Differences of Biochemical Components Between the Skin Tissues of Normal and Black-speckled 'd'Anjou' Pears after Prolonged Low-oxygen Storage

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Abstract. A proportion of 'd'Anjou' pear fruit (*Pyrus communis* L.) developed a disorder, "black speck" or "skin speckling", after prolonged controlled atmosphere (CA) storage (1% O₂, -0.5 C). A comparative study of biochemical components revealed that there was no significant difference in succinic, citric, fumaric, and pyruvic acids between the speckled and normal skin tissues. The content of malic acid in the affected tissue was almost three times lower than that in the normal tissue. The specific activity of NADP-malic enzyme (EC 1.1.1.40) in the affected tissue was also lower, but the total activities were similar. The affected tissue contained higher percentages of dry matter and soluble proteins than the normal tissue. Two-dimensional gel electrophoresis of proteins showed that two groups of novel polypeptides appeared only in the affected skin tissue. This study indicated that a certain proportion of 'd'Anjou' pear fruit might have been exposed to unfavorable preharvest environmental stresses, and, therefore, could no longer tolerate the subsequent semi-anaerobic and chilling stresses during prolonged CA storage.

A certain proportion of mature-green 'd'Anjou' pear fruit stored in controlled atmosphere (CA) storage is, somehow, vulnerable to a disorder called "skin speckling" or "black speck" (Fig. 1) (Chen and Varga, 1989; Kupferman, 1988). The actual etiology of the disorder is not known. However, the symptom and its relationship with the O₂: CO₂ ratios in CA storage have been reported (Chen and Varga, 1989). The disorder is not caused by pathogens or chemicals and is closely associated with low oxygen concentration (<2%) in CA storage (Chen and Varga, 1989). Fruit with the disorder become unmarketable due to the unpleasant appearance, even though they are capable of ripening normally.

Fig. 1. Symptom of black speck disorder of 'd'Anjou' pear fruit after 8 months of storage in 1% O₂ at -0.5C.

There is ample evidence that interference with normal respiration, or injury to the respiratory mechanism in fruit tissue, is related to the development of certain functional disorders of pome fruit during storage. Hulme (1956) and Williams and Patterson (1964) reported that apple and pear fruit stored in high-CO₂ concentration increased succinic acid content to toxic levels, which resulted in 'brown core' disorder. An accumulation of oxalacetic acid was found to be associated with low-temperature breakdown of apple fruit (Hume et al., 1964). An abnormal type of organic acid metabolism in apple fruit was associated with the development of Jonathan spot disorder (Richmond et al., 1964). The spotted fruit tissue had a higher pH and total acid content than normal fruit tissue. The increase in organic acid content in the spotted tissue was due to K⁺ ion accumulation. To compensate for the influx of K⁺ ion, malic acid in the vacuole was dissociated into malate and even further into malate⁻, which resulted in an increase in pH (Richmond et al., 1964).

Fruit stored in either low O₂, high CO₂, or a combination of both, are under semi-anaerobic stress. 'd'Anjou' pear fruit stored in CA storage at -1C, the optimum storage temperature (Porritt, 1964), are probably subjected to both semi-anaerobic and chilling stresses. Alterations in protein synthesis in response to anaerobic (Sachs and Freeling, 1978; Sachs et al., 1980) and low temperature (Tseng and Li, 1987) stresses in plants have been well-documented. However, the effects of such stresses on the protein synthesis of pear fruits is not known.

The objective of this study was to compare the differences in organic acid contents, malic enzyme activity, and protein patterns between normal and black-speckled skin tissue of 'd'Anjou' pears after 8 months of storage in 1% O₂ at -0.5C (± 0.5C) in an attempt to elucidate the possible etiology of the disorder.

Materials and Methods

Normal and black-speckled 'd'Anjou' pears were obtained from three commercial warehouses (Borton & Sons, Inc., Yakima, Wash.; Diamond Fruit Growers, Inc., Hood River, Ore.; Duckwall-Pooley Fruit Co., Hood River, Ore.) in the Pacific Northwest and from the Mid-Columbia Agricultural Research and Extension Center (MCAREC), Hood River, Ore. They were

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designated as fruit source #1, #2, #3, and #4, respectively. All fruit had been stored in the CA conditions with 1% O₂ and <0.1% CO₂ at -0.5C ($\pm$ 0.5C) for 8 months. At the end of storage, both normal and affected fruits were collected individually on the packing line from the three commercial warehouses and were sub-sampled into four lots, with each lot consisting of 100 fruits. Fruits stored at MCAREC were manually segregated. Normal and affected fruits were also sub-sampled into four lots, with each lot consisting of about 50 fruits.

Fifteen fruit from each lot were selected randomly for sample preparation. Each selected fruit was washed, rinsed, and surface-wiped to dryness with a paper towel. For each lot, the skin tissue of each fruit was peeled with a potato peeler. To avoid excessive oxidative browning during sample preparation, the peeled skin tissue was covered immediately with a piece of cheese cloth wetted with 0.5 % aqueous solution of sodium bisulfite. After weighing, the peel samples were wrapped in two layers of cheese cloth, immediately frozen in liquid N₂, and were then lyophilized. The lyophilized skin tissues were weighed to obtain the dry weight and then pulverized into fine powder with a Waring blender at high speed. The tissue powders were stored in glass vials that were kept in a desiccator at -20C until use.

Analysis of organic acids. Lyophilized pear skin powder (2 g) was suspended in 50 ml of 80% ethanol and boiled for 2 min while being stirred. After being cooled to room temperature, the ethanol extract was filtered through Whatman #1 filter paper. The residue was washed at least five times with 80% ethanol. The volume of combined filtrates was brought to 100 ml with 80% ethanol. Fifty milliliters of the ethanol extract (equivalent to 1 g of dry powder) was evaporated at 60C to $\approx$ 3 ml in an air-draft oven to remove the ethanol. The aliquot was filtered further through Whatman #4 filter paper to remove the precipitated material, washed at least five times, 1 ml each, with distilled water. Combined filtrates were adjusted to pH 7.8 with 0.5 N NH₄OH and passed through a Bio-Rex 5 ion exchange (Cl⁻ form; 100-200 mesh, 0.8 x 4 cm, Bio-Rad, Richmond, Calif.) column. Organic acids trapped in the resin were eluted from the column with 1.5 ml of 10% H₂SO₄ followed by 3 x 2 ml of distilled water into a 10-ml volumetric flask. The eluent was brought to a volume of 10 ml with distilled water and filtered through a 0.45- μ m membrane (RC55, S&S). A 10- μ l aliquot was introduced into a high-performance liquid chromatography (Varian Model 5000, Palo Alto, Calif.) by the loop-valve injector. Organic acids were separated by a crosslinked polystyrene strong cation exchange column (Bio-Rad Aminex PHX-87, 7.8 x 300 mm) using 0.01 N H₂SO₄ eluent (flow rate 0.5 ml·min⁻¹, column temperature 30 C). The chromatographic profiles of individual organic acids were detected by a variable wave-length detector (Varian Varichrom) at 210 nm. Each peak was identified by the retention times of known organic acid standard solutions and also by spiking each known organic acid standard individually into the unknown sample. The quantity of each identified acid was estimated by the internal standard method (formic acid as internal standard) using the Varian CDS 111L chromatography data system.

Malic enzyme extraction and assay. Dry powder (1 g) was mixed with 1 g of quartz sand (acid-washed) and 1 g of insoluble polyvinylpyrrolidone (M_w 700,000) in a prechilled mortar. After mixing, 10 ml of pre-chilled extraction buffer containing 0.25 M Tris-HCl (pH 7.5), 0.01 M DIECA (diethyldithio-carbamate), 0.5% (v/v) 2-mercaptoethanol (2-ME), and 6% (w/v) PEG 4000 (Hartman and Drouet, 1979; Hartman et al., 1977) were added

and then homogenized with a mortar and pestle for 2 min at 2C. The homogenized slurry was centrifuged at 20,000 x g for 30 min at 2C and the supernatant obtained was passed through a BioGel P60 desalting column (Bio-Rad Econo-Pac 10 DG column). The desalted crude extract was used for the assay of malic enzyme activity.

Malic enzyme activity was determined spectrophotometrically by measuring the reduction of absorbance of nicotinamide adenine dinucleotide phosphate (NADP) at 340 nm at 25C. The assay mixture contained 80 mM Tris-HCl buffer (pH 7.5), 1 mM MnSO₄, 0.33 mM NADP, 7.3 mM sodium malate, and 0.1 ml desalted crude extract in a total volume of 3 ml (Hartman and Drouet, 1979; Hartman et al., 1977). The reaction was started by the addition of NADP. Without the addition of Mn²⁺ or Mg²⁺ ion, the enzyme activity was not detected. Thus, the crude extract itself did not exhibit any NADP malic dehydrogenase activity (Hartman and Drouet, 1979; Hartman et al., 1977). Under these conditions, there was a linear relation between reaction rate and enzyme concentration. One unit of enzyme activity was defined as the reduction of 1 μ mol of NADP per minute at 25C.

Protein content in the crude extract was determined by a modified Lowry's method (Peterson, 1977).

Protein characterization by gel electrophoresis. Freeze-dried powder (1 g) was homogenized (five 30-sec pulses with a Tekmar Tissumizer set at 90) in 4 ml of extraction buffer, containing 50 mM Tris-HCl (pH 8.6), 2% NaDodSO₄ (SDS), 5% 2-ME, and 10 mM phenylmethylsulfonyl fluoride (Bethesda Research Laboratories, Bethesda, Md.). The slurry was centrifuged at 15,000 x g at 4C for 30 min. The supernatant was obtained and then filtered through a glass-wool-plugged funnel into a 30-ml Corex tube. Five volumes of cold acetone (-20C) were added to the supernatant and incubated at -20C for 30 min to precipitate proteins. After centrifuging at 9000 x g, 4C, for 30 min, the supernatant was discarded and the protein pellet was washed twice with cold acetone and air-dried. About 500 μ l of lysis buffer containing 9.5 M urea, 3% (w/v) 3-[(3-cholamidopropyl)-diethylammonio]-l-propanesulfonate (CHAPS) (Sigma, St. Louis), 3% (w/v) ampholytes (2 parts pH 3-10 and 1 part pH 5-7) (Bio-Rad), and 5% (v/v) 2-ME was added to redissolve the protein pellet. The protein solution was spun in a microcentrifuge (10,000 x g, 30 min) to remove any remaining particulate matter. Two-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was based on modification of O'Farrell's method (1975), as reported by Holloway and Arundel (1988) and Bio-Rad (Technical Bulletin #1144). A volume of the protein solution equivalent to 200 μ g of protein was mixed with an equal volume of glycerol and 0.5 part (w/v) solid urea and vortexed until the urea dissolved. The mixture was loaded on a 2.5 x 140-mm isoelectric focusing tube gel consisting of 9.5 M urea, 4.5% acrylamide, 3% CHAPS, and 3% ampholytes (same ratio as in the lysis buffer). Gels were focused in 0.1 M NaOH cathode and 0.01 M H₃PO₄ anode buffers for 14 hr at 500 V, followed by 1 hr at 1000 V. Methods for the second-dimension SDS-PAGE run were essentially those by O'Farrell (1975), with stacking and running gel concentrations at 4% and 11%, respectively. Slab gels were stained with Coomassie Blue R-250 (Sigma).

Statistical analysis. All the variables were analyzed as a randomized block design with subsampling. The main plots were two types of fruit (normal and black-speckled) and replicated four times (i.e., four fruit sources). Subplots were four subsampled experimental units. Analysis of variance was obtained

to test the effect of fruit source (i.e., four warehouses), types of fruit (normal vs. speckled), and the interaction between the two. Means were separated by the LSD method at the 5% level.

Results and Discussion

Similar to the flesh tissue (Li and Hansen, 1964; Chen et al., 1981, 1982), the major organic acid in the skin tissue of 'd'Anjou' pears was malic acid, followed by succinic and citric acids (Table 1). Pyruvic and fumaric acids were present at considerably lower concentrations. Although malic, succinic, and citric acids are involved in the Krebs cycle in mitochondria, they also accumulate in the vacuole as "extracyclic" acids (Biale, 1960). The content of malic acid in the normal skin tissue was consistently about three times higher than that in the speckled peel tissue based on either 1 g dry weight or 100 g fresh weight (Table 1). The contents of succinic, citric, and pyruvic acids in the normal peel tissue were also slightly higher than those in the speckled peel, with an exception of fruit source #4. The content of fumaric acid was not significantly different between the two fruit conditions. The predominant difference in organic acid content between the two conditions of skin tissue was due to malic acid. No analysis was made at time of harvest, as it was impossible to separate the potential speckled fruits from normal ones at that time. Net changes in individual acids before and after storage were not determined. Succinic and pyruvic acids in the skin tissue of 'd'Anjou' fruit may not be the cause of black speck disorder because the levels of these two acids in the skin tissue of speckled fruit were not significantly different from normal peel tissue, regardless of fruit source (Table 1).

There are two possible hypotheses for the differences in malic

Table 1. Organic acid content in the peel tissue of normal and black-speckled 'd'Anjou' pear fruit after 8 months of CA storage in 1% O₂ at -0.5C.

Fruit source	Type of fruit	Organic acid (mg·g ⁻¹) ^z				
		Malic	Succinic	Citric	Pyruvic	Fumaric
<i>Dry-weight basis</i>						
#1	Normal	7.77	2.4	0.40	0.05	0.005
	Speckled	2.51	1.2	0.33	0.04	0.005
#2	Normal	7.94	2.6	0.23	0.05	0.004
	Speckled	2.68	2.0	0.13	0.02	0.002
#3	Normal	8.56	1.8	0.26	0.09	0.001
	Speckled	3.02	1.4	0.15	0.05	0.002
#4	Normal	9.11	1.1	0.27	0.03	0.004
	Speckled	2.46	1.8	0.42	0.08	0.001
	LSD _{0.05} ^y	0.49	0.31	0.11	0.01	0.002
<i>Fresh-weight basis</i>						
#1	Normal	219	66.7	11.2	1.4	0.15
	Speckled	73.0	35.7	9.6	1.1	0.15
#2	Normal	210	67.9	6.1	1.2	0.10
	Speckled	79.5	60.3	3.7	0.7	0.66
#3	Normal	227	49.0	7.4	2.5	0.03
	Speckled	84.4	39.6	4.3	1.4	0.07
#4	Normal	240	36.7	7.0	0.8	0.10
	Speckled	69.1	49.4	11.7	2.3	0.03
	LSD _{0.05}	13.1	8.4	3.2	0.3	0.06

^aOrganic acid contents were calculated as milligram acid per gram dry or fresh weight of skin tissue.

^yLSD at *P* = 0.05.

acid content between the two fruit conditions. First, we assume that the initial contents of malic acid in the peel tissue of both fruit conditions were similar at harvest. However, during CA storage, fruit tissue becomes susceptible to black speck, and may metabolize malic acid faster than the normal peel tissue. If so, the activity of NADP-malic enzyme in the affected skin tissue should be higher than that in the normal one. However, the specific activity of this enzyme in the affected tissue was actually lower than that in the normal tissue, while the total activities were not significantly different between the two types of tissues (Table 2). NADP-malic enzyme catalyzes the conversion of malic acid to pyruvic acid. Pyruvic acid, in turn, can either enter the Krebs cycle via acetyl-CoA or decarboxylize to acetaldehyde, which can be reduced further to ethanol by fermentation. Both acetaldehyde and ethanol are extremely toxic to the plant cells (Wade, 1979; Smagula and Bramlage, 1977). The lower malic acid content in the affected skin tissue might have resulted from a higher rate of fermentation during storage. The levels of acetaldehyde, ethanol, and the related enzyme activities in the skin tissue of both fruit conditions were not determined in this study. Further study is needed to judge the validity of this hypothesis.

Second, we assume that the initial content of malic acid in the affected skin tissue was significantly lower than that in the normal tissue at the time of harvest. The "development of black speck may only reflect that a certain proportion of harvested fruit has been exposed to unfavorable environmental stresses before harvest. This portion of fruit, in turn, can no longer tolerate the subsequent semi-anaerobic (low O₂) and chilling (-0.5C) stresses during CA storage. 'd'Anjou' pear fruits grown in an unfavorable temperature regime (> 20.0 or <11.7C) during 6 weeks before harvest always accumulated more protein and had a significantly lower malic acid content than those grown in a favorable temperature regime (between 13.9 and 17.2C) when measured either at the time of harvest or after 8 months of storage in air at -1.1C (Mellenthin and Wang, 1976). In our study, the affected skin tissue had significantly higher contents of soluble proteins and dry matters (Table 3), but a lower level of malic acid (Table 1) than the normal skin tissue. The results support the concept that the speckled fruit might indeed have suffered unfavorable environmental stresses during the growing season.

The comparison of the SDS soluble proteins between normal and black-speckled skin tissues by two-dimensional electropho-

Table 2. Malic enzyme activities in the peel tissue of normal and black-speckled 'd'Anjou' pear fruit after 8 months of CA storage in 1% O₂ at -0.5C.

Fruit source	Type of fruit	Specific activity (units/mg of protein)	Total activity	
			(units/g dry wt)	(units/100 g fresh wt)
#1	Normal	0.83	1.37	38.7
	Speckled	0.58	1.24	36.4
#2	Normal	0.81	1.30	34.3
	Speckled	0.62	1.24	36.7
#3	Normal	0.90	1.74	44.8
	Speckled	0.64	1.85	48.6
#4	Normal	0.79	1.34	35.3
	Speckled	0.59	1.32	37.6
	LSD _{0.05}	0.07	0.15	4.3

Table 3. Dry matter, water, and soluble protein contents in skin tissue of normal and black-speckled 'd'Anjou' pear fruit after 8 months of in 1% O₂ at -0.5°C.

Fruit source	Type of fruit	Dry matter	Moisture	Soluble protein	
		(g/100 g fresh wt)	(g/100 g fresh wt)	(mg·g ⁻¹ dry wt)	(mg/100 g fresh wt)
#1	Normal	26.7	73.3	1.65	46.7
	Speckled	29.1	70.9	2.14	62.1
#2	Normal	26.4	73.6	1.60	42.2
	Speckled	29.7	70.3	2.01	59.6
#3	Normal	25.8	74.3	1.93	49.6
	Speckled	28.2	71.8	2.90	81.7
#4	Normal	26.4	73.6	1.70	44.8
	Speckled	28.6	71.4	2.24	64.2
LSD _{0.05}		0.4	0.4	0.11	3.3

Fig. 2. Coomassie blue-stained two-dimensional SDS-PAGE proteins from 'd'Anjou' pear skin tissue. (A) Normal fruit. (B) Black-speckled fruit. Open circles indicate the appearance of novel polypeptides or the increased quantities of polypeptides, resulting from black speck.

resis indicates the presence of novel peptides resulting from the disorder (Fig. 2). Some peptides were present in both tissues, but are much more abundant in black-speckled fruit skin. However, the appearance of two groups of peptides at ≈ 29 –43 kDa ($pI = 5.1$) and at 14.3–18.4 kDa ($pI = 6.2$) are the most obvious. They occurred in all the disordered fruit, but never in any of the normal skin samples (data not shown). Visual inspection of the gels does not seem to indicate any noticeable decrease or disappearance of peptides due to the disorder. Protein patterns of the speckled and clear skin portion of the same fruit were also compared (data not shown). Of the 15 black-speckled fruits tested, all clear skin portions contained the same protein pattern as in the diseased portions. This result suggests that fruits that contain these unique proteins have a greater tendency to develop black speckling and, second, even if the fruits do not exhibit visual speckling symptoms, the novel protein may already be present.

Whether these new polypeptides that appeared in the affected skin tissue were induced by the preharvest stresses or by the low O₂ and chilling stresses during storage remain to be studied further. By using immunochemical technology, these new polypeptides can be used for the preparation of antibodies. If these novel polypeptides in the skin tissue have been induced by either preharvest or postharvest stresses before the appearance of black speck, then the percentage of 'd'Anjou' fruit that carries these groups of polypeptides in any storage lot can be estimated easily. Thus, it would be possible to predict the potential susceptibility to black speck of fruit within a storage lot before or during storage. The economical loss caused by this type of disorder could, therefore, be reduced.

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