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Postharvest Changes in the Total Phenolic Content, Antioxidant Capacity and L-Phenylalanine Ammonia-Lyase Activity of Strawberries Inoculated with *Botrytis cinerea*

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Abstract

A comparative study of the total phenolic content (TPC), antioxidant capacity (AC) and L-phenylalanine ammonia-lyase (PAL) activity of non-inoculated and inoculated fruits of five strawberry cultivars ('Clé des Champs', 'Darselect', 'Jewel', 'Kent' and 'Veestar') with varying degrees of resistance or susceptibility to *Botrytis cinerea* was performed during five days of storage in 2007 and 2008. During storage, the TPC and AC of the non-inoculated fruits remained steady, whereas the TPC and AC changed significantly in the inoculated fruits, with the exception of the AC of the 'Veestar' fruits. For the susceptible cultivar 'Kent', a quadratic pattern of PAL activity was observed in both the non-inoculated and the inoculated fruits, similar to the pattern in the non-inoculated fruits of 'Clé des Champs'. In general, linear reductions in the TPC and AC were observed in the inoculated fruits.

Keywords: *Fragaria×ananassa*, *Botrytis cinerea*, total phenolic content, antioxidant capacity, L-phenylalanine ammonia-lyase activity

1. Introduction

Strawberry (Fragaria×ananassa (Weston) Duchesne ex Rozier) is a nonclimacteric fruit (Given, Venis, & Gierson, 1988) that is characterized as nutritious but delicate and perishable, with a short shelf-life due to its chemical composition, delicate surface texture and potential for postharvest disease. Gray mold caused by *Botrytis cinerea* Pers. (teleomorph: Botryotinia fuckeliana [de Bary] Whetzel) is one of the most widespread fungal diseases, inflicting serious losses in food and ornamental crops (Jarvis, 1977). It occurs mainly on mature fruits after harvest but also appears on flowers as well as on immature and mature fruits before harvest (Maas, 1984; Snowdon, 1990). *Botrytis cinerea* is especially important as a postharvest pathogen because it causes decay and extensive losses up to 55% in untreated strawberry crops (Daugaard, 1999), even at low temperatures (Elad, Williamson, Tudzynski, & Delen, 2004; Martínez-Romero et al. 2007).

The susceptibility of plants to *Botrytis cinerea* varies between different cultivars, and it has been shown that phenolics are responsible for plants' resistance (Soylu, 2006; Lee, Chang, Su, Huang, & Jang, 2007). Strawberries vary in their inherent susceptibility to *Botrytis cinerea* according to their physiological status (Gilles, 1959) and genotype (Daugaard, 1999; Hébert et al., 2002), but, none strawberry cultivar is highly resistant to gray mold. In our laboratory, we have already established a positive correlation between phenolic content, antioxidant capacity (AC), shelf-life and disease resistance for several cultivars (Khanizadeh, Cousineau, Gauthier, Buszard, & Hébert, 2002; Khanizadeh et al., 2005). Hébert et al. (2002) also reported a higher degree of resistance to gray mold growth in six strawberry cultivars, concluding that 'Seascape' proanthocyanidin extracts provided the highest inhibition of mold radial growth (76.2%), and that during storage that cultivar was

also the most resistant to the appearance of mold. Tao et al. (2010) reported that several polyphenolic antioxidants inhibited the development of *Botrytis cinerea in vitro*. The phenolic compounds are originated from the general phenylpropanoid metabolism, which consists of three early steps in the conversion of L-phenylalanine to various hydroxycinnamic acids. The enzymes catalyzing the individual steps in this sequence are, respectively, L-phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), cinnamate 4-hydroxylase (C4H, EC 1.14.13.11) and 4-coumarate:CoA ligase (4CL, EC 6.2.1.12) (Haslam, 1998). The first, PAL, is considered to be the key enzyme in phenolic biosynthesis because it catalyzes the reductive deamination of L-phenylalanine to form trans-cinnamic acid (Hao, Charles, Yu, & Simon, 1996), the first step in the biosynthesis of plant phenylpropanoid compounds, including polyphenolics, lignin, suberin, flavonoids, coumarins and amides (Rösler, Krekel, Amrhein, & Schmid, 1997; Solecka & Kacperska, 1995). The activity of PAL varies with the plant development stage, during cell and tissue differentiation, and under various stresses such light, temperature, irradiation, growth regulators, tissue damage, nutrient deficiencies, fungal and insect attacks, and fungicide and herbicide applications (Jones, 1984; Chalker-Scott & Fuchigami 1989; Waterman & Mole, 1994; Ruiz et al., 1998; Ruiz, Garcia, Rivero, & Romero, 1999).

In spite of PAL activity and its derivatives in various stresses, including tissue damage and fungal attacks, no literature has been published on its relationship to gray mold in strawberries and its use as a chemical marker for selecting resistant genotypes. Therefore, an experiment was conducted in 2007 and repeated in 2008 in order to evaluate the TPC, AC and PAL activity of fruits inoculated and non-inoculated with gray mold during storage. Meanwhile, it's also an attempt to understand the mode of action of and fruit responses to gray mold inoculation in selected disease-resistant and disease-susceptible cultivars, The aim of this study was to obtain more details on the progress of diseases and to develop a relationship to be used in our breeding program.

2. Materials and Methods

2.1 Preparation of Botrytis cinerea Conidia

Botrytis cinerea isolates were originally obtained and purified from infected strawberry plants, with the stock cultures maintained on potato dextrose agar (PDA) in the dark at 24°C until use, as described previously (Tao et al., 2010). The *Botrytis cinerea* conidia were obtained by washing PDA slant cultures with sterile 5% glycerol aqueous solution. The conidia concentration was adjusted to 1×10^6 conidia per milliliter using a hemacytometer (Fuchs-Rosenthal counting chamber, Hausser Scientific, Horsham, PA, USA).

2.2 Preparation of Samples

Fruits of five strawberry genotypes ('Clé des Champs', 'Darselect', 'Jewel', 'Kent' and 'Veestar') were collected at the Agriculture and Agri-Food Canada experimental farm located at L'Acadie, QC, Canada (longitude 73.35 W; latitude 45.32 N). Fresh fruits were harvested at optimum maturity and brought to the laboratory, where they were cleaned and surface-disinfected with 0.1% sodium hypochlorite and then divided into two groups for each cultivar. One group was kept as a control (non-inoculated), while the other was inoculated by spraying *Botrytis cinerea* conidial suspension (1×10^6 mL^{-1}). Both groups were then stored in a dark chamber at room temperature (20°C) with 95% relative humidity. Then, 500 g inoculated and non-inoculated fruits were sampled internally over five days (day 0 = first day, day 4 = fifth day), frozen in liquid nitrogen and stored at −80°C until extraction.

2.3 Chemicals

All chemicals were analytical-grade. The Folin-Ciocalteu phenol reagent, 2, 4, 6-Tri(2-pyridyl)-s-triazine (TPTZ), iron(II) sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$), and ferric chloride ($FeCl_3$) were purchased from Sigma Chemical Co. (Oakville, ON, Canada). The gallic acid and sodium carbonate (Na_2CO_3) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The PDA was obtained from Difco Laboratories (Franklin Lakes, NJ, USA).

2.4 Extraction and Analysis of the Phenolics

Using a Polytron blender (Brinkmann Instruments, New York, NY, USA), 10 g fresh-frozen strawberries were blended in 50 mL 50% methanol. The mixture was filtered through filter paper (Whatman No.1) and then through a 0.45-µm Acrodisc syringe filter (Gelman Sciencies, Ann Arbor, MI, USA). The final filtrate was then stored at -20°C prior to analysis. The extracts resulting from the procedure were used for TPC and ferric reducing/antioxidant power (FRAP) assays.

The TPC was determined according to the Folin-Ciocalteu (FC) method (Slinkard & Singleton, 1997) with slight modifications. The standard or sample extract (0.2 mL) was mixed with 1.0 mL FC reagent and 0.8 mL) Na_2CO_3 (7.5%) in a 20 mL vial and allowed to stand for 30 min at room temperature (20°C). Absorption was measured at 765 nm in a Varian Cary 3C spectrophotometer (Varian Analytical Instruments, Harbor City, CA, USA). Gallic

acid was used as a standard, and the TPC was expressed as gallic acid equivalent (GAE) in micrograms per gram fresh-frozen weight. Concentrations beyond the highest point (500 $\mu g \cdot mL^{-1}$) of the linear range of the standard curve were diluted before final analysis.

The AC was measured using the FRAP assay according to the method of Benzie and Strain (1996), which was modified for the 96-well microplate reader (Tsao et al., 2003). The standard ($FeSO_4 \cdot 7H_2O$) or sample extract (10 µL) was mixed with 300 µL ferric-TPTZ reagent (prepared by mixing 300 $mmol \cdot L^{-1}$ acetate buffer [pH 3.6], 10 $mmol \cdot L^{-1}$ TPTZ in 40 $mmol \cdot L^{-1}$ HCl and 20 $mmol \cdot L^{-1}$ $FeCl_3$ at a ratio of 10:1:1 [v/v/v]) and added to the wells. The plate was incubated at 37°C for the duration of the reaction. The absorbance readings were immediately taken at 593 nm and after 4 min, using a visible-UV microplate kinetic reader (EL 340, Bio-Tek Instruments Inc., Winooski, VT, USA). The FRAP value of the samples was calculated on the basis of 500 µM Fe^{2+} ($FeSO_4 \cdot 7H_2O$).

2.5 PAL Assay

The PAL was extracted according to the method of Morelló, Romero, Ramo, and Motilva (2005). The material was homogenized for 30 s in chilled 0.05 $mol \cdot L^{-1}$ potassium phosphate buffer (pH 6.6) with 0.8% (v/v) Triton X-100 and 0.1% (w/v) polyvinylpolypyrrolidone (PVPP), and then the suspension was centrifuged at 4°C for 15 min at 25900×g. The supernatant, stored on ice, was filtered through glass wool and used as a source of crude enzyme.

The activity of PAL in the crude enzyme extracts was assayed using an adaptation of the methods of Zucker (1965) and McCallum and Walker (1990). The assay mixture consisted of 0.06 $mol \cdot L^{-1}$ sodium borate buffer (4.1 mL; pH 8.8) and crude enzyme (0.4 mL), and the reaction was initiated by the addition of 1 ml L-phenylalanine solution (10 $mg \cdot mL^{-1}$; final concentration 11 $mmol \cdot L^{-1}$). The tubes were incubated at 37°C for 1 h. The reaction was stopped by adding 6 N HCl (0.5 mL), and the tubes were centrifuged for 5 min at 5000×g to pellet the denatured protein. The cinnamic acid yield was estimated by measuring the absorbance of the supernatant at 290 nm (A290) in 1-cm quartz cuvettes. Triplicate assays were performed for each extract, both with and without substrate in order to compensate for increases in absorbance, even in the absence of added L-phenylalanine. One unit of enzyme activity (U) was defined as the increase of 0.01 units of absorbance per hour under the assay conditions; the enzyme activity was referred to as fresh weight ($U \cdot g^{-1}$ FW).

2.6 Statistics

A randomized complete block design was used in this experiment, and all data were subjected to an analysis of variance (ANOVA) using the GLM and CORR procedures of SAS (SAS Institute, 1989). The means were separated using the least significant difference (LSD) test at the 0.05 level, when the variable was significant.

3. Results

3.1 TPC Assay

The changes of the TPC in non-inoculated and inoculated fruits of each genotype are shown in Table 1. The changes of the TPC during the five-day storage period were not significant in the non-inoculated fruits but significant in the inoculated fruits of all the tested genotypes. The effects on the TPC of the inoculated fruits of all tested genotypes were the same, namely linear, indicating that the development of the disease significantly decreases the TPC of fruits during storage. On the first day (day 0), the value order of TPC was as follow: 'Darselect' > 'Jewel' > 'Veestar' > 'Kent' > 'Clé des Champs'. After inoculation, the decrease in the TPC of fruits was extremely significant and linear in 'Clé des Champs' (from 1176.8 $\mu g \cdot g^{-1}$ to 654.6 $\mu g \cdot g^{-1}$), 'Darselect' (from 1600.7 $\mu g \cdot g^{-1}$ to 744.3 $\mu g \cdot g^{-1}$), 'Jewel' (from 1546.4 $\mu g \cdot g^{-1}$ to 723.3 $\mu g \cdot g^{-1}$) and 'Kent' (from 1361.8 $\mu g \cdot g^{-1}$ to 536.3 $\mu g \cdot g^{-}$); but was different in 'Veestar', where it decreased from day 0 (1445.7 $\mu g \cdot g^{-1}$) to day 1 (1337.5 $\mu g \cdot g^{-1}$), then increased on day 2 (1429.0 $\mu g \cdot g^{-1}$), decreased on day 3 (957.8 $\mu g \cdot g^{-1}$), and finally increased slightly on day 4 (1030.7 $\mu g \cdot g^{-1}$). In each cultivar, the mean of the TPC was lower in the inoculated fruits than in the non-inoculated ones.

Table 1. The total phenolic content (TPC) of the fruits of selected strawberry genotypes inoculated or non-inoculated with *Botrytis cinerea* during the five-day storage period

	'Clé des Champs'		'Darselect'		'Jewel'		'Kent'		'Veestar'	
Days	Non-inoculated	Inoculated	Non-inoculated	Inoculated	Non-inoculated	Inoculated	Non-inoculated	Inoculated	Non-inoculated	Inoculated
0	1176.8 a	1176.8 a	1600.7 a	1600.7 a	1546.4 a	1546.4 a	1361.8 a	1361.8 a	1445.7 a	1445.7 a
1	1143.8 a	1163.9 a	1507.8 a	1514.6 a	1223.4 a	1428.3 a	1367.4 a	1340.2 a	1656.0 a	1337.5 ab
2	1111.0 a	1044.0 a	1624.8 a	1479.3 a	1202.1 a	1374.4 a	1205.6 a	1302.2 ab	1564.3 a	1429.0 a
3	1015.7 a	860.2 b	1479.3 a	1229.3 ab	1388.9 a	938.7 b	1365.2 a	987.0 b	1510.6 a	957.8 b
4	1121.5 a	654.6 c	1497.3 a	744.3 b	1329.0 a	723.3 b	1391.1 a	536.3 c	1470.5 a	1030.7 b
Mean	1113.8	979.9	1542.1	1313.6	1337.9	1202.2	1338.2	1105.5	1529.4	1240.1
$LSD_{0.05}$	202.9	147.3	649.4	504.9	422.4	382.6	376.3	335.4	477.7	389.9
[a]OPC	ns	L***	ns	L***	ns	L***	ns	L***	ns	L**

Values are the means of eight replicates and expressed as micrograms gallic acid equivalent (GAE) per gram fresh-frozen weight.

$LSD_{0.05}$: least significant difference at the 0.05 level.

*P<0.05; **P<0.01; ***P<0.001; ns = not significant, P>0.05.

[a]OPC: orthogonal polynomial contrast; L = linear.

Table 2. The antioxidant capacity (AC) of the fruits of selected strawberry genotypes inoculated or non-inoculated with *Botrytis cinerea* during the five-day storage period

	'Clé des Champs'		'Darselect'		'Jewel'		'Kent'		'Veestar'	
Days	Non-inoculated	Inoculated	Non-inoculated	Inoculated	Non-inoculated	Inoculated	Non-inoculated	Inoculated	Non-inoculated	Inoculated
0	3257.2 a	3257.2 ab	4168.3 a	4168.3 ab	4369.3 a	4369.3 a	4041.3 ab	4041.3 a	4087.9 ab	4087.9 a
1	3149.0 a	3160.3 ab	4066.3 a	5158.6 a	3839.9 a	3853.8 ab	4179.0 a	3371.3 a	4782.4 a	4000.7 a
2	3198.1 a	3539.9 a	4037.2 a	2865.2 bc	3676.8 a	3548.7 ab	2165.9 b	3716.4 a	4929.8 a	4069.9 a
3	2990.8 a	1812.5 bc	4545.8 a	3113.1 b	3654.6 a	1975.2 b	3745.2 ab	2595.3 a	3843.5 ab	2701.0 a
4	3060.3 a	1129.7 c	3079.3 a	1112.8 c	3827.6 a	2693.3 ab	3553.6 ab	816.8 b	2464.0 b	3257.9 a
Mean	3131.1	2579.9	3979.4	3283.6	3873.6	3288.1	3536.988	2908.22	4021.5	3623.5
$LSD_{0.05}$	1737.5	1697.0	1867.2	1870.6	2075.8	1979.4	1906.6	1608.7	2112.4	2244.0
[a]OPC	ns	L**	ns	L***	ns	L*	ns	L***	ns	ns

Values are the means of eight replicates and expressed as micrograms ascorbic acid equivalent (AAE) per gram fresh-frozen weight.

$LSD_{0.05}$: least significant difference at the 0.05 level.

*P<0.05; **P<0.01; ***P<0.001; ns = not significant, P>0.05.

[a]OPC: orthogonal polynomial contrast; L = linear.

3.2 FRAP Assay

On the first day (day 0), the value order of AC was 'Jewel' > 'Darselect' > 'Veestar' > 'Kent' > 'Clé des Champs'. Both in the non-inoculated fruits of 'Clé des Champs', 'Darselect', 'Jewel' and in the inoculated fruits of 'Veestar', no significant effect on the AC was found for disease infection (Table 2). However, in the inoculated fruits of 'Clé des Champs', 'Darselect', 'Jewel', 'Kent', there was a significant negative linear relationship between disease development and the AC. Similar to the TPC, the mean of the AC was lower in the

inoculated fruits than in the non-inoculated ones in each cultivar. On the second day (day 1) of storage, the AC of the inoculated fruits was higher than that of the non-inoculated fruits in 'Clé des Champs', 'Darselect', and 'Jewel'; that also happened with the TPC.

Table 3. The L-phenylalanine ammonia-lyase (PAL) activity of the fruits of selected strawberry genotypes inoculated or non-inoculated with *Botrytis cinerea* during the five-day storage period.

	'Clé des Champs'		'Darselect'		'Jewel'		'Kent'		'Veestar'	
Days	Non-inoculated	Inoculated	Non-inoculated	Inoculated	Non-inoculated	Inoculated	Non-inoculated	Inoculated	Non-inoculated	Inoculated
0	341.3 a	341.3 b	465.8 ab	465.8 ab	345.0 b	345.0 bc	430.4 bc	430.4 b	434.6 c	434.6 b
1	385.2 a	521.5 a	407.1 ab	401.6 b	488.5 a	488.4 a	526.7 a	394.9 b	541.3 ab	549.9 a
2	349.5 a	359.8 b	371.0 b	503.5 ab	322.6 b	458.8 ab	438.9 b	595.5 a	386.3 c	454.3 b
3	452.1 a	302.4 b	500.4 ab	650.5 a	494.1 a	334.8 c	386.7 c	473.3 b	589.6 a	394.3 b
4	128.3 a	377.8 b	515.6 a	477.1 ab	437.1 ab	377.6 abc	572.3 a	410.5 b	471.1 bc	464.3 b
Mean	331.3	380.6	452.0	499.7	417.5	400.9	471.0	460.9	484.6	459.5
$LSD_{0.05}$	114.8	108.8	133.6	185.6	124.7	116.0	51.4	88.5	95.1	72.7
[a]OPC	Q***	ns	ns	ns	ns	ns	Q**	Q**	ns	ns

Values were the means of six replicates and expressed as units (U) per gram fresh-frozen weight.

$LSD_{0.05}$: least significant difference at the 0.05 level.

*P<0.05; **P<0.01; ***P<0.001; ns = not significant, P>0.05.

[a]OPC: orthogonal polynomial contrast; Q = quadratic.

3.3 PAL Activity

As shown in Table 3, no significant relationship was observed between storage duration and PAL activity in both non-inoculated and inoculated fruits of 'Darselect', 'Jewel' and 'Veestar', while the relationship showed a very significant quadratic pattern in both non-inoculated and inoculated fruits of the susceptible cultivar 'Kent' during storage. In 'Clé des Champs', a mid-season cultivar (1 to 2 days after 'Kent'), the storage duration showed an extremely significant quadratic effect on the PAL activity in the non-inoculated fruits, but no significant effect on the PAL activity in the inoculated ones. Slightly different from those of the TPC and AC, the means of the PAL activity were higher in inoculated fruits than in non-inoculated ones of 'Clé des Champs' and 'Darselect'. For the remaining three cultivars, however, the means of the PAL activity were lower in inoculated fruits than in non-inoculated ones. The PAL activity of both non-inoculated and inoculated fruits was also observed to be higher on day 4 than on day 0, with the exception of the non-inoculated 'Clé des Champs' fruits and the inoculated 'Kent' fruits. From the experiment results, we also found that on the first day (day 0) the value order of PAL was 'Darselect' > 'Veestar' > 'Kent' > 'Jewel' > 'Clé des Champs'.

4. Discussion

All the tested cultivars, namely 'Clé des Champs', 'Darselect', 'Jewel', 'Kent' and 'Veestar', are June-bearing strawberry varieties. 'Clé des Champs' is a productive mid-season cultivar and reported to have a good shelf-life and qualities including very good firmness, attractive luster, and no symptoms of gray mold, fruit rot or powdery mildew disease (Khanizadeh et al. 2006a; Rekika et al. 2005). 'Darselect' is an early-mid-season cultivar and very productive, with large, attractive, firm, bright red fruits, but is very susceptible to powdery mildew. 'Jewel' is a mid- to late-mid-season cultivar and semi-vigorous with good yield and large, glossy, bright red fruits that are good for freezing; And it is sensitive to Sinbar herbicide and susceptible to black root rot, leaf spot, red stele, powdery mildew and *Verticillium* but moderately resistant to *Botrytis* rot. 'Kent', a mid-season cultivar, has a long fruiting season and provides high yields of medium-sized, bright red fruits, but it is very susceptible to leaf spot, leaf scorch, angular leaf spot, *Botrytis* and anthracnose fruit rot and very sensitive to Sinbar. 'Veestar', an early-season cultivar, provides high yields of medium-sized, dark red berries. It is tolerant to Sinbar and moderately resistant to *Botrytis* rot but susceptible to red stele. The differences in the TPC, AC and PAL activity

could also be related to the agronomic characteristics of the strawberries, considering their respective time of ripening, except for their resistance to diseases.

Based on the results of this study, we have concluded that the *Botrytis cinerea* resistance and agronomic characteristics of strawberry cultivars affect the TPC, AC and PAL activity of strawberries. The changing trends in the TPC and AC were slightly similar in fruits with or without fungus inoculation during the five-day storage period, indicating that there was a certain correlation between them. These results are in agreement with the work of other researchers (Wang, Cao, & Prior, 1996; Wang & Jiao 2000; Wang & Lin 2000; Kalt et al. 2003; Rekika et al. 2005; Khanizadeh, Ehsani-Moghaddam, & Levasseur, 2006b). Although PAL is considered to be the key enzyme in phenolic biosynthesis (Hao et al., 1996), we did not find any significant correlation between the PAL activity and the TPC of strawberries with or without fungus inoculation. The reason may be that there are many kinds of phenolic compounds (sucu as cyanidin-3-glucoside, pelargonidin-3-glucoside, *p*-hydroxybenzoic acid, gallic acid, *p*-coumaric acid, ferulic acid, quercetin-3-galactoside, ellagic acid, kaempferol, myricetin, etc), and different compounds have different antifungal mechanisms. Furthermore, as a new cultivar, 'Clé des Champs' concluding the lowest TPC, AC and PAL needs further research regarding to its resistance by combination of field identification, field inoculation and laboratory isolated inoculation.

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