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Influence of lipoxygenase activity and calcium and potassium contents on bitter pit occurrence in commercial apple cultivars

Marzena Wińska-Krysiak, Barbara Łata

Laboratory of Basic Sciences in Horticulture
Warsaw University of Life Sciences
Nowoursynowska 159, 02-776 Warsaw, Poland
e-mail: marzena_winska_krysiak@sggw.pl

ABSTRACT

The content of calcium and potassium and lipoxygenase (LOX) activity as well as the expression of LOX encoding gene in the fruit of apple cultivars that are both susceptible and resistant to bitter pit were evaluated. Fruits were collected and analyzed after harvest in the 2004 growing season and again after four months of common cold storage. The main reason was the explanation of relationship between those LOX activity and K:Ca ratio on bitter pit occurrence during storage. The biggest differences between the tested cultivars were noted in LOX activity and Ca content, followed by K:Ca ratio and the lowest potassium content. The cultivars that were resistant and susceptible but without symptoms of bitter pit exhibited higher calcium content compared to cultivars with signs of bitter pit, and with a drop in LOX activity after storage. A negative correlation was found between LOX activity and Ca content and a positive correlation was found between K:Ca ratio and LOX activity.

Key words: *Malus domestica* Borkh., calcium, K:Ca ratio, lipoxygenase activity

INTRODUCTION

The chemical composition of fruit is determined by several factors, both environmental and genetic (Chiu and Bould 1977, Coccuci et al. 1990, Saure 1996, Drazeta et al. 2004). Fruits of low Ca content and high K content, and consequently, a high K:Ca ratio, are susceptible to bitter pit occurrence. Moreover, the development of this disorder is also influenced by storage conditions and the activity of the enzymes involved in fruit respiration. Special attention should be paid to LOX, which increase is frequently observed during storage, especially in cultivars with high K:Ca ratios (Marcelle 1989).

The aim of this study was to investigate the relationship between Ca content, K:Ca ratio and LOX activity and bitter pit development in cultivars of low and high susceptibility to that disease.

MATERIAL AND METHODS

Plant material and sample preparation

The research was performed on apples harvested in the 2004 vegetation season in an orchard located in central Poland and cultivated according to standard horticultural practices. The soil was maintained as herbicide strips with rows of trees and sward between them. Mean temperatures and rainfall in 2004 season are presented in Table 1. The plant materials used were the fruits of eight cultivars, both susceptible (‘Šampion’, ‘Cortland’, ‘Mutsu’, ‘Ligol’) and resistant (‘Gala’, ‘Idared’, ‘Alwa’, ‘Gloster’) to bitter pit. Fruits for analysis were collected twice: at harvest and after four months of common cold storage. Each sample consisted of 8-mm thick vertical slices derived from five fruits (one replication). Samples were immediately frozen in liquid nitrogen and stored at -80°C until biochemical and molecular analyses were

Table 1. The air temperatures (°C) and rainfall (mm) for the field of the Warsaw-Wilanow Station in the year 2004

	Year	Month					
		IV	V	VI	VII	VIII	IX
Mean monthly temperature (°C)	2004	9.3	12.8	16.6	18.6	19.8	14.5
	Mean for 1971-2000	7.9	13.7	16.5	18.1	17.7	13.0
Rainfall (mm)	2004	60	53	39	58	52	20
	Mean for 1971-2000	35	51	71	73	59	49

conducted. Directly before analysis, apple tissue was ground to a fine powder in liquid nitrogen. Chemical and molecular analyses were performed in 4 replicates for each cultivar.

Calcium and potassium contents and mean fruit weight

Samples were dried at 65°C for 24 h and then at 105°C for 3 h, and mineralized in a combustion furnace. Calcium content was determined by an inductively-coupled plasma spectrometer, whereas potassium content was determined by photometer. The Ca and K concentrations were reported on a dry-mass basis. The mean fruit weight of the cultivar was obtained from 10 apples.

LOX activity measurement

LOX activity was measured by monitoring the increase in absorbance at 234 nm. The reaction was carried out for 4 min at 25°C. The plant tissue was homogenized in a 0.2 M boric buffer, pH 7.0 containing 0.2% PVPP and centrifuged at 15,000 g for 20 minutes at 4°C. The reaction mixture contained 100 µl of plant extract, 0.5 ml linoleic acid as substrate (25 µl of linoleic acid dissolved in 0.5 ml of methanol and 37.5 µl of Tween 20, diluted to 50 ml with water; prior to analysis, 4.2 ml of linoleic acid/methanol solution was mixed with 20.8 ml of 0.2 M boric buffer pH 8.0) and 0.2 M boric buffer pH 8.0 in a final volume of 1 ml. The results were calculated from a standard curve standardized with soybean Lipoxygenase EC 1.13.11.12 Type V (Sigma-Aldrich). LOX activity was expressed as U mg^{-1} protein.

Statistical analysis

The obtained results were elaborated using a one-way factorial analysis of variance (ANOVA 1) separately for harvest and after storage. The significance of difference between means was tested using Tukey's honestly procedure at $\alpha = 0.05$.

RNA isolation and RT-PCR analysis

The RNA was extracted, as described by Zeng and Yang (2002), and quantified spectrophotometrically. Reverse transcription reactions were carried out as recommended by the manufacturer (Reverse Transcription System, Promega). The products of reverse transcription were used as templates for PCR analysis. The presence of genes encoding lipoxygenase in all cultivars was confirmed by a polymerase chain reaction (PCR) with a primer pair: 1F CTTTCATGTATGAAGAGTTGGA, 1R ACCCTTCTGCTGGAGTCT. Cycle number and primer hybridization temperature − essential for PCR gene amplification − were 28 cycles at 37°C.

RESULTS

Calcium content

The fruits of the studied cultivars differed significantly in their calcium content (Tab. 2). Bitter pit-resistant cultivars revealed the calcium content in the range of 571 ('Gloster') to 705 ('Gala') µg g^{-1} d.m. after harvest, and a considerable decrease of calcium after long storage was expressed only in the 'Gala' and 'Idared' apples. Calcium content at harvest in bitter pit-susceptible cultivars varied between 451 ('Ligol') and 669 ('Mutsu') µg g^{-1} d.m. After storage, the lowest concentration of Ca was exhibited by 'Ligol' with bitter pit and the highest in 'Alwa' apples. Symptoms of bitter pit were observed only in fruits of the susceptible cultivars. Ca content determined in the 'Šampion', 'Ligol', 'Mutsu', and 'Cortland' fruits with bitter pit was reduced up to 38.3% as compared to fruits of the same cultivar, but with no signs of bitter pit.

Potassium content

The potassium content in both sampled times was nearly the same, on average: 17.4 and 16.7 mg g^{-1} d.m., respectively (Tab. 2). The concentration of potassium ranged from 12.9 ('Cortland' with bitter pit after long term cold storage) to 19.5 ('Gloster' at harvest), respectively.

Ratio K:Ca

The mean of the K to Ca ratio was 33.2. The highest value of this parameter was displayed by 'Ligol' (with bitter pit in fruits after storage), whereas the lowest one was characterized by 'Alwa' apples at harvest. The K to Ca ratio, equalling 28.8 on average, was noted at harvest in cultivars without symptoms of bitter pit. Four months of cold storage influenced the K:Ca ratio; however, this effect was highly cultivar-dependent. The highest decrease in the K:Ca ratio was noted for the 'Gloster'

Table 2. The concentration of potassium and calcium in fruits and K:Ca ratio depending on the cultivar and the time of analysis

Cultivar	K (mg g^{-1} d.m.)		Ca (mg g^{-1} d.m.)		K:Ca ratio	
	At harvest	After long-term cold storage	At harvest	After long-term cold storage	At harvest	After long-term cold storage
'Idared'[1]	16.1 b*	16.9 ab	0.638 ab	0.567 bc	25.2 d	29.9 cd
'Alwa'[1]	14.8 b	16.9 ab	0.700 a	0.728 a	21.2 d	23.3 d
'Gala'[1]	18.0 ab	18.7 a	0.705 a	0.565 bc	25.6 d	33.2 bc
'Gloster'[1]	19.5 a	18.2 a	0.571 ab	0.604 b	34.3 bc	30.0 cd
'Šampion'[2]	17.1 ab	15.3 ab	0.553 b	0.538 bc	31.1 c	28.4 cd
'Ligol'[2]	16.8 ab	18.1 a	0.451 bc	0.468 bc	37.2 b	39.1 b
'Mutsu'[2]	18.6 ab	17.7 a	0.669 ab	0.571 bc	27.9 cd	30.9 c
'Cortland'[2]	17.6 ab	18.1 a	0.643 ab	0.658 ab	28.1 cd	27.9 cd
'Šampion'[3]	nd	15.1 ab	nd	0.431 c	nd	35.0 bc
'Ligol'[3]	nd	16.4 ab	nd	0.265 d	nd	62.6 a
'Mutsu'[3]	nd	15.8 ab	nd	0.428 c	nd	36.9 bc
'Cortland'[3]	17.9 ab	12.9 b	0.320 c	0.332 cd	55.8 a	38.7 b
Average	17.4	16.7	0.583	0.513	31.8	34.7

*Means followed by the same letter do not differ significantly at $\alpha = 0.05$
nd – lack symptoms of bitter pit on the fruits in orchard
[1] cultivars resistant to bitter pit
[2] cultivars susceptible to bitter pit but without symptoms
[3] cultivars susceptible to bitter pit with symptoms

Table 3. LOX activities in fruits and mean weight of fruit depending on the cultivar and the time of analysis

Cultivar	LOX activities (U mg^{-1} protein)		Mean weight of fruit (g)	
	At harvest	After long-term cold storage	At harvest	After long-term cold storage
'Idared' [1]	0.93 bc*	0.48 c	188.4 f	176.4 f
'Alwa' [1]	1.09 bc	0.32 c	209.4 e	239.8 d
'Gala' [1]	2.45 a	0.70 bc	184.1 f	164.6 f
'Gloster' [1]	1.00 bc	0.44 c	271.1 c	253.2 d
'Šampion' [2]	1.02 bc	1.12 a	203.2 ef	205.0 e
'Ligol' [2]	1.00 bc	0.48 c	311.7 b	198.1 e
'Mutsu' [2]	0.78 c	0.28 c	349.8 a	282.8 c
'Cortland' [2]	1.24 b	0.48 c	202.9 ef	202.0 e
'Šampion' [3]	nd	1.17 a	nd	346.9 a
'Ligol' [3]	nd	0.61 bc	nd	240.4 d
'Mutsu' [3]	nd	0.73 bc	nd	301.6 b
'Cortland' [3]	1.29 b	0.79 b	246.9 d	238.5 d
Average	1.2	0.6	240.8	237.4

*Explanations: see Table 2

(12.4%), followed by 'Šampion' (8.6%) and 'Cortland' (0.6%). In the case of the other cultivars, an increase of K to Ca was observed. The mean K:Ca ratio in cultivars without symptoms of bitter pit (after storage) was 33.4, while it rose to 43.3 in the fruits of the same cultivars but with bitter pit.

LOX activity and fruit weight

The mean fruit weight varied between 164.6 g ('Gala') and 349.8 g ('Mutsu') (Tab. 3). The mean fruit weight of cultivars with symptoms of bitter pit was significantly higher than for the same cultivars without bitter pit.

At harvest, LOX activity varied between 0.78 and 2.45 U mg^{-1} protein (Tab. 3). After four months of storage this value decreased 2.75 times in apples not displaying signs of bitter pit, on average. The occurrence of this physiological disorder was accompanied by a 40% increase of lipoxygenase activity. The lowest LOX activity was recorded for the 'Mutsu' cultivar without

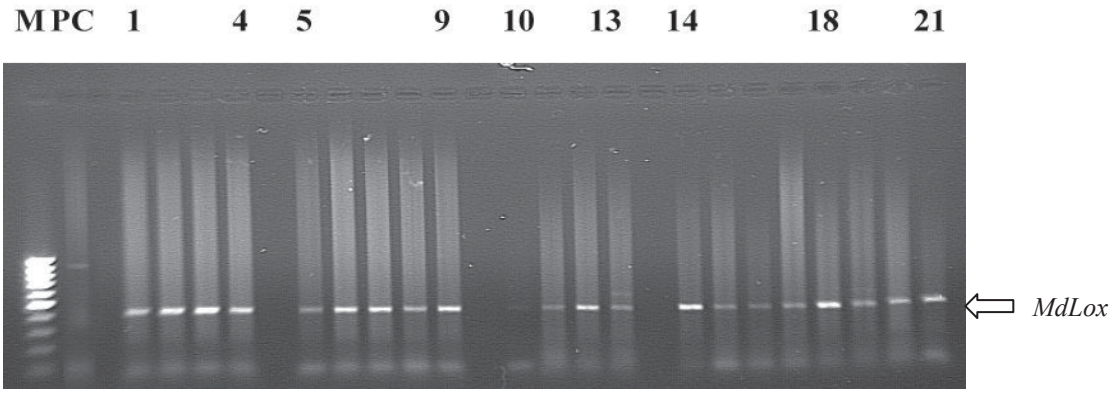

Figure 1. Lipoxygenase gene expression in various fruit developmental stages analysed by RT-PCR; PC) Positive control – polyA. 1) 'Alwa', 2) 'Gloster', 3) 'Gala', 4) 'Idared', 5) 'Šampion', 6) 'Ligol', 7) 'Mutsu', 8) 'Cortland', 9) 'Cortland' with bitter pit, 10) 'Alwa', 11) 'Gloster', 12) 'Gala', 13) 'Idared'; 14) 'Šampion', 15) 'Ligol', 16) 'Mutsu', 17) 'Cortland', 18) 'Šampion' with bitter pit, 19) 'Ligol' with bitter pit, 20) 'Mutsu' with bitter pit, 21) 'Cortland' with bitter pit, 2-9) – after harvest; 10-21) − after long-term storage under standard conditions; amplification was performed with a 1 F and R primers pair

bitter pit after long term cold storage, and the highest for 'Gala' apples analysed at harvest.

A significantly negative correlation was found between the Ca content and LOX activity (R = -0.55). The negative correlation coefficient increased after storage (R = -0.66). A negative correlation was also found between apple weight and LOX activity in all of the studied cultivars (R = -0.34). A positive correlation between K to Ca ratio and LOX activity (R = 0.39) was recorded.

Expression and nucleotide sequence of LOX

Expression of *MdLOX* measured as the amount of mRNA depended on the cultivar and sampling time (Fig. 1). It was higher in fruits analysed immediately after harvest compared to fruits subjected to four months of storage. An increase of the lipoxygenase gene transcript in fruits with bitter pit, as compared to the same cultivar without signs of bitter pit, was noted. Compared to LOX activity, there were no evident differences in the case of pattern expression of gene-encoded LOX and only a few examples ('Šampion' at harvest with a lower expression, and 'Mutsu' with a higher expression compared to LOX activity).

DISCUSSION

Bitter pit is one of the most common physiological disorders observed during storage. It is known that apple storability potential is influenced by the contents of calcium and potassium as well as their ratio. An insufficient calcium content causes bitter pit occurrence in apple fruits (Sadowski et al. 1967, Tomala and Sadowski 1989). The current study also revealed that cultivars with symptoms of bitter pit were characterized by a low calcium content (355 μg g^{-1} d.m.) and high LOX activity. LOX activity observed in fruits of the 'Šampion', 'Ligol', 'Mutsu' and 'Cortland' cultivars with visible signs of bitter pit was 38% higher than LOX activity of apples not expressing symptoms of bitter pit. The Ca content was also related to the fruit size: bigger fruits contained smaller amounts of calcium, what is explained as a result of its dilution.

Ratkowsky and Martin (1974) and Tomala (1995) proved in their experiments that apples with bitter pit symptoms contained more potassium than healthy ones. This relationship was not observed in the current study. Fruits of the 'Šampion', 'Ligol', and 'Mutsu' cultivars with bitter pit were characterized by a lower K content in comparison to the same cultivars without symptoms of bitter pit. Schumacher et al. (1978) stated that a high K:Ca ratio (above 36) is a more important indicator of bitter pit occurrence than K concentration. That finding was supported by further observations by Schumacher (1982) and de Jager (1994). Here, fruits with symptoms of bitter pit showed a K:Ca ratio above 35. It is noteworthy that in the bitter pit-free fruits of the 'Ligol' cultivar, that ratio was above 35 in both fruits sampled after harvest and after storage. Fruits of the same cultivar, but with developed symptoms of bitter pit, showed a K:Ca ratio above 62. Clearly, this supports the previous findings of de Jager (1994) in different cultivars.

The increase in the K:Ca ratio in fruits as well as the expression of bitter pit symptoms could be correlated with an increase of lipoxygenase activity during storage (Marcelle 1989). However, we observed this phenomenon only in fruits of the 'Šampion' cultivar. Moreover, due to the significant decrease of LOX activity (59.4% on average) in all of the other cultivars tested, it must be

considered that the period of storage was longer than in previous studies (four months vs. three months).

CONCLUSIONS

Cultivars without symptoms of bitter pit were characterized with higher calcium content as compared to cultivars expressing this disorder, and a decrease in LOX activity after storage. The expression of the LOX-encoding gene in fruits was different and depended on the cultivars and sampling time. High LOX activity was assigned to low calcium content and high K:Ca ratio.

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AKTYWNOŚĆ LIPOXYGENAZY, ZAWARTOŚĆ WAPNIA I POTASU A GORZKA PLAMISTOŚĆ PODSKÓRNA W OWOCACH WYBRANYCH ODMIAN JABŁONI

Streszczenie: Zawartość wapnia, potasu oraz aktywność lipoksygenazy jak również ekspresja genu kodującego LOX w owocach odmian jabłoni podatnych na występowanie gorzkiej plamistości podskórnej i tych, u których objawy tej choroby nie występują były zróżnicowane. Owoce pobrano i analizowano bezpośrednio po zbiorze w sezonie 2004 oraz po długim przechowywaniu w chłodni zwykłej. Główną przesłanką badań było wyjaśnienie roli powyższych parametrów w przechowywaniu jabłek. Największe zróżnicowanie między badanymi odmianami odnotowano w przypadku aktywności LOX, zawartości wapnia, następnie stosunku K:Ca, a najmniejsze dla zawartości potasu. Owoce bez objawów gorzkiej plamistości podskórnej charakteryzowały się wysoką zawartością wapnia i spadkiem aktywności LOX po przechowywaniu. Wykazano negatywną korelację między aktywnością LOX a zawartością wapnia, oraz pozytywną korelację między stosunkiem K:Ca a aktywnością LOX.

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