Botrytis cinerea differentially induces postharvest antioxidant responses in ‘Braeburn’ and ‘Golden Delicious’ apple fruit

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Abstract

BACKGROUND: The fruit of two apple cultivars – ‘Braeburn’, which is susceptible to inoculation with Botrytis cinerea, and the less susceptible cv. ‘Golden Delicious’ – were investigated with respect to their response to inoculation with B. cinerea. Successful infection by B. cinerea leads to an oxidative burst and perturbation of plant redox homeostasis. To investigate the interaction between apple fruit and B. cinerea, antioxidant metabolism in fruit samples from sun-exposed and shaded sides of different tissue types was measured over time.

RESULTS: The sun-exposed tissue of ‘Braeburn’ had higher initial levels of total vitamin C in the peel and phenolic compounds in the flesh than ‘Golden Delicious’, despite its greater susceptibility to gray mold. A substantial antioxidant response was recorded in diseased ‘Braeburn’ fruit 14 days after inoculation, which involved an elevated superoxide dismutase activity and ascorbate peroxidase activity, a progressive oxidation of total vitamin C, and a decrease in peroxidase activity and phenolic content. Disease development was slower on the sun-exposed sides than on the shaded sides.

CONCLUSION: The two cultivars appeared to utilize different strategies to defend themselves against B. cinerea. ‘Golden Delicious’ almost entirely escaped infection. Preharvest exposure of apple fruit to high light / temperature stress appears to prepare them to better resist subsequent postharvest attack and disease.

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Keywords: Malus × domestica; postharvest storage; Botrytis cinerea; antioxidant metabolism

INTRODUCTION

Apples (Malus × domestica Borkh.) are a major source of antioxidants, such as ascorbic acid (AsA), also known as vitamin C, and phenolic compounds.1 Preharvest conditions during the cultivation of apples have an impact on the levels of phytochemicals and these can relate to the postharvest resistance of apple fruit to pathogens. Botrytis cinerea is a necrotrophic pathogen, which causes preharvest and postharvest disease (gray mold) in apple.2,3

Stress conditions during the postharvest period can lead to the accumulation of reactive oxygen species (ROS), such as superoxide (O2−), singlet oxygen (¹O2), hydroxyl radicals (OH−), and hydrogen peroxide (H2O2), which can have a detrimental effect on cellular metabolism.4 However, plant cells are to some extent protected from ROS by their antioxidant system, consisting of radical scavengers and detoxifying agents.5 The first redox reaction is catalyzed by the enzyme superoxide dismutase, which converts superoxide (O2−) into H2O2. An excess of H2O2 is toxic to cells, so H2O2 needs to be metabolized further. Flavonoid peroxidases

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(POX; EC 1.11.1.7), ascorbate peroxidase (APX; EC 1.11.1.11), and catalase (CAT; EC 1.11.1.6) all have the ability to convert \( \text{H}_2\text{O}_2 \) to water. Flavonoid peroxidases detoxify \( \text{H}_2\text{O}_2 \) by using flavonoids as substrates. Catalase is thought to metabolize \( \text{H}_2\text{O}_2 \) to water and oxygen via the iron-heme groups that are attached to the enzyme. Ascorbate peroxidase detoxifies \( \text{H}_2\text{O}_2 \) by using AsA as a reducing agent, yielding monodehydroascorbate (MDHA), a radical form, and dehydroascorbate (DHA) – both oxidized and reduced back to AsA by monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR), respectively. The chemical balance between the reduced and oxidized forms of ascorbic acid is maintained through the AsA-glutathione cycle.6 Maintaining a large pool of AsA and an efficient recycling mechanism is crucial for the protection of cells against oxidative stress. Dehydroascorbate and MDHA are transient species, and these oxidized forms thus typically constitute minor proportions of the total vitamin C content in plant tissues.7 It is generally accepted that preharvest exposure and adaptation to stress, for instance via high light / high-temperature regimes, increase the antioxidant content in apples.8,9 Apple tissues with high levels of antioxidants, and in particular of AsA, are less susceptible to inoculation with \( B. \text{cinerea} \).10 In the early stages of gray mold infection, \( \text{H}_2\text{O}_2 \) accumulates and is converted to ROS, resulting in host cell death.11 The protocol to colonize the plants successfully.11,12 The functions of ROS in plants and \( B. \text{cinerea} \) are multiple, including signaling aspects.13,14 During pathogenesis, there is histochimical evidence that \( B. \text{cinerea} \) produces \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) in hyphal germ tubes at the infection site,15 and that its superoxide dismutases (SOD; EC 1.15.1.1) may be involved in pathogenicity.16 Two apple cultivars, ‘Braeburn’ and ‘Golden Delicious’ were selected to elucidate further the influence of preharvest sun exposure and antioxidant metabolism on subsequent postharvest disease development caused by \( B. \text{cinerea} \). The relationships between the antioxidant content and antioxidant enzyme activity in both peel and flesh were analyzed in relation to the susceptibility of fruit to \( B. \text{cinerea} \), studying both the sun-exposed and shaded sides of apple fruit.

MATERIALS AND METHODS

Fruit

The ‘Braeburn’ (‘Br’) and ‘Golden Delicious’ (‘GD’) apple cultivars were selected based on their differences in color and susceptibility to \( B. \text{cinerea} \). ‘Br’ has a bicolor fruit with a clear difference between the red, sun-exposed and the green, shaded side of the fruit, whereas both sides of the ‘GD’ fruit are different shades of yellow. Random samples of healthy apple fruit of comparable size were harvested in the fall of 2008 (22 September 2008 for ‘GD’ and 22 October 2008 for ‘Br’) from trees grown at the Research Station for Fruit Cultivation (Proefcentrum Fruiteente, Sint-Truiden, Belgium (50° 46’ 21.82” N, 5° 09’ 36.10” E)). Apple fruit were stored at Proefcentrum Fruiteente under controlled atmosphere conditions (for ‘Br’ 0.5% \( \text{CO}_2, \) 1–2% \( \text{O}_2, \) 2–2.5% \( \text{CO}_2, \) 95% RH, whereas for ‘GD’ 1% \( \text{CO}_2, \) 2.5–3.0% \( \text{O}_2, \) < 8% \( \text{CO}_2, \) 95% RH), until July 2009, when inoculation was carried out at KU Leuven, Belgium.

Sampling for biochemical analyses

For the analysis of antioxidant metabolism and of antioxidant enzyme activity, samples of apple tissue taken from the center of the point of inoculation were excised, using a 0.5 cm diameter cork-borer, weighed, and immediately frozen in liquid nitrogen. The upper 0.2 cm of the fruit plug was considered peel sample, and the remainder, at 0.2 to 2.0 cm from the surface, was considered flesh (hypanthium – fruit cortex) sample.

Inoculation with \( B. \text{cinerea} \)

\( B. \text{cinerea} \) strain B05.10 was obtained from the Center of Microbial and Plant Genetics (CMPG unit PFI) at KU Leuven. Cultivation and spore harvesting were performed as described by Broekaert et al.17 Round apple wounds, 6 mm deep and 3 mm wide, were created by pressing a sterile metal tool into the equators of each apple cheek. The apples were treated on both the sun-exposed and the shaded sides of the fruit by introducing either 10 \( \mu \text{L} \) of sterile water (mock treatment) or 10 \( \mu \text{L} \) of spore suspension (inoculated treatment) into each wound. The spore suspension contained \( B. \text{cinerea} \) spores at a concentration of \( 1.5 \times 10^5 \text{ mL}^{-1} \). Controls were neither wounded nor inoculated. Following treatment, fruit were stored at room temperature (20 ± 2 °C), at 100% RH for the first 24 h, and then at 80% RH for the remainder of the experiment by placing them in sealed plastic bags. At the start of the experiment, 46 similar fruit per cultivar were selected. Ten fruit per cultivar were immediately employed as non-inoculated control fruit at 0 days. Of the 36 remaining fruit per cultivar, 12 were designated as non-inoculated controls for 5 days and 14 days post inoculation. Similarly, 12 fruit were mock-inoculated and 12 were inoculated with \( B. \text{cinerea} \). For each treatment, six fruit per cultivar were evaluated 5 days after treatment and the remaining six fruit per treatment 14 days after treatment. When scoring the infection response, the inoculated fruit were classified either as tolerant, apples without disease symptoms, or as susceptible, apples with symptoms.

Evaluation of disease progress

The quantification of disease symptom development was carried out by measuring the diameter of the lesion (in mm) emerging from the point of inoculation in the peel. Three treatments (control, mock-inoculated, and \( B. \text{cinerea}-\)inoculated) were scored on both the sun-exposed and shaded sides of the two apple cultivars at the three time points of the experiment: 0 days, 5 days and 14 days post inoculation. The number of apples with characteristic symptoms of gray mold were counted 5 and 14 days post inoculation.

Biochemical measurements

Apple tissue extraction

Extraction of fruit tissue was carried out according to Ahn et al.18 Protein content was determined according to Bradford,19 using bovine serum albumin as a standard.

Analysis of antioxidant enzyme activity

All enzymatic kinetic analyses were implemented on quartz 96-well microtiter plates (Hellma™, VWR, Belgium) in a total assay volume of 200 \( \mu \text{L} \), consisting of 20 \( \mu \text{L} \) of enzyme extract and 180 \( \mu \text{L} \) of reaction mixture. Readings were recorded every 30 s using a Multiskan spectrum-microplate spectrophotometer (Thermo Lab Systems, Helsinki, Finland). Enzyme activity was expressed as units per g of fresh weight.

The activity of SOD was assayed following Banowetz et al.20 The commercial standard of superoxide dismutase from bovine liver (15 000 units) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). A stock solution of the SOD standard was prepared of 2 g L\(^{-1}\)
in water, equivalent to 3277 units per mg protein, and stored at 
−20 °C. A standard curve was generated for the range of 1 to 
100 μM L−1.

The activity of POX was analyzed essentially as described by 
Chance and Maehly,21 with slight modifications. First, 180 μL of the 
reaction mixture, containing 50 mmol L−1 potassium phosphate 
buffer pH 7.0, 0.01 mol L−1 EDTA (ethylene-diamine-tetra-acetic 
acid), 0.02 mol L−1 pyrogallol and 1.47 mmol L−1 H2O2, was added 
to the wells of a 96-well plate. Subsequently, 20 μL of enzyme 
extract was added to the wells, with triplicates for each treatment 
replicate. Finally, the plate was agitated briefly and incubated in 
the microplate spectrophotometer for 30 s before absorbance was 
recorded at 470 nm.

The activity of APX was monitored by using a slightly modified 
version of Nakano and Asada’s method.22 First, 180 μL of the 
reaction mixture, containing 50 mmol L−1 potassium phosphate buffer 
ph 7.0, 0.1 mmol L−1 EDTA, 0.88 mmol L−1 AsA, and 0.1 mmol L−1 
H2O2, was added to the wells of a 96-well plate. Subsequently, 
20 μL enzyme extract was added to the wells, using triplicates for 
each sample. Finally, the plate was agitated slightly and incu-
bated in the microplate spectrophotometer for 30 s, after which 
absorbance was recorded at 290 nm (extinction coefficient of 
2.8 mM−1 cm−1). The reaction rate was measured during the first 
30–90 s.

The activity of CAT was measured based on Aebi’s method,23 
with slight modifications. The reaction mixture contained 10 mmol 
L−1 H2O2 in 50 mM potassium phosphate buffer at pH 7.0. First, 
180 μL of reaction mixture was distributed into each well of a 
quartz 96-well microtiter plate (VWR), then 20 μL of CAT standard 
at eight different concentrations, was added in triplicate for each 
concentration of CAT standard, in three columns of the 96-well 
plate. Then, 20 μL enzyme extract was added to separate wells 
in another part of the plate, with triplicates for each treatment 
replicate. Finally, the plate was agitated briefly and incubated in 
the microplate spectrophotometer for 30 s before the disappear-
ance of hydrogen peroxide was monitored at 240 nm. The rate 
was calculated from the initial linear portion of the curve. A stan-
dard of CAT from bovine liver was purchased from Sigma-Aldrich 
(Steinheim, Germany).

Analysis of antioxidants

The phenolic content in apple fruit tissues was measured accord-
ing to a modified version of the Folin–Ciocalteu assay.24 Total 
cvitamin C was extracted by transferring approximately 0.2 g of 
tissue to a 1.5 mL reaction tube followed by addition of 1 mL of 
reaction buffer containing 3% metaphosphoric acid and 1 mmol 
L−1 EDTA. The tubes were vortexed and stored on ice until cen-
trifugation for 10 min at 10 000 x g in a precooled microcentrifuge. 
The supernatant was filtered through a PVDP filter with 0.45 μm 
pore size (Millipore, Brussels, Belgium) and analyzed immediately 
for total vitamin C content, consisting of AsA and DHA. Total 
cvitamin C content was quantified by high-performance liquid 
chromatography (HPLC) analysis, as described by Franck et al.,25 
and expressed on a fresh weight basis.

Tissues used in the analysis

Antioxidant enzyme activity and antioxidant content were mea-
sured in both the peel and flesh tissues in the three treatments 
at the three time points of the experiment. An exception was the 
measurement of phenolic content, for which only flesh tissue was 
used. The peel tissue could not be analyzed for phenolic com-
ounds, due to the limited amount of tissue available.

Statistical analysis

Statistical analyses were carried out using R (version 3.5.1, R devel-
opment core team, Vienna, Austria, 2018). The response variables 
were continuous, so the errors were assumed to follow a Gaussian 
distribution. Overall statistical analysis was by multivariate analysis 
of variance (MANOVA) using the manova function in R. Pairwise 
tests for mean differences were carried out with the emmeans func-
tion of the emmeans (previously lsmeans) package in R, followed 
by Tukey’s test to control the family-wise error rate at a prede-
termined α = 0.05. Alternatively, after a significant ANOVA, pair-
twise t-tests were performed using the pairwise.t.test function in 
package stats in R, with non-pooled standard deviation and no 
correction for multiple comparisons. Both procedures were con-
sidered suitable for unbalanced designs. Letters for indicating sig-
nificant differences among multiple comparisons were generated 
using the CLD function (package emmeans), or the multcompLet-
ters function of the multcompView package.

RESULTS

Overall statistical analysis

The focus of this study was to examine the effect of treatment 
on a set of dependent components, in particular, on lesion size, 
which was analyzed by MANOVA (Table 1). The treatment factor 
consisted of three levels: B. cinerea inoculation, mock inoculation, 
and control. Other categorical factors included in the MANOVA 
were: cultivar (levels ‘Br’ and ‘GD’), side (levels sun-exposed and 
shaded), tissue (levels peel and flesh), and day (levels 0 days, 
5 days and 14 days). Dependent components were lesion size, 
AsA, phenolics, and enzyme activities (SOD, POX, APX, and CAT).

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AsA, phenolics, and enzyme activities (SOD, POX, APX, and CAT).

All categorical factors were significant in the overall MANOVA. 
However, many significant interaction factors were also observed, 
calling for further analysis.

Development of disease

The MANOVA demonstrated that treatment, cultivar, and day 
significantly influenced lesion size (Table 1). The factor ‘side’ was 
also significantly different. The lesion sizes appeared smaller on 
the sun-exposed sides than on the shaded sides (Fig. 1). The shaded 
sides of ‘Br’ fruit were almost all displaying symptoms at 5 days, 
whereas a similar situation did not appear until 14 days for the 
sun-exposed sides (data not shown). At 5 days post inoculation, 
disease symptoms were observed on both sides of ‘Br’ apple 
fruit inoculated with B. cinerea (Fig. 1(A), (B)). The lesions were 
tiny on the sun-exposed sides (Fig. 1(A)), but they had grown 
significantly in size by 14 days post inoculation (Fig. 2). For ‘GD’ 
apples, disease symptoms were observed only on the shaded 
sides of a few ‘GD’ apple fruit (Fig. 1(D)). The sun-exposed sides 
of ‘GD’ apples did not develop disease (Fig. 1(C)), even at 14 
days. For ‘Br’ shaded sides, the high incidence recorded at 5 
days remained for 14 days, indicating early onset of disease. 
However, for both ‘Br’ sun-exposed and ‘GD’ shaded fruit, the low 
disease incidence doubled between 5 days and 14 days (data not 
shown), indicating a slow progression over time. The ranking 
of susceptibility for the four tested tissues was identical, regardless 
of whether disease incidence (percentage infected fruit) or severity 
(lesion size) was used as measure: ‘Br’ shaded (most susceptible) > 
‘Br’ sun exposed > ‘GD’ shaded > ‘GD’ sun exposed (least suscepti-
ble). The Spearman rank correlation coefficient between 
disease incidence and lesion size was equal to 1. In conclusion, ‘Br’ 
was more susceptible to gray mold than ‘GD’.

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Table 1. Summary of MANOVA for components tested. The dataset was analyzed in two separate MANOVAs with the categorical variables (source of variation) as independent variables (predictors) and the continuous variables (components) as dependent variables.

| Source of variation | Interactions |
|---------------------|--------------|
| Treatment (A)       | 2            |
| Cultivar (B)        | 1            |
| Side (C)            | 1            |
| Tissue* (D)         | 1            |
| Day (E)             | 1            |
|                     | 2            |
|                     | 2            |
|                     | 2            |
|                     | 2            |
|                     | 1            |
|                     | 1            |
|                     | 1            |
|                     | 2            |
|                     | 2            |
| dfb                 | 21           |
| Overall MANOVA      | ***          |
| Componentsb         | ***          |
| Lesion size         | ***          |
| V C                 | ***          |
| Phe                 | NS           |
| SOD                 | ***          |
| POX                 | *            |
| APX                 | ***          |
| CAT                 | NS           |

The summary table is based on the MANOVA in which tissue is included as predictor and phenolic content was excluded, because phenolic data was only available for flesh tissue (Table S1a). The second MANOVA included phenolics and excluded tissue (Table S1b); from which the results for the analysis of phenolics were transferred into the summary table. The analysis was done in R using the MANOVA function of package stats.

* Tissue is not a relevant predictor for the dependent variable lesion size, because peel and flesh have the same lesion size (thus, indicated NA).

b Abbreviations: df, degrees of freedom; Vit C, total vitamin C content; Phe, phenolic content; SOD, superoxide dismutase activity; POX, flavonoid peroxidase activity; APX, ascorbate peroxidase activity; CAT, catalase activity; NS, not significant (P > 0.05); NA, not applicable.

P < 0.05. **P < 0.01. ***P < 0.001.

Figure 1. Symptoms of B. cinerea at 5 days post inoculation; representative fruit of 'Braeburn' sun-exposed sides (A), and shaded sides (B), and fruit of 'Golden Delicious' showing the absence of symptoms on the sun-exposed sides (C), and occasional symptoms on shaded sides (D).

Antioxidant metabolite content

Total vitamin C Content

The MANOVA demonstrated that all factors significantly influenced total vitamin C content (Table 1). Total vitamin C content tended to be higher in peel as compared to flesh tissues, for both ‘Br’ and ‘GD’ (Table S2). The total vitamin C content of sun-exposed peel tissue of ‘Br’ was approximately tenfold higher than that of sun-exposed flesh tissues. Sun-exposed peel of ‘Br’ had significantly higher total vitamin C content than any other tissue examined (Fig. 2 and Table S2); as much as fivefold or sixfold higher than sun-exposed peel tissue of ‘GD’. Over the course of the experiment, the total vitamin C content in...
Figure 2. The change over time of total vitamin C content in peel (bars) and in lesion sizes (lines) of apples of the cultivars ‘Braeburn’ (‘Br’) and ‘Golden Delicious’ (‘GD’), at 5 and 14 days after inoculation with \( B. \text{cinerea} \). The inoculated, sun-exposed sides of ‘GD’ were symptomless at all time points; thus, the data is not presented. The treatments were: control (neither wounded, nor inoculated) at 0 days post inoculation, and \( B. \text{cinerea} \)-inoculated (wounded and inoculated with 10 µL of a \( 1.5 \times 10^5 \) mL\(^{-1} \) \( B. \text{cinerea} \) spore suspension). The results from separate t-tests of total vitamin C content and lesion size without pooled standard deviations are shown as capital letters (total vitamin C content) and as small letters (lesion size), respectively. Measurements with the same letter were not significantly different at \( P \leq 0.05 \).

the peel tissues of \( B. \text{cinerea} \)-inoculated ‘Br’ apples was reduced (Fig. 2). At 14 days post inoculation, when rot of gray mold was extensive on the shaded side of inoculated ‘Br’ fruit, the total vitamin C content in peel was no longer detectable, whereas it was significantly reduced in sun-exposed apple peel (Fig. 2, and Table S3).

**Disease development and vitamin C content**

The relationship between total vitamin C content and lesion size over time is presented in Fig. 2. Overall, the total vitamin C content in the apples decreased as the lesion size increased. However, the total vitamin C content at 0 days was significantly different among different apple sides and cultivars (Fig. 2). At 5 days post inoculation, the total vitamin C content of the sun-exposed side of ‘Br’ was still significantly higher than those of the shaded sides of ‘Br’ and ‘GD’. The largest increase in lesion sizes from 5 to 14 days post inoculation was seen on the shaded side of ‘Br’ (Fig. 2).

**Total phenolic content**

In the MANOVA (Table 1), a significant effect on phenolic content was found only for factor day. At the start of the experiment, the phenolic content was significantly higher in flesh tissue of ‘Br’ as compared to the flesh tissue of ‘GD’, especially on the sun-exposed side (Table S2). In general, sun exposure did not have a significant influence on phenolic content, as seen in samples taken at 0 days (Table S2), and as also indicated by the MANOVA results. There was no difference in phenolic content in ‘Br’ flesh tissues at 14 days when the content in \( B. \text{cinerea} \)-inoculated treatments was compared by t-tests to that of the mock-inoculated and the non-inoculated controls. However, by dividing the \( B. \text{cinerea} \)-inoculated ‘Br’ apples at 14 days into susceptible and tolerant reactions, it was apparent that the phenolic content was lower at 14 days only in the susceptible flesh tissue \( (0.67 \pm 0.16 \text{ mg kg}^{-1}) \), as compared to the control at day 0 \( (0.92 \pm 0.10 \text{ mg kg}^{-1}) \), or to the controls at day 5 (non-inoculated control, \( 1.03 \pm 0.13 \text{ mg kg}^{-1} \); mock-inoculated control \( 1.07 \pm 0.15 \text{ mg kg}^{-1} \)). The phenolic content also decreased in susceptible ‘Br’ flesh from 5 days \( (0.98 \pm 0.20 \text{ mg kg}^{-1}) \) to 14 days post inoculation.

**Antioxidant enzyme activity**

*Superoxide dismutase (SOD) activity*

The MANOVA demonstrated that the factors treatment, cultivar, tissue and day significantly influenced SOD activity (Table 1). A significant increase in SOD activity in ‘Br’ was recorded 14 days post inoculation in \( B. \text{cinerea} \)-inoculated peel tissue, when compared to any other treatment on any day (Table S3, Table S4a), which, in fact, reflected the response in susceptible apples at 14 days post inoculation (Table S4b). When susceptible apples were examined further at 14 days post inoculation, the peels displayed significantly higher SOD activity on the sun-exposed sides as compared to the shaded sides (data not shown). Control or mock-inoculated treatments showed no significant differences over time in SOD activity in peel tissue for either cultivar (Table S3). When the cultivars were compared, e.g. at 0 days, SOD activity was significantly ~ sixfold higher in flesh of ‘Br’ as compared to flesh of ‘GD’ (Table S2).
higher POX activity than that of ‘GD’. Flesh tissue generally had higher POX activity than peel tissue for both cultivars (Table S2). At 14 days after inoculation, ‘GD’ did not display any difference in POX activity for control and inoculated treatments, whereas for ‘Br’, POX activity was significantly reduced in the *B. cinerea*-inoculated (treated and susceptible) tissue, flesh and peel combined, when compared to the control or mock-inoculated tissue (Table S4a). Furthermore, only susceptible apples, and not tolerant ones, had lowered POX activity (Table S4b). Specifically, this lowered POX activity was located in flesh tissues. The POX activity in the peel tissue of ‘Br’ did not change significantly over time (data not shown).

**Ascorbate peroxidase (APX) activity**
The MANOVA demonstrated that the factors tissue and treatment significantly influenced APX activity (Table 1). At 0 days, APX activity was similar in both tissues for both cultivars, and across the sun-exposed and shaded tissues (Table S2). Significant differences were, however, found at 14 days post inoculation in ‘Br’. At that time, APX activity had increased in the inoculated treatment, as compared to the control (Table S4a). This increase was due to an increase in APX activity in susceptible fruit 14 days post inoculation (Table S4b).

**Catalase (CAT) activity**
The MANOVA demonstrated that the factors tissue and day significantly influenced CAT activity (Table 1). The two apple cultivars displayed similar levels of CAT activity. CAT activity was also similar for sun-exposed and shaded sides of fruit tissues. Similar CAT activity was observed among the three treatments (Table 1, S1a and S1b). CAT activity was slightly different in peel and flesh of ‘Br’ (Table S2). In flesh tissue, it was significantly higher than in peel tissues on the shaded side (Table S2). CAT activity changed over time (Table 1), increasing steadily in all treatments (data not shown). At 14 days post inoculation, CAT activity was not significantly different between the *B. cinerea*-inoculated treatment and the non-inoculated control (Table S4a). The CAT activity of susceptible apples at 14 days post inoculation did not differ from that of the control apples (Table S4b).

**Overall antioxidant enzyme activity**
In the MANOVA (Table 1), the factor treatment strongly influenced SOD and APX activities, and also POX activity, whereas there was no significant effect on CAT activity. The antioxidant enzyme activities of inoculated ‘Br’ at 14 days post inoculation (Table S4a) were analyzed further by pairwise t-tests, separately comparing the activity in tolerant and susceptible apples with that of control apples at 14 days (Table S4b). It was the susceptible ‘Br’ apples that displayed the observed increase in SOD and APX activity and the decrease in POX activity that had been observed in inoculated apples. The flow chart presented in Fig. 3 summarizes the significant changes reported in Table S4b. The antioxidant enzyme activity in peel and flesh tissues (both sun exposed and shaded sides) of ‘Br’ fruit susceptible to inoculation with *B. cinerea* at 14 days post inoculation are compared with the antioxidant enzyme activity of control fruit at 14 days post inoculation. The colors demonstrate the change in enzyme activity and are based on the analysis presented in Table S4b. Following inoculation of apples with *B. cinerea*, SOD activity increased by 4.1-fold, APX activity increased by 2.8-fold, and POX activity decreased by 2.0-fold.

**DISCUSSION**
In the present study, two apple cultivars, ‘Golden Delicious’ (‘GD’) and ‘Braeburn’ (‘Br’), were analyzed with respect to antioxidants and selected antioxidant enzyme activity after inoculation with *B. cinerea*. The increase in SOD and APX, as well as the decrease in POX in inoculated tissue (Table S4a) are a result of the response in apple during disease, because these changes were only observed in susceptible tissue (Table S4b). The results are summarized in a flow chart (Fig. 3).

As SOD is an important part of plant detoxification of ROS, the increase in SOD activity observed is an attempted plant defense strategy. Superoxide dismutase activity could possibly also originate from *B. cinerea* because BCSOD1 was found to be a virulence factor for *B. cinerea* in *Phaseolus vulgaris*, and in *Arabidopsis thaliana* and tomato plants. However, *B. cinerea* SOD activity has also been described as redundant, because a *bap1* deletion strain of *B. cinerea*, defective in the transcription of several genes for ROS detoxification systems, displayed no change in virulence on bean, *A. thaliana*, apple, or tomato; nor did the absence of antioxidant enzymes affect its ability to survive in infected plants. Inoculation with *B. cinerea* led to a strong increase in ‘Br’ APX activity in heavily diseased tissue, particularly in the flesh of the shaded side. The increase in APX activity may suggest an attempt by the host to metabolize the H$_2$O$_2$ generated from other ROS during disease. The increase in APX activity was accompanied by a decrease in total vitamin C levels both in flesh and peel tissues over the course of the experiment. This presumably occurred because disease perturbed the redox equilibrium. Thus, as the disease became more severe, vitamin C was no longer recycled but was being consumed as a consequence of APX activity.

The POX activity was higher in ‘Br’ than in ‘GD’ tissues, in accordance with previous results. Flavonoid peroxidase activity on the shaded side of ‘Br’ at 14 days was lower in the diseased flesh tissue than in control and mock-inoculated fruit. The low POX expression correlated well with the low amount of phenolics and progression of disease that was found at 14 days in the susceptible ‘Br’. This could possibly indicate that the availability of the enzyme is regulated by, or dependent on, the availability of its substrate. Alternatively, the POX pathway may have been down-regulated by *B. cinerea*, as was previously suggested from inoculation experiments with *B. cinerea* on bean (*Phaseolus vulgaris*) leaf discs.

Plant CAT activity is essential to plant health, particularly during gray mold infection, but also when plants are not infected. CAT activity remained unchanged in ‘Br’ apples after inoculation and during the course of disease development. Some of the measured activity could have been of *B. cinerea* origin, as seven CAT genes are present in its genome and CAT activity has been reported from growth on culture media. However, it is unlikely, because deletion of one of the catalase genes did not affect virulence and because of the importance of other detoxification systems – see below.

In theory, the enzyme activities analyzed here have also been detected in *B. cinerea* when grown on culture media. These are needed for pathogen survival on artificial media supplemented with H$_2$O$_2$, at levels encountered during infection and disease development. Some of the antioxidant activity that was measured in the fruit samples could therefore have originated from *B. cinerea*. However, the pathogen does not appear to need all these enzymes when invading a number of different plant species.
Instead, recent evidence suggests that thioredoxin and thioredoxin reductase have more crucial roles in _B. cinerea_ pathogenicity because they enable the pathogen to survive and respond to high levels of oxidative stress.\(^{31}\) The elevated antioxidant enzyme activities detected in ‘Br’ during onset of disease proved unsuccessful as a defense strategy to _B. cinerea_.

‘Br’ apple fruit display red, sun-exposed, and green, shaded sides, respectively. The sun-exposed side contains several antioxidant pigments that accumulate in the apple peel as a consequence of sun exposure.\(^{32,33}\) Common pigments found in apples belong to groups of compounds, such as anthocyanins, flavanols, and carotenoids.\(^{3}\) The anthocyanins contribute with red, purple and blue colors.\(^{33}\) The flavanols consist mostly of quercetin glycosides that accumulate in the fruit during ripening in the field and are also stably maintained in the fruit after a long storage period in the dark.\(^{34}\) Some of these compounds may also have antifungal activity, such as, for instance, quercetin-3-galactoside, which inhibits germ tube elongation of _B. cinerea_.\(^{35}\)

Quercetin is one of the most potent plant antioxidants.\(^{36}\) ‘GD’, the less susceptible cultivar, was richer than ‘Br’ in quercetin and its glucosides in both flesh and peel.\(^{1}\) These compounds are classified as phenolics, and were found to have been consumed in the diseased apples of ‘Br’, at 14 days post inoculation. Ascorbic acid is the most abundant, water-soluble plant antioxidant. It is very important for maintaining redox homeostasis when plants are subjected to environmental stressors that involve ROS.\(^{7}\) Thus, it could play a role in plant defense against necrotrophs. Across eight Belgian commercial apple cultivars that had been artificially inoculated with _B. cinerea_, there was a correlation between low AsA levels at harvest and disease development, and between high levels of AsA and less severe disease development.\(^{10}\) Similarly, in the present study, a higher accumulation of total vitamin C was detected in the sun-exposed, less susceptible side than in the shaded side. Total vitamin C levels were also higher in peel than in flesh, which has been observed previously, and this difference persists throughout periods of cold storage.\(^{37}\) Total vitamin C levels decreased during the course of disease development in ‘Br’ apples, presumably, because they were being consumed in the decaying tissue while acting as a substrate for APX in the detoxification of H₂O₂. In healthy tissue AsA is recycled, and appears to accumulate gradually in response to high light regimes.\(^{28}\)

‘Br’ was susceptible to _B. cinerea_ whereas ‘GD’ was essentially tolerant or even resistant, which has been observed previously.\(^{10,39}\) Significant changes in enzyme activity and antioxidant content were found in ‘Br’ over time. In contrast, there was no antioxidant enzyme induction in ‘GD’ and no change in total vitamin C or phenolic content as a result of inoculation. The initial phenolic content was higher in ‘Br’ compared to ‘GD’ in flesh tissue. Nonetheless, ‘Br’ was more susceptible. Similarly, ‘Br’ displayed higher initial total vitamin C content in peel and higher SOD and POX activities in flesh tissues as compared to ‘GD’. The distinct spectra and quantities of specific phenolic compounds present in the two cultivars may have influenced their susceptibility. But overall, the resistance of ‘GD’ must depend on other factors than those investigated here.

__Botrytis cinerea_ produces phytoxic compounds, such as botrydial and botcinolides, as well as cell-wall-degrading enzymes.\(^{40,41}\) _B. cinerea_ like other postharvest pathogens modulate the host pH environment during infection, and low pH is conducive to gray mold development.\(^{42,43}\) _Botrytis cinerea_ secretes a number of organic acids, of which citric acid appears to play an important role in its interaction with host plant tissue.\(^{44}\) Oxalic acid, another organic acid that is implicated in virulence in _Sclerotinia sclerotiorum_, a close relative to _B. cinerea_, does not appear to be involved in the gray mold-apple interaction.\(^{45,44}\)

Based on the current view of the strategy of _B. cinerea_ to deal with ROS during pathogenesis, it is reasonable to suggest that the observed increase in antioxidant enzyme activity (SOD and APX) in the susceptible apple tissues were of apple origin. Particularly at the last time point, both host and pathogen would have attempted to maintain cellular redox homeostasis and they would therefore have tried to activate effective antioxidant systems. Future studies could continue to address the nature of the antioxidant enzyme activities, which originate from fruit or fungus, respectively. It would also be desirable to clarify the importance of the timing of the various antioxidant responses that operate during different steps of pathogenesis.

**CONCLUSIONS**

The changes detected in antioxidant enzyme activity over time are consistent with the need for ROS detoxification in diseased

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**Figure 3.** Summary of the antioxidant enzyme responses in ‘Braeburn’ (‘Br’) apple fruit as a consequence of infection by _B. cinerea_. The antioxidant enzyme activity is collectively analyzed for shaded and sun-exposed tissue, as for peel and flesh tissue. The colors represent the changes in antioxidant enzyme activity of susceptible fruit at 14 days post inoculation, compared to noninoculated control fruit at the same point in time. The changes in enzyme activity were analyzed by ANOVA, followed by pairwise t-tests. Abbreviations: APX, ascorbate peroxidase; AsA, vitamin C (L-ascorbic acid); CAT, catalase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; H₂O₂, hydrogen peroxide; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; O₂⁻, superoxide anion; POX, flavonoid peroxidase; SOD, superoxide dismutase.
tissue. The results indicate that high exposure of apple fruit to sun stress in the field might lead to an improved tolerance to postharvest B. cinerea infection. Genetic differences in disease tolerance underlie the relative difference in susceptibility between the two cultivars, but the tolerance mechanism does not appear to involve antioxidant metabolism. Further studies are needed to investigate the nature of cultivar differences in their tolerance and susceptibility to postharvest gray mold.

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SUPPORTING INFORMATION
Supporting information may be found in the online version of this article.

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