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Relationship of Superficial Scald Related Fruit Maturity with Polyphenoloxidase and Superoxide Dismutase Activities in Red Spur Delicious Apples

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The paper demonstrates the possible relationship between scald and scald related maturity factors to the changes in activities of superoxide dismutase (SOD), peroxidase (POD) and polyphenol oxidase (PPO) in apple fruits during storage. Fruits from red spur delicious apples at early mature, full mature and over mature stages were harvested and shortly after harvest, stored at -1 °C. Fruits from each maturity stage were analyzed for enzymes activities (SOD, POD, PPO), ethylene synthesis, flesh firmness, soluble solids and starch contents at the time of harvest, during ripening at 2, 4 and 8 weeks intervals and then after 3, 4 and 5 months of storage. Higher scald index in immature fruits than mature fruits was recorded and that was increased with increase in storage duration, which corresponds with increased fruit softening and senescence. No change was found in POD activity with change in maturity at the time of harvest, but higher POD activity in immature fruits during storage compared to mature ones conflicts with such conclusion and may suggest no relationship between POD and scald development. However, the results of high scald incidence in immature fruits which were also high in PPO activity during storage and low in antioxidants (SOD) activity in immature fruits suggests a possible correlation between scald, PPO activity and antioxidant level.

Key Words: *Malus domestica*, Enzymes, Antioxidants, Soluble solids, Scald.

INTRODUCTION

Red spur delicious apple fruits are susceptible to superficial scald (scald) that is a postharvest physiological disorder and causes considerable economic loss to the growers, shippers, handlers and consumers. This malady is characterized by progressive internal browning of the hypodermal cells and in

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severe cases, the brown discolouration of skin extends through five or six layers of hypodermis and epidermal cells also turn brown[1]. Scald is induced at low temperatures (-1 to 5 ºC) during storage, but symptoms generally appear a few days after removal of the fruit from storage and held at ambient temperature[2]. This disorder has been strongly affected by fruit maturity and cultivar in addition to several other factors[3]. Earlier studies[2,4] have reported that oxidation of volatiles, lipids and amino acids and associated formation of free radicals induce scald in apples and pears. Phenolics are oxidized by enzyme polyphenol oxidase (PPO; EC 1.14.18.1) to quinones which are then polymerized to cause browning of the plant tissue[5,6].

To cope with some of the harmful effects of a generally beneficial aerobic environment, plants possess a protective mechanism and free radicals produced in oxidative reaction, are scavenged by a number of naturally occurring antioxidant molecules and enzymes. Superoxide dismutase (SOD; EC 1.115.1.1) is a ubiquitous antioxidant enzyme involved in the first line of cellular defense against oxidative stress and it catalyses the dismutation[7-9] of O_2 to H_2O_2 and O_2. Scald is primarily an autoxidation process and there is possibility that SOD level is affected by scald induction. There is also evidence that under stress conditions POD can lead to polymerization of phenolics into brown pigments[3,10,11]. As far as scald is concerned, lower POD activity was observed in scalded tissue than in healthier one[5]. It is clear that more attention should be focussed to determine the possible role of free radical scavenging enzymes in scald development. Present work was conducted to clarify if any relationship exists between scald and scald related maturity factors to the changes in activities of SOD, POD and PPO in apple fruits during storage.

EXPERIMENTAL

Fruits for this research work were harvested from 25 years old apple (*Malus domestica* Borkh cv. red spur delicious) trees grafted on M-26 root-stock grown at the Pomology Research Centre, University of Illinois at Urbana-Champaign, USA. The fruits were harvested from 15 trees, which were randomly divided into 5 replicates *i.e.*, 3 trees per replicate. 35 Fruits per tree *i.e.*, 105 fruits per replicate were harvested at early mature (immature), commercially mature and over mature stages and were immediately stored at -1 ºC. Maturity at harvest and ripening during storage were determined. Five fruits per replicate from each maturity stage were analyzed for enzyme activities (SOD, POD, PPO), ethylene synthesis, flesh firmness, soluble solids and starch contents at the time of harvest, during ripening period at 2, 4 and 8 weeks intervals and then for senescence after 3, 4 and 5 months of storage.

Fruit firmness was measured with an Effigi® pressure tester (Effigi Co. Alfonsine, Italy) using an 11 mm plunger tip. Soluble solids concentration was measured with hand refrectrometer® (Atago N1 Co., Japan) and starch contents were also measured[12].

Additional nine fruits per replicate were removed from storage after 3 months and kept at ambient temperature (25 ºC) for 7 d and then evaluated for scald. Scald incidence was rated[5,13]. The samples were rated for slight (less than about 10 % of the surface affected), moderate (10-50 % of surface affected) and severe scald (50-100 % of surface affected). The following equation was used to calculate scald index:

$$\text{Scald index} = [(\%\ \text{slight scald} \times 1) + (\%\ \text{moderate scald} \times 2) + (\%\ \text{severe scald} \times 4)]/4$$

Preparation of cell free extracts: A 5-6 g fresh weight sample from 5-9 fruits peel was ground in liquid nitrogen using a mortar pestle. The powdered tissue was suspended in 15 mL of 100 mM KPO_4 buffer (pH 7.8), containing 0.5 % (v/v) Triton-X-100 and 1 g PVPP. The mixture was centrifuged at 18,000 × g at 4 ºC for 0.5 h and the supernatant was collected and stored at -80 ºC for further analysis.

Protein concentration: The protein concentration was determined using bovine serum albumin as a standard[14].

Superoxide dismutase assay (SOD), peroxidase (POD), polyphenol oxidase (PPO): The activity of SOD was assayed spectrophotometrically by measuring its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT), similarly in respect of POD and PPO[11].

RESULTS AND DISCUSSION

Effect of harvest maturity on fruit firmness, soluble solids, starch contents, ethylene synthesis, POD, PPO and SOD activities in red spur delicious apples after 8 weeks of storage at -1 ºC: The fruits were harvested at early stage (immature), commercially mature and over-mature stages by using a combination of maturity indices, flesh firmness, soluble solids concentration and starch-iodine test. Flesh firmness decreased as the maturity proceeded while the total soluble solids concentration, starch contents and ethylene synthesis increased with the maturity of fruits after 8 weeks of storage at -1 °C (Table-1). Fruits at early stage of maturity showed lowest soluble solids concentration compared to the over-mature fruits at harvest and this difference was maintained even during storage. The POD, PPO and SOD activity was observed during the present study and the results showed statistically significant difference ($p < 0.05$) during storage. The POD and PPO activity was maximum in fruits which were harvested at early-mature stage and the minimum in full-mature fruits. On the other hand, SOD activity was higher in over mature fruits as compared with early and full mature fruits (Table-1).

Effect of harvest maturity on fruit firmness, soluble solids, scald index, POD, PPO and SOD activities in red spur delicious apples after 5 months of storage at -1 ºC: The results regarding fruit firmness, soluble solids concentration, scald index, PPO and SOD activities depicted statistically significant differences ($p < 0.05$) while the POD activity showed non-significant results after 5 months of storage at -1 °C (Table-2). It was observed that the fruits which were harvested at early mature stage had higher firmness as compared with full mature and over-mature fruits, while the soluble solids concentration increased as the maturity proceeded. On the other hand, scald index was found maximum in early mature fruits and the minimum in over mature fruits. The POD activity showed non-significant results even after 5 months of storage at -1 °C. The PPO activity was maximum in early mature fruits while the minimum in over-mature fruits. The SOD activity was higher in over mature fruits after 5 months of storage at -1 °C (Table-2).

Changes in fruit firmness, soluble solids, starch contents, ethylene synthesis, POD, PPO and SOD activities in red spur delicious apples during ripening: The flesh firmness and starch contents (starch contents are negatively correlated with starch index) decreased while soluble solids increased with increase in storage duration and with the stage of maturity (Table-3). The endogenous ethylene synthesized by fruits was also measured at different stages of maturity and during storage. Ethylene synthesis increased during storage and there was a sharp rise after 2nd week of storage where the full mature and over mature fruits showed clearly higher levels of ethylene contents (7.1 and 6.5 µL/kg/h, respectively) than immature ones (5.1 µL/kg/h) at the end of 8 weeks of ripening period in cold storage. The POD activity was observed maximum in over mature fruits after 2 weeks of storage while minimum in early and full mature fruits after 8 weeks of storage at -1 °C (Table-3). In the present study, PPO activity showed significant change with change in fruit maturity during ripening. A relatively higher activity was assayed at weekly intervals, it significantly increased in immature fruits and its activity remained at the highest level from 2nd week and the maximum activity was found after 8 weeks of storage during ripening. However, SOD activity in stored apple fruits significantly increased with increase in storage duration and with the increase in maturity level. The maximum SOD activity was observed in over mature fruits after 8 weeks of storage.

Changes in fruit firmness, soluble solids, scald development, POD, PPO and SOD activities in red spur delicious apples during senescence: The fruit firmness decreased with the increase in storage duration and maturity level while soluble solids concentration increased with increase in storage duration along with the stages of maturity (Table-4). The scald development

TABLE-1
CHANGES IN FRUIT FIRMNESS, SOLUBLE SOLIDS CONCENTRATION, STARCH CONTENTS, ETHYLENE SYNTHESIS, POD, PPO AND SOD ACTIVITIES IN RED SPUR DELICIOUS APPLES AT DIFFERENT STAGES OF MATURITY AFTER 8 WEEKS OF STORAGE AT -1 ºC

Stage of maturity	Firmness (N)	Soluble solids (%)	Starch[a]	Ethylene (µL/kg/h)	POD ΔO.D. min^{-1} mg^{-1} protein	PPO ΔO.D. min^{-1} mg^{-1} protein	SOD U g^{-1} fr. Wt.
Early-mature	80.90 ± 0.2a	10.35 ± 1.0c	2.95 ± 0.13c	2.15 ± 0.07c	1.18 ± 0.00a	3.28 ± 0.07a	41.75 ± 0.13b
Full-mature	78.93 ± 0.13b	11.18 ± 0.13b	4.73 ± 0.20b	2.83 ± 0.07b	0.75 ± 0.07c	2.48 ± 0.13c	40.35 ± 0.67c
Over-mature	75.06 ± 0.67c	11.73 ± 0.07a	5.91 ± 0.00a	2.95 ± 0.00a	0.83 ± 0.01b	3.03 ± 0.40b	44.35 ± 0.40a

[a]1 = 100 % starch and 9 = 0 % starch; ± = Standard error of means.
Different letters within the column denote significant difference at $p < 0.05$; means were separated according to LSD test.

TABLE-2
CHANGES IN FRUIT FIRMNESS, SOLUBLE SOLIDS CONCENTRATION, SCALD INDEX, POD, PPO AND SOD ACTIVITIES IN RED SPUR DELICIOUS APPLES AT DIFFERENT STAGES OF MATURITY AFTER 5 MONTHS OF STORAGE AT -1 ºC.

Stage of maturity	Firmness (N)	Soluble solids (%)	Scald index[a]	POD ΔO.D. min^{-1} mg^{-1} protein	PPO ΔO.D. min^{-1} mg^{-1} protein	SOD U g^{-1} fr. Wt.
Early-mature	82.18 ± 0.13a	10.38 ± 0.67b	15.40 ± 0.12a	1.30 ± 0.00NS	3.08 ± 0.67a	48.72 ± 0.21b
Full-mature	71.53 ± 0.10b	10.56 ± 0.05b	3.50 ± 0.25b	1.05 ± 0.01NS	2.38 ± 0.07b	61.11 ± 0.12a
Over-mature	66.93 ± 0.17c	11.43 ± 0.00a	2.02 ± 0.32c	1.12 ± 0.02NS	1.78 ± 0.07c	66.21 ± 0.10a

[a]Scald index = {(% light scald × 1) + (% medium scald × 2) + (% severe scald × 4)}/4; NS = Non-significant; ± = Standard error of means.
Different letters within the column denote significant difference at $p < 0.05$; means were separated according to LSD test.

TABLE-3
CHANGES IN FRUIT FIRMNESS, SOLUBLE SOLIDS CONCENTRATION, STARCH CONTENTS, ETHYLENE SYNTHESIS, POD, PPO AND SOD ACTIVITIES IN RED SPUR DELICIOUS APPLES AT DIFFERENT STAGES OF MATURITY DURING RIPENING

Stage of maturity	Storage period (weeks)	Firmness (N)	Soluble solids (%)	Starch[a]	Ethylene (μL/kg/h)	POD ΔO.D. min^{-1} mg^{-1} protein	PPO ΔO.D. min^{-1} mg^{-1} protein	SOD U g^{-1} fr. Wt.
Early mature	0	87.1 ± 0.01a	9.6 ± 0.60h	1.5 ± 0.20j	0.3 ± 0.21h	1.18 ± 0.50c	2.8 ± 0.51e	29.8 ± 0.21i
	2	82.4 ± 0.00b	10.1 ± 0.20gh	2.9 ± 0.64i	0.1 ± 0.20i	0.75 ± 0.20gh	3.3 ± 0.30b	40.0 ± 0.23g
	4	80.0 ± 0.67cd	10.6 ± 0.01efg	3.6 ± 0.60h	3.1 ± 0.61e	0.83 ± 0.25fgh	3.2 ± 0.21bc	47.1 ± 0.25e
	8	82.1 ± 0.13b	11.1 ± 0.21cde	3.8 ± 0.30gh	5.1 ± 0.10c	0.02 ± 0.12i	3.8 ± 0.12a	50.1 ± 0.21d
Full-mature	0	80.8 ± 0.12c	10.5 ± 0.15fg	3.9 ± 0.20g	0.5 ± 0.00g	0.83 ± 0.13fg	2.3 ± 0.06f	20.8 ± 0.12k
	2	80.1 ± 0.20cd	11.1 ± 0.01cde	4.5 ± 0.45f	0.2 ± 0.10hi	1.10 ± 0.07cd	2.9 ± 0.08de	28.4 ± 0.07j
	4	79.6 ± 0.21d	11.3 ± 0.30bcd	4.9 ± 0.06e	3.5 ± 0.01d	1.00 ± 0.01de	2.3 ± 0.12f	45.5 ± 0.67f
	8	75.2 ± 0.31fg	11.8 ± 0.21b	5.6 ± 0.04cd	7.1 ± 0.20a	0.03 ± 0.20i	2.4 ± 0.06f	66.7 ± 0.32b
Over-mature	0	76.6 ± 0.02e	11.0 ± 0.20def	5.5 ± 0.01d	0.9 ± 0.10f	0.90 ± 0.02ef	2.8 ± 0.01e	20.1 ± 0.24k
	2	74.4 ± 0.24gh	11.6 ± 0.01bc	5.7 ± 0.20bc	0.8 ± 0.20f	1.60 ± 0.04 a	3.1 ± 0.01bcd	33.0 ± 0.20h
	4	75.4 ± 0.07f	11.7 ± 0.01b	5.9 ± 0.07b	3.6 ± 0.30d	1.40 ± 0.67 b	3.0 ± 0.25cde	51.2 ± 0.01c
	8	73.8 ± 0.04h	12.6 ± 0.10a	6.5 ± 0.06a	6.5 ± 0.10b	0.70 ± 0.13h	3.2 ± 0.45bc	73.1 ± 0.64a

Different letters within the column denote significant difference at $p < 0.05$; means were separated according to LSD test.
± = Standard error of means

TABLE-4
CHANGES IN FRUIT FIRMNESS, SOLUBLE SOLIDS CONCENTRATION, STARCH CONTENTS, ETHYLENE SYNTHESIS, POD, PPO AND SOD ACTIVITIES IN RED SPUR DELICIOUS APPLES AT DIFFERENT STAGES OF MATURITY DURING SENESCENCE

Stage of maturity	Storage period (months)	Firmness (N)	Soluble solids (%)	Scald index[a]	POD ΔO.D. min^{-1} mg^{-1} protein	PPO ΔO.D. min^{-1} mg^{-1} protein	SOD U g^{-1} fr. Wt.
Early-mature	0	87.1 ± 0.13a	9.6 ± 0.23e	0.0 ± 0.00h	0.77 ± 0.12c	2.8 ± 0.20b	29.6 ± 0.01f
	3	81.0 ± 0.20b	10.7 ± 0.14cd	12.2 ± 0.01c	1.63 ± 0.67a	3.4 ± 0.14a	51.5 ± 0.02cd
	4	80.1 ± 0.01c	10.6 ± 0.25d	18.3 ± 0.10b	1.20 ± 0.60a	3.5 ± 0.012a	52.3 ± 0.01cd
	5	80.5 ± 0.21bc	10.6 ± 0.27d	31.1 ± 0.12a	0.77 ± 020abc	2.6 ± 0.02b	61.5 ± 0.20c
Full-mature	0	77.0 ± 0.45d	9.2 ± 0.10e	0.0 ± 0.00h	1.10 ± 0.01bc	2.1 ± 0.01c	33.3 ± 0.10ef
	3	72.9 ± 0.60e	10.9 ± 0.20bcd	1.3 ± 0.10g	1.10 ± 0.10abc	2.1 ± 0.01c	45.1 ± 0.04de
	4	68.1 ± 0.06f	10.8 ± 0.10cd	4.4 ± 0.21e	1.10 ± 0.01abc	2.7 ± 0.00b	78.1 ± 0.06b
	5	68.1 ± 0.07f	11.3 ± 0.20abcd	8.3 ± 0.01d	0.90 ± 0.02bc	2.6 ± 0.00b	87.9 ± 0.01ab
Over-mature	0	72.9 ± 0.05e	11.0 ± 0.01abcd	0.0 ± 0.00h	1.50 ± 0.02abc	2.0 ± 0.20cd	30.1 ± 0.01f
	3	66.4 ± 0.10g	11.6 ± 0.21ab	1.1 ± 0.10gh	1.50 ± 0.21abc	1.8 ± 0.13d	64.1 ± 0.02c
	4	64.8 ± 0.52h	11.4 ± 0.14abc	2.8 ± 0.01f	1.17 ± 0.012abc	1.8 ± 0.01d	78.7 ± 0.01b
	5	63.6 ± 0.14i	11.7 ± 0.24a	4.2 ± 0.02e	0.90 ± 0.20bc	1.5 ± 0.10e	91.9 ± 0.10a

Different letters within the column denote significant difference at $p < 0.05$; means were separated according to LSD test.
± = Standard error of means

was higher in early mature fruits as compared with full mature and over mature fruits and it increased with the increase in storage duration and the maximum scald index was observed after 5 months of storage at -1 °C. The POD activity was observed maximum in early mature fruits after 3 months of storage, after which a slight decrease in POD activity was recorded in full mature and early mature fruits (Table-4). In the present study, PPO activity showed significant change with change in fruit maturity during senescence. A relatively higher activity was assayed in early mature fruits as compared with full mature and over mature fruits and the highest activity was observed in early mature fruits after 4 months of storage while the lowest activity was found in over mature fruits after 5 months of storage (Table-4). However, SOD activity in stored apple fruits significantly increased with increase in storage duration and with the increase in maturity level. The maximum SOD activity was observed in over mature fruits after 5 months of storage at -1 °C.

Fruits after harvest started ripening rapidly which is accompanied by softening of texture, starch conversion into sugars and rise in ethylene synthesis[15,16]. In immature fruits less soluble solids percentage after storage might be due to less starch stored in fruits, which is to be converted to increase soluble solids[17]. Similarly, lower ethylene levels produced by immature fruits during storage showed that immature fruits might not have developed enough ability to produce as much ethylene as mature fruits. However, the role of ethylene in enhancement of the ripening process could be attributed to the higher level of soluble solids and softening of fruits in case of mature and over mature fruits recorded during storage[18].

As usual scald incidence was observed in fruits after longer time in storage and increased with increase in storage duration, which corresponds with increased fruit softening and senescence[2,19]. The observation of higher scald incidence in immature fruits than mature fruits is also in accordance with the findings of other researchers[3,20,21]. Scald is thought to be result of oxidation process because of its close association with the oxidation products of α-farnesene[10]. Its control by antioxidant diphenyl amine and increased susceptibility of immature fruits to scald is thought to be related with the accumulation of smaller amount of antioxidants and hence less resistance to oxidation products[3,5,11].

Generally higher POD activity was observed in immature fruits than mature fruits during storage. Results similar to the present study were reported in Golden delicious apples[3]. It is also reported that POD activity is correlated with ethylene production[22]. On comparison of POD activity to the development of scald in fruits, it was found that POD activity decreased later in storage whereas scald incidence significantly increased. Low POD activity was also observed in scalded peel of Granny Smith apples[5]. The

present observation of low POD activity in mature fruits during storage and a general decline in POD activity late in storage support the result of Lurie *et al.*[5] that scald development is inversely related with POD[23,24] but higher POD activity in immature fruits during storage compared to mature ones conflicts with such conclusion and may suggest no relationship between POD and scald development.

The increased PPO activity during storage in immature fruits might be in response to the release of phenolic substrate[25] resulting from the membrane breakdown and cell decompartmentallization because of senescence. Immature fruits had high phenolic compounds as compared to mature fruits as previous studies have shown that chlorogenic acid, major phenolic compound in apples, decreases rapidly during the early stages of development to reach an almost steady level at maturity[21,26] and total phenolic concentration stays at relatively constant level during storage[26]. Immature fruits which are high in phenolic compounds, at the same time have less efficient antioxidant system compared to mature fruits[26]. Release of phenolic compounds from the vacuole activates PPO[25] which might be the reason of increased PPO activity in immature fruits during storage. PPO when gets in contact with phenolics, oxidizes them which results in tissue browning[4,21]. Scald which is also characterized for browning of tissue has also been found to be correlated with PPO activity[5,21]. The present study result of high scald incidence in immature fruits which are also high in PPO activity during storage suggests a possible correlation between scald and PPO activity[3]. The lower level of antioxidants[20] and low SOD activity in immature fruits seem to have some additive effect on the frequency of the scald incidence (Table-5).

TABLE-5
PEARSON CORRELATION (R) BETWEEN
SOD, POD, PPO AND SCALD INDEX

	SOD	POD	PPO
POD	-0.84		
PPO	0.42	-0.02	
Scald Index	1.00*	-0.86	0.34

*Significant at $p < 0.05$

The SOD activity in stored apple fruits significantly increased with increase in storage duration. In these studies, the higher SOD activity in immature fruits might be due to younger tissues of fruits or it might be the result of higher amount of chloroplast and one of the SOD isoenzyme Cu-Zn SOD which is mostly localized in chloroplast[27] might had contributed to higher total SOD activity in immature fruits compared to mature fruits[28]. The increase in SOD activity during cold storage of fruits might be in

response to enhance free radicals production during ripening and senescence[29]. Relatively lower increments in SOD activity in immature fruits compared to mature fruits during cold storage and higher scald incidence in immature fruits which is related to oxidative reactions[5,23] suggests that immature fruits must have higher free radicals production, however they might be lacking the ability to produce as much antioxidant enzyme SOD as mature fruits. Previous studies on antioxidants, other than SOD, in apples have also suggested the inability of immature apple fruits to produce enough antioxidants during storage as mature ones[10]. An oxidation related physiological disorder, scald of apple and pear fruits was found to be reduced by naturally occurring antioxidants in fruits[24] and by treating with synthetic antioxidant diphenyl amine[5,11]. Various studies on transgenic plants over expressing SOD activity were found to have enhanced oxidative stress protection[30]. The present results of relatively lower activity of antioxidant enzyme SOD in immature fruits which also developed more scald during storage confirm that fruits lower in antioxidant level are more susceptible to scald incidence and suggest that mature fruits by expressing higher SOD activity can better protect themselves from scald. Even though mature fruits were higher in SOD activity but still developed some sort of scald which shows that even this much activity is not sufficient to prevent fruits from oxidative injury, however, immature fruits where activity was low, got higher scald incidence. These results suggest a possible relationship between scald, free radicals and SOD activity.

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