, Hampsch-Woodill M, Prior RL (2001) Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. J Agric Food Chem 49: 4619-26.

34. R Core Team (2017) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

35. Oksanen J, Guillaume BF, Friendly M, Kindt R, Legendre P, et al. (2017) Vegan: Community Ecology package. R package version 2.4-4.

36. Bates D, Maechler M, Bolker B, Walker S (2015) Fitting Linear Mixed-Effects Models Using lme4. J Stat Softw 67: 1-48.

37. Kuznetsova A, Bruun Brockhoff PB, Bojesen Christensen RHB (2016) lmerTest: Tests in Linear Mixed Effects Models. R package version 2.0-33.

38. Fox J (2003) Effect Displays in R for Generalised Linear Models. J Stat Softw 8: 1-27.

39. De Rosario-Martinez H (2015) Phia: Post-hoc Interaction Analysis.

40. Fox J, Weisberg S (2011) An {R} Companion to Applied Regression, Second Edition. Thousand Oaks CA: Sage.

42. Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48: 909-30.

43. Chinnici F, Bendini A, Gaiani A, Riponi C (2004) Radical Scavenging Activities of Peels and Pulps from cv. Golden Delicious Apples as Related to Their Phenolic Composition. J Agric Food Chem 52: 4684-89.

44. Drogoudi P, Michailidis Z, Pantelidis G (2008) Peel and flesh antioxidant content and harvest quality characteristics of seven apple cultivars. Sci Hortic 115: 149-53.

45. Carbone K, Giannini B, Picchi V, Lo Scalzo R, Cecchini F (2011) Phenolic composition and free radical scavenging activity of different apple varieties in relation to the cultivar, tissue type and storage. Food Chem 127: 493-500.

46. Michielse CB, Becker M, Heller J, Moraga J, Collado IG, Tudzynski P (2011) The Botrytis cinerea Reg1 Protein, a Putative Transcriptional Regulator, Is Required for Pathogenicity, Conidiogenesis, and the Production of Secondary Metabolites. Mol Plant Microbe Interact 24: 1074-1085.

47. Shi Y, Jiang L, Zhang L, Kang R, Yu Z (2014) Dynamic changes in proteins during apple (*Malus x domestica*) fruit ripening and storage. Hortic Res 1: 6.

48. Qin G, Wang Q, Liu J, Li B, Tian S (2009) Proteomic analysis of changes in mitochondrial protein expression during fruit senescence. Proteomics 9: 4241-53.

49. Rysman T, Van Hecke T, De Smet S, Van Royen G (2016) Ascorbate and Apple Phenolics Protein Oxidation in Emulsion-type Sausages during Storage and in Vitro Digestion. J Agric Food Chem 64: 4131-8.

50. Tomás-Barberán F, Espin JC (2001) Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. J Sci Food Agric 81: 853-876.

51. Adyanthaya I, Kwon YI, Apostolidis E, Shetty K (2009) Apple postharvest preservation is linked to phenolic content and superoxide dismutase activity. J Food Biochem 33: 535-56.

52. Muckenschnabel I, Goodman BA, Williamson B, Lyon GD, Deighton N (2002) Infection of leaves of Arabidopsis thaliana by Botrytis cinerea: changes in ascorbic acid, free radicals and lipid peroxidation products. J Exp Bot 53: 207-14.

53. Smirnoff N (2005) Ascorbate, tocopherol and carotenoids: metabolism, pathway engineering and functions. In: Smirnoff N (ed) Antioxidants and Reactive Oxygen Species in Plants. Blackwell, UK, pp. 53-86.

54. Jones MA, Smirnoff N (2005) Reactive oxygen species in plant development and pathogen defense. In: Smirnoff N (ed) Antioxidants and Reactive Oxygen Species in Plants. Blackwell, UK, pp. 197-214.

55. Bentayeb K, Vera P, Rubio C, Nerín C (2014) The additive properties of Oxygen Radical Absorbance Capacity (ORAC) assay: The case of essential oils. Food Chem 148: 204-208.