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Title: Mineral Nutrition, Pyruvate Kinase Activity, and Protein Concentration in 'd'Anjou' Pear Fruit Affected by Cork Spot

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Cork spot is the most serious physiological disorder in pear (Pyrus communis L. cv. d'Anjou) production in the United States. The disorder is recognized by a dimpled or bumpy surface on the calyx end of the fruit. Beneath the dimples, which are sometimes yellowish in color, is found brown, necrotic tissue. Fruit affected by cork spot are not marketable.

A review of the literature indicated cork spot is generally linked with low calcium concentration in the fruit. The ratio of nitrogen to calcium in the fruit has also been correlated with the disorder. The review further showed that the results of fruit mineral analysis can vary widely.

Mineral analyses were conducted in 1987 and 1988 on soil, leaves, and fruit peel from normal trees and trees prone to cork spot. Soil tests and leaf analysis did not provide measureable differences (5% level) between the two groups of trees. Fruit analysis did provide measureable differences, but the results were subject to variation. Contrary to the findings of previous researchers, cork spot was not

related to low levels of fruit calcium or a high ratio of nitrogen to calcium in the fruit, instead it was not closely associated with any single nutrient or ratio of nutrients. Of the nutrients examined, high potassium levels in the fruit were most often linked with the disorder.

The literature review and the results of the mineral analysis illustrated that fruit analysis can give diverse results. This variability led to a search for alternative methods of identifying cork spot. The present study evaluated an assay for the enzyme pyruvate kinase (PK) as a diagnostic tool for cork spot.

First, PK activity was closely examined in peel of normal and cork spotted fruit at one point in time, i.e. 56 days after the 1988 harvest. The assays provided measureable differences between normal and disordered fruit. Second, PK activity was measured during selected months of 1987 and 1988 in peel of normal and affected fruit. In the 12 months the fruit were assayed, significant differences between normal and cork spotted fruit in total PK activity were revealed in only two months. Compared to fruit potassium concentration, which was different in five of the 12 months, the PK assay is a weak diagnostic tool for cork spot.

An important finding of this study was to learn cork spotted fruit had higher soluble protein concentrations then normal fruit. In six out of the 12 months fruit were assayed protein concentration was significantly higher in disordered fruit than normal fruit. The assay for protein concentration merits further study for its potential to diagnose cork spot.

Mineral Nutrition, Pyruvate Kinase Activity, and Protein Concentration in 'd'Anjou' Pear Fruit Affected by Cork Spot

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DEDICATION

I dedicate this dissertation to my three sons: Michael, Jack and Jeremy. They were my inspiration.

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CONTRIBUTIONS OF AUTHORS

Four scientists made significant contributions to this study of cork spot of 'd'Anjou' pear fruit which was carried out at the Mid-Columbia Agricultural Research and Extension Center (MCAREC). Eugene A. Mielke, my major professor and MCAREC Superintendent, supervised all aspects of the study. He took special interest in structuring an outline for the literature review in Chapter 2. Timothy J. Facteau advised on sampling techniques, statistical analysis, table preparation, and interpretation of results. He also conducted the fruit mineral analysis on the 1988 post-harvest samples presented in Chapters 3 and 4. The study was carried out in the laboratory of Paul M. Chen who provided guidance in developing the methods employed for assaying proteins and pyruvate kinase in pear fruit. He had an instrumental role in identifying the major factors, presented in Chapter 4, that affect pyruvate kinase activity in pear fruit. Ruth Lavon, an exchange scientist from Israel, introduced and demonstrated the assay for pyruvate kinase to the MCAREC staff. She also prepared the electrophoretic gels presented in Appendix B.

The chapters are written in the format of manuscripts for the Journal of the American Society for Horticultural Science.

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CHAPTER 1.

INTRODUCTION

Cork spot is the most serious physiological disorder in pear (Pyrus communis L. cv. 'd'Anjou') production in the United States. It is assumed to be a calcium related disorder similar to bitter pit in apple and blossom end rot in tomato and watermelon.

Cork spot is recognized (Fig. 1.1) by a dimpled or bumpy surface on the calyx end of the fruit (Burkhart, 1980; Raese, 1980; 1984; 1987). Beneath the dimples, which are sometimes yellowish in color, is found brown, necrotic tissue. The visual symptoms become evident three weeks before harvest. Fruit affected by cork spot are not marketable.

Pears are the most important tree fruit in the State of Oregon. The state produces 26% of the United States' pear crop, and is ranked second or third in pear production among the 50 states (Weiser and Crabtree, 1987). In 1985, Oregon's pear crop had a farm gate value of $50 million (Weiser and Crabtree, 1987). Approximately 32% of the pear crop is of the cultivar d'Anjou, which is susceptible to cork spot. The incidence of the disorder varies from year to year. For example, in 1987, 1988, and 1989, in the test plots associated with this study, the average incidence of cork spot at harvest was 14%, 9%, and 13%, respectively. Applying these figures to the value of the 1985 crop makes it clear that the impact of cork spot can be measured

Fig. 1.1. Cork spot is the principal physiological disorder of 'd'Anjou' pear in the United States. It is recognized by bumps or dimples on the surface of the fruit and brown, necrotic tissue beneath the peel.

in millions of dollars of lost income to farmers.

Cork spot is generally associated with low calcium concentration in the fruit (Mason and Welsh, 1970; Woodbridge, 1971; Al-Ani, 1978; Richardson and Lombard, 1979; Richardson and Al-Ani, 1982; Raese, 1980; 1984; 1986; 1987; 1988; 1989; Raese and Stahly, 1982; Vaz, 1984; Fallahi, et al., 1988; Curtis, 1988). Despite this correlation between the disorder and low calcium, attempts to use fruit mineral analysis to diagnose the problem have led to conflicting or inconclusive results (Kupferman, 1988). This is because calcium content can vary from fruit to fruit on the same tree (Brun et al., 1985). Calcium content can also vary among trees in the same orchard, between orchards, and from year to year (Kupferman, 1988). This wide variability in fruit calcium levels has prevented scientists from agreeing on a threshold or absolute level below which the fruit becomes susceptible to cork spot. Thus, fruit mineral analysis for calcium has not proven to be a reliable tool for diagnosing the disorder.

The search for an alternative tool to diagnose cork spot led to the present study. The approach centers on measuring enzyme or protein activity as an indication of nutrient status in the plant tissue (Bar-Akiva, 1971). The present study evaluates an assay for the enzyme pyruvate kinase as a technique for the identification of cork spot.

Pyruvate kinase is an enzyme that catalyzes the metabolic reaction in glycolysis, where phosphoenolpyruvate (PEP) and ADP are converted to pyruvate and ATP. The reaction occurs in the cytosol.

The enzyme requires K^+ and Mg^{+2} as cofactors or activators, and is strongly inhibited by Ca^{+2}. These same three cations are believed to be important factors in the development of cork spot.

The value of an assay for pyruvate kinase as an indicator of physiological disorders due to imbalances of K^+, Mg^{+2}, and Ca^{+2} was discovered by Bar-Akiva (et al., 1976) in Israel. Bar-Akiva showed that K^+ and Mg^{+2} deficiencies decreased pyruvate kinase activity in lemon leaves, whereas Ca^{+2} deficiency caused an increase in enzyme activity. Lavon (et al., 1988a; 1988b) conducted a detailed study of the effect of K^+, Mg^{+2}, and Ca^{+2} on pyruvate kinase activity in citrus. Together, Lavon's and Bar-Akiva's results demonstrated the inhibitory effect of Ca^{+2} on pyruvate kinase activity, and confirmed Bar-Akiva's suggestion that the enzyme possessed promise as a diagnostic tool for calcium related disorders. The present study transfers these biochemical techniques from citriculture to pomology.

The present study tests three hypotheses. The first hypothesis is that cork spot is associated with low Ca^{+2} and high K^+ and Mg^{+2} concentrations in the fruit. These cations are thought to be important factors in the development of the disorder, and they also exert great influence on the activity of pyruvate kinase. The second hypothesis is that cork spot is associated with high levels of pyruvate kinase activity. The testing of this hypothesis will verify the theoretical basis for using the assay as a diagnostic tool. The third hypothesis is that cork spot is associated with unique proteins or polypeptides. If unique proteins exist, they may have value as markers for defining or identifying the disorder.

The overall goal of this study is to lay the foundation for using the assay for pyruvate kinase for diagnosing cork spot in pear fruit.

CHAPTER 2.

LITERATURE REVIEW

A. Description of cork spot.

Cork spot is a physiological disorder of the fruit of 'd'Anjou' pear. The disorder appears 1 to 2 weeks before the fruit reaches harvest maturity (Mason and Welsh, 1970). Affected fruit are characterized by bumps or dimples on the external surface. Beneath the bumps are found sub-epidermal lesions (Richardson and Al-Ani, 1982) or necrotic spots that are 'corky' in texture. These external and internal symptoms are typically found at the calyx end of the fruit (Mason and Welsh, 1970; Burkhart, 1980; Raese, 1980; 1984; 1987; 1988). The browning and necrosis of the tissue is apparently induced by physiological injury to the cells (Wang and Mellenthin, 1973). The disorder continues to develop and expand while the fruit are in storage (Richardson and Al-Ani, 1982). Approximately 10 to 25% of the disordered pears are detected at harvest, the remainder appear during or after storage (Richardson and Lombard, 1979).

B. Similarities between cork spot and other disorders.

Cork spot is generally assumed to be similar to bitter pit of apple. Both disorders share a similar breakdown of the fruit flesh and both are associated with low calcium concentration in the fruit (Mason and Welsh, 1970; Shear, 1975; Richardson and Lombard, 1979; Richardson and Al-Ani, 1982).

Shear (1975) includes pear with 21 other horticultural crops that

are prone to disorders related to inadequate levels of calcium. The most well known of these disorders is blossom-end rot of tomato.

In the literature, cork spot is sometimes identified by the synonyms cork, drought spot, pit, 'd'Anjou' pit and pear bitter pit.

C. Calcium deficiency as the origin of cork spot and five theories for the mechanism of induction.

Cork spot was first linked to low calcium concentration in the fruit by Mason and Welsh (1970). Numerous authors have confirmed this initial finding and attributed cork spot to a localized calcium deficiency of the fruit (Woodbridge, 1971; Al-Ani, 1978; Richardson and Lombard, 1979; Richardson and Al-Ani, 1982; Raese, 1980; 1984; 1986; 1987; 1988; 1989; Raese and Stahly, 1982; Vaz, 1984; Brun et al., 1985; Fallahi et al., 1988; Gerasopoulos, 1988; Curtis, 1988).

Few authors have offered theories on the mechanisms that actually cause cork spot. In the following sections five theories are presented on the induction of the disorder. The theories are drawn from interpretations of research on bitter pit apple, which has a much longer and deeper history of research than cork spot.

1. Loss of membrane integrity theory.

Calcium stabilizes membranes by cross-linking phospholipids with proteins. When calcium is replaced by hydrogen or certain other ions the membranes become more permeable. This substitution allows for the enlargement of membrane pores and the passage of ions and low molecular weight molecules. The greater the replacement of calcium by other ions the more leaky the membrane becomes (Mengel and Kirkby, 1982).

The loss of membrane integrity is accompanied by a loss of compartmentation which leads to the death of the cell (Van der Boon, 1970; Bangerth, 1974; Hilkenbaumer and Naumann, 1974).

2. Crystal impediments theory.

Stebbins et al. (1972) found crystals containing calcium in the pedicils and petioles of apple trees and theorized that the crystals impede the movement of calcium into the fruit and predispose the fruit to disorders. Organic acids, such as oxalic acid, can inactivate calcium ions by chelation (Bangerth, 1974). Oxalic acid binds to calcium to form crystals of calcium oxalate. When the crystals form in the xylem of pedicils they may impede calcium movement into the fruit. When crystals form in the phloem in leaf petioles the calcium movement from leaves to fruit may be obstructed.

This theory provides support for another avenue of inquiry into calcium-related disorders. The source of nitrogen fertilizer can affect the level of organic acids, such as oxalic acid, which in turn affects the amount of calcium tied up as crystals (Link, 1974). Thus, nitrogen fertilizers, for example nitrates, that favor the chelation of calcium may promote fruit disorders.

3. Acceleration of respiration theory.

Low calcium concentration in apple fruit is associated with high rates of respiration (Faust and Shear, 1972; Bangerth et al., 1972). 'D'Anjou' pears affected by cork spot had higher and accelerated rates of respiration when compared with unaffected fruit (Wang and Mellenthin, 1973). Wang and Mellenthin (1973) theorized that accelerated respiration leads to an accumulation of respiratory

products such as acetaldehyde, alcohol, and other potentially damaging products. When not compartmentalized by membranes these products damage the cell and increases its permeability to oxygen. The oxygen could then react with chlorogenic acid and induce tissue browning. Chlorogenic acid is the principal phenolic compound in pears and is the primary substrate in the enzymic browning of fruit tissue which is catalyzed by polyphenoloxidase (Wang and Mellenthin, 1973). Support for this theory comes from the observation that high chlorogenic acid levels were associated with 'd'Anjou' pear tissues affected with cork spot.

4. Calcium withdrawal theory.

Perring (1986) theorized that bitter pit is induced by the withdrawal of calcium from cells in close proximity to the vascular system. If the fruit is on the tree, calcium could be withdrawn via the vascular system when the fruit is subjected to water stress in periods of hot weather or drought. Under these conditions bitter pit may develop prior to harvest. In stored apples, calcium moves from the peel region to the core via the vascular system. The lowered calcium concentration leads to weakened membranes or impaired biochemical activity that is followed by necrosis.

Advantages to this theory are that it partly accounts for the wide variability in calcium concentration found in fruit and it recognizes the core as the site of major biochemical activity where a demand for calcium arises.

5. Calmodulin inactivation theory.

Calmodulin is a protein that binds calcium ions and regulates

biochemical processes in plants at the cellular level. Experimental evidence summarized by Poovaiah (1985) indicates changes in cell wall rigidity, membrane permeability, and enzyme activation are regulated, in part, by calcium and calmodulin. Fukumoto (1985) theorized that bitter pit occurs when calmodulin fails to be activated due to a lack of calcium. He suggested that under normal conditions calmodulin is activated at sites on membranes. Further study is needed to identify the biochemical processes that are directly affected when calmodulin fails to activate.

The most convincing evidence for this theory is that apples treated with the calmodulin antagonists, chlorpromazine and fluphenazine, developed bitter pit symptoms (Fukumoto, 1985; Fukumoto and Venis, 1986). These antagonists inactivated the calmodulin and the disorder appeared in two to seven days.

D. Calcium nutrition and cork spot.

The following sections review the salient features of calcium's relationship to cork spot. Attention is focused on calcium's deficiency symptoms, its effects on physiological and biochemical processes, its interactions with other nutrients, its link to orchard practices, and the roles these factors play in the development of cork spot.

1. Calcium deficiency symptoms in plants.

Calcium deficiency in plants is characterized by a reduction in growth of meristematic tissues (McMurtrey, 1950). The deficiency is usually first observed in growing shoot tips and the youngest leaves. In extreme cases the deficiency results in the death of the

meristematic tissue.

Most soils have sufficient calcium for plant growth and deficiency symptoms as described above are rarely encountered. For example, Shear (1975) reported that recognizable foliar symptoms of calcium deficiency are seldom observed on field-grown fruit crops. What is common is symptoms of indirect calcium deficiency which result from a reduced supply of calcium to the fruit. Thus cork spot is not directly due to a deficiency of soil calcium, but may be the result of diminished amounts of calcium being transported to the fruit.

2. Role of transpiration in calcium nutrition.

The evaporation of water through the stomates on the surface of the leaf provides the 'pull' for the transpirational movement of water and nutrients, such as calcium, upward through the xylem to the fruit (Himelrick and McDuffie, 1983; Peryea, 1987). Calcium is a relatively immobile nutrient in this transport system. Leaves and fruit compete for transpirational water and nutrients. Since leaves transpire much more water than do fruit, most of the calcium is directed to leaf tissue (Peryea, 1987). The result is fruit are more prone to localized calcium deficiency. Atmospheric humidity and temperature effect the rate of transpiration (Rost et al, 1979), possibly explaining how weather patterns can affect calcium nutrition and the development of calcium-related disorders.

3. Effect of calcium on cell membranes.

A key role of calcium is the maintenance of cell membrane permeability (Rost et al., 1979). An important function of the membranes is to provide control over metabolic pathways. The

membranes do this by compartmentalizing or confining enzymes to organelles so they are separated from the enzymes of other pathways. This compartmentation permits the membrane to exert control over the flow of molecules between pathways (Rost et al., 1979). Calcium probably stabilizes the membrane by linking with neighboring phospholipid phosphate groups in the membrane and pulling the membrane tighter together (Bangerth, 1974).

Calcium deficiency in the cell leads to leaky membranes (Marinos, 1962). Bangerth (1974) reports that the membranes will begin to leak long before microscopically visible breakdown is evident.

Tomato fruit with low calcium had high tissue permeability preceding the occurrence of blossom-end rot (Van Goor, 1968). Plasma membranes in pitted tissue of apple and in tomatoes with blossom-end rot are severely disturbed if not destroyed (Bangerth, 1974). Low calcium levels in pear fruit may favor membrane leaking which damages the cell and tissue and leads to the development of cork spot.

4. Effect of calcium on organic acids.

Apple tissue with bitter pit contains higher concentrations of organic acids, protein, and the amino acids asparic acid and glutamic acid (Faust and Shear, 1969; Bangerth, 1974). The predominant organic acid in bitter pit is citric acid, while malic acid is the major constituent in normal tissue (Faust and Shear, 1968). Organic acids can remove calcium ions from their binding sites on cell membranes by acting as a calcium chelator (Bangerth, 1974). Removal of calcium from the membrane by organic acids can increase permeability and favor the development of cork spot.

Oxalic acid can bind with calcium to form insoluble crystals of calcium oxalate (Rost et al., 1979). These crystals can accumulate in the fruit pedicel and impede further calcium movement into the fruit (Peryea, 1987).

5. Effect of calcium on fruit firmness.

Firmness is an important component of quality for the long term storage of pear fruit. Flesh firmness is related to the calcium content of the fruit (Mason and Welsh, 1970; Richardson, 1976; Raese and Stahly, 1982; Gerasopoulos, 1988; Raese, 1989). Fruit which is low in calcium generally softens prematurely and cannot be stored for long periods of time. Preharvest sprays of calcium increase fruit firmness and reduce the incidence of disorders (Al-Ani, 1978; Richardson and Lombard, 1979; Richardson and Al-Ani, 1982; Raese and Stahly, 1982; Raese, 1984; 1987; 1988; 1989). Attempts have been made to use post-harvest dips of calcium solutions to increase the calcium content of the fruit, but the results have been inconsistent (Al-Ani, 1978; Richardson and Lombard, 1979; Richardson and Al-Ani, 1982).

6. Effect of calcium on ethylene biosynthesis.

Calcium suppresses the ethylene synthesis systems in 'd'Anjou' pear fruit (Richardson and Al-Ani, 1982; Gerasopoulos, 1986; 1988; Vaz, 1984). The effects of calcium sprays on storage and ripening behavior of 'd'Anjou' pears was studied by Gerasopoulos (1986, 1988). He found calcium-treated pears had significantly lower internal ethylene. Wang and Mellenthin (1973) reported that 'd'Anjou' pears affected by cork spot had higher and accelerated rates of ethylene synthesis. The mechanism by which calcium suppresses ethylene

synthesis in pears is not known. Gerasopoulos (1986) suggested calcium may have a direct effect on ethylene synthesis or it may indirectly affect synthesis through its role in maintaining the intregrity of cell walls and membranes.

Calcium enhances ethylene production in senescent apple tissue slices (Lieberman and Wang, 1982) and stressed apple protoplasts (Anderson et al., 1979). These may be considered examples of wounded tissue. The effect of adding calcium is to stabilize the membranes and, thus, preserve the ethylene-producing system. In normal apple tissue, calcium inhibits ethylene synthesis (Faust and Shear, 1973).

In summary, calcium suppresses ethylene synthesis in normal fruit, but in stressed or wounded fruit added calcium can increase ethylene synthesis.

7. Effect of calcium on respiration.

Increasing the calcium concentration in 'd'Anjou' pear fruit causes a reduction in respiration rate (Al-Ani, 1978; Richardson and Al-Ani, 1982; Vaz, 1984). Al-Ani (1978) reported a highly significant negative correlation between fruit calcium concentration and respiration rate ($r = -0.83$) during ripening at 20°C. Fruit affected by cork spot had higher rates of respiration when compared with unaffected fruit (Wang and Mellenthin, 1973).

In a study on the effect of calcium on respiration in apple fruit Faust and Shear (1972) found respiration was inversely related to calcium content in the fruit. They interpret the effect of calcium on respiration as explaining the relation between disorders, such as bitter pit, and calcium level in the fruit. In a similar study on the

effect of postharvest dips of calcium solution on respiration and internal breakdown, a physiological disorder, of apple fruit Bangerth et al. (1972) reported calcium retarded respiration and inhibited internal breakdown. They suggest calcium inhibits the disorder by enhancing the uptake and compartmentation of damaging substrates. In other words, calcium depresses respiration by limiting substrate diffusion from the vacuole into the cytoplasm.

8. Effect of calcium on protein synthesis.

There are no reports of calcium having a direct effect on protein synthesis (Trewavas, 1986).

Faust and Shear (1968; 1969) reported that increased protein synthesis was associated with bitter pit and other calcium-related disorders of apple.

9. Effect of calcium on portharvest disorders.

The principal cause of decay in pear fruit is fungal infection. The two fungal diseases that are responsible for the greatest economic losses are grey mold (Botrytis cinerea) and blue mold (Pencillium expansum) (Spotts, personal communication).

A secondary source of economic loss in 'd'Anjou' pear production is physiological disorders, such as cork spot, green stain, freckle pit, black end, and stoney pit. Raese (1984) has shown that each of these disorders is associated with a deficiency of calcium in the fruit.

The effect of increasing calcium in the fruit is to increase the fruit's resistance to postharvest disorders, both pathogenic (Vaz, 1984) and physiological (Raese, 1984; 1987; 1988; 1989).

10. Interaction between calcium and other elements in the development of cork spot.

Other elements can influence the uptake, translocation, or metabolic activity of calcium in the plant (Shear, 1975). In the following sections nitrogen, phosphorus, potassium, magnesium, and boron are discussed in terms of their interactions with calcium and their possible roles in the development of cork spot.

a. Nitrogen.

Nitrogen is the nutrient, after calcium, that is most closely related with cork spot of 'd'Anjou' pear. An unusual feature of this relationship is that nitrogen alone is weakly correlated with the disorder, but the ratio of nitrogen to calcium provides a strong correlation and is the most useful diagnostic tool for cork spot (Al-Ani, 1978; Vaz, 1984; Fallahi et al., 1988; Curtis, 1988; Raese, 1988). Shear (1975) reported a similar relationship between nitrogen and calcium in the development of bitter pit in apple.

Excessive nitrogen fertilization contributes to cork spot development (Raese, 1987). Nitrogen promotes vegetative growth. During the early stage of fruit development, the fruit are competing with rapidly growing shoots for calcium. The demands of the leafy shoots supersede those of the developing fruit (Shear, 1975) and the fruit become prone to calcium deficiency.

In a study on the effect of calcium on respiration in apple fruit Faust and Shear (1972) found that high calcium levels reduced respiration, but high nitrogen levels increased respiration. Furthermore, high calcium levels successfully counteracted the

nitrogen effect and kept the respiration at a low level.

b. Phosphorus.

There is no evidence of a major role for phosphorus in the development of cork spot (Vaz, 1984; Raese, 1984).

c. Potassium and magnesium.

Reports conflict on the relationship of calcium, potassium, and magnesium to cork spot. Vaz (1984) found cork spotted fruit had higher levels of potassium and magnesium than normal fruit. Woodbridge (1971) reported cork spotted fruit had higher potassium and lower magnesium levels than normal fruit. Raese (1988) found affected fruit had lower magnesium levels than normal fruit. Other studies revealed no difference in potassium and magnesium levels between disordered and normal fruit (Mason and Welsh, 1970; Raese, 1980; Richardson and Lombard, 1982; Raese, 1984).

Bangerth (1974) suggested that potassium and magnesium ions antagonize calcium in the development of bitter pit of apple. He explains that these cations are able to replace calcium on membrane binding sites. Potassium cannot crosslink membrane components as calcium does. The substitution alters the behavior of the membrane. Magnesium, on the other hand, can crosslink with probably very little physical change; however, the small change may be important with respect to membrane function.

d. Boron.

It is unlikely boron has a major role in cork spot development. Woodbridge (1971) and Raese (1980) found cork spotted fruit had higher levels of boron than normal fruit, whereas other authors reported no

difference in boron level between disordered and normal fruit (Richardson and Lombard, 1979; Richardson and Al-Ani, 1982; Vaz, 1984; Fallahi et al., 1988).

There are mixed reviews on the relationship between bitter pit and boron. While the mechanism is not understood, boron has been shown to increase the movement of calcium into apple leaves and fruit (Shear and Faust, 1971). Faust and Shear (1968) found ten authors who concluded boron was not related to the disorder and eleven authors who reported that boron treatment was beneficial for the treatment of bitter pit.

11. Orchard factors affecting calcium nutrition and cork spot.

Orchard practices, particularly ones that promote vigorous vegetative growth, can have a pronounced effect on the incidence of cork spot (Raese, 1984; 1987). The next sections discuss the effect of soil moisture, tree vigor, crop load, and harvest maturity on calcium nutrition and cork spot.

a. Soil moisture.

Soil moisture has a profound effect on calcium nutrition. Calcium is relatively immobile in the soil and inadequate or excessive moisture levels can favor cork spot or bitter pit.

A high incidence of cork spot was positively correlated with high levels of irrigation in a study conducted by Brun et al. (1985). To control cork spot Raese (1984; 1987) recommends moderate levels of irrigation.

The much longer history of research on bitter pit provides a more complete picture of the effect of soil moisture on calcium nutrition.

An adequate and regular water supply insures the best calcium supply to the fruit (Van der Boon et al., 1970). The uptake of calcium is primarily by mass flow (Van der Boon et al., 1974; Himelrick and McDuffie, 1983; Peryea, 1987). The uptake of calcium is regulated by the tree's water consumption. Thus the availability of calcium for the tree is determined not only by the calcium level in the soil, but also by the water content of the soil (Van der Boon, 1970). The rate of calcium uptake depends on the rate of water uptake, which in turn is affected by weather factors that affect transpiration, such as temperature, humidity, and rainfall or irrigation (Peryea, 1987). Dry weather in the final months of fruit development is apt to increase bitter pit (Van der Boon, 1970).

Cork spot may be associated with growing seasons where the first half is wetter than average and the second half is dryer and hotter than average (Facteau et al., 1986), though Mason and Welsh (1970) were not able to correlate temperature and precipitation with the disorder.

b. Tree vigor.

Vigorous tree growth favors the uptake of calcium. The vigor can be the result of excessive nitrogen fertilizer, over-irrigation, severe dormant pruning, certain rootstocks, favorable weather patterns, and other factors that promote vegetative growth. These same factors contribute to the development of cork spot (Richardson and Lombard, 1979; Raese, 1984; 1987; 1988; 1989; Brun et al., 1985) and bitter pit (Faust and Shearer, 1968; Van der Boom et al., 1970; Hilkenbaumer and Nauman, 1974; Shear, 1975). Pear trees on Japanese

rootstocks are prone to cork spot (Mason and Welsh, 1970).

c. Crop load.

The size and number of fruit on a tree can influence the fruit's susceptibility to calcium-related disorders. Raese (1988) reports a trend for cork spot to be related to large fruit and light crop loads, though fruit size can vary considerably each year (Raese, 1984). Vaz (1984) identified the ratio of fruit weight to fruit calcium concentration as a consistent predictive tool for cork spot.

A similar pattern is apparent for bitter pit in apple. The disorder can be related to large fruit and light crop loads. There is a tendency for larger fruit to have a lower calcium content per unit fresh weight and this is related to increased susceptibility to bitter pit (Van der Boon et al., 1970; Sharples, 1974). In lightly cropping trees, where there is a high leaf to fruit ratio, there is a high incidence of bitter pit. Vigorous vegetative growth favors the movement of calcium into the leaves rather than the fruit (Van der Boon et al., 1970; Sharples, 1974).

d. Harvest maturity.

Picking date can influence the incidence of calcium-related disorders in fruit. Early picking discourages cork spot in 'd'Anjou' pear and late picking increases the problem (Richardson, 1976). The opposite is true of apple. Apples are more susceptible to bitter pit if harvested at too early maturity and less prone to pitting if harvested at a late maturity (Bunemann, 1974; Perring, 1986). The relationship between harvest maturity and the disorders is one of the few important differences between cork spot of 'd'Anjou' pear and

bitter pit of apple. Richardson (1976) attributes the difference to diverging rates of fruit growth at the time of harvest. Pears are harvested while the fruit are still rapidly growing. Thus harvesting at later maturity allows for greater dilution of fruit calcium and more opportunity for cork spot. The growth of apples, on the other hand, is leveling off at harvest.

E. Drawbacks to calcium deficiency as the origin of cork spot.

There are two major drawbacks to calcium deficiency as the origin of cork spot. First, if the disorder was the result of a simple deficiency of calcium then the addition of calcium fertilizer should correct the problem. This is not the case. Orchard sprays of calcium solution reduce the incidence of cork spot, but they do not completely control the problem (Al-Ani, 1978; Richardson and Lombard, 1979; Richardson and Al-Ani, 1982; Raese and Stahly, 1982; Raese, 1984; 1987; 1988; 1989). This suggests the problem is more complex than a simple deficiency of calcium.

The second drawback relates to mineral analysis for calcium concentration in affected fruit. If cork spot is due to simple calcium deficiency it should be possible to use mineral analysis o diagnose the disorder. This is not the case. Despite the correlation between the disorder and low calcium levels, attempts to use fruit mineral analysis to diagnose the problem can lead to conflicting or inconclusive results (Kupferman, 1988). This is because calcium content can vary from fruit to fruit on the same tree (Brun et al., 1985). Calcium content can also vary among trees in the same orchard, between orchards, and from year to year (Kupferman, 1988). This wide

variability in fruit calcium levels has prevented scientists from agreeing on a threshold or absolute level below which the fruit becomes susceptible to cork spot. For example, it is possible to have two fruit with the same calcium concentration, but one can be a normal fruit and the other can be a cork spotted fruit. Thus, fruit mineral analysis for calcium has not proven to be a reliable tool for diagnosing the disorder. This is additional evidence that the cause of cork spot is more complex than a simple deficiency of calcium.

F. Pyruvate kinase and calcium-related disorders.

Pyruvate kinase (PK) is an enzyme whose activity issensitive to the interactions of calcium, potassium, and magnesium. In glycolysis PK catalyzes the conversion of phosphoenolpyruvate and ADP to pyruvate and ATP (Lehninger, 1982). The enzyme requires potassium and magnesium ions as cofactors and is inhibited by calcium ions (Tomlinson and Turner, 1973; Besford, 1975; Bar-Akiva et al., 1976; Ireland et al., 1980).

An important theme to the literature on pyruvate kinase is the role of cations on its activity and its value as an indicator of nutritional status in plants (Evans, 1963; Duggleby and Dennis, 1973; Tomlinson and Turner, 1973; Besford and Maw, 1975; Besford, 1975; 1978; Bar-Akiva, 1976; Lavon, 1988a, 1988b).

The value of an assay for pyruvate kinase as an indicator of physiological disorders due to imbalances of potassium, magnesium, and calcium was discovered by Bar-Akiva (et al., 1976) in Israel. Bar-Akiva showed that potassium and magnesium deficiencies decreased pyruvate kinase activity in lemon leaves, whereas calcium deficiency

caused an increase in enzyme activity. Lavon (et al., 1988a; 1988b) conducted a detailed study of the effect of potassium, magnesium, and calcium ions on pyruvate kinase activity in citrus. Together, Lavon's and Bar-Akiva's results demonstrated the inhibitory effect of calcium on pyruvate kinase activity, and confirmed Bar-Akiva's suggestion that the enzyme possessed promise as a diagnostic tool for calcium related disorders.

CHAPTER 3.

MINERAL NUTRITION AND CORK SPOT IN 'D'ANJOU PEAR FRUIT

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Additional index words: Pyrus communis, disorder, nutrition, calcium, potassium, postharvest physiology

Abstract

Mineral analyses were conducted in 1987 and 1988 on soil, leaves, and fruit peel from normal pear (Pyrus communis L. cv. d'Anjou) trees and trees prone to cork spot. Soil tests and leaf analysis did not provide measureable differences between normal trees and trees prone to cork spot. In 1987, fruit analysis at harvest revealed no difference in mineral content between normal and disordered fruit, while in 1988 fruit with cork spot had higher concentrations of potassium, calcium, and magnesium than normal fruit at harvest. In 1987, analysis of fruit after 144 days of storage in air at -0.5° C (+/- 0.5° C) indicated cork spotted fruit had higher levels of phosphorus and potassium than normal fruit. In 1988, after 138 days under the same storage conditions, affected fruit had higher potassium concentration than normal fruit. Based on the evidence gathered in our two year study, we concluded cork spot cannot be consistently related to any nutrient, but of the nutrients examined, high concentrations of potassium in the fruit was most often linked with the disorder.

Introduction

Cork spot is a serious physiological disorder in 'd'Anjou' pear production in the United States. It is assumed to be similar to bitter pit in apple and blossom end rot in tomato and watermelon. Cork spot is generally associated with low calcium concentration in the fruit (Mason and Welsh, 1970; Woodbridge, 1971; Richardson and Lombard, 1979; Richardson and Al-Ani, 1982; Raese, 1984; 1986; 1987; 1988; Vaz, 1984; Fallahi et al., 1988; Curtis, 1988). Nevertheless, attempts to use fruit calcium analysis to diagnose the disorder have often given conflicting or inconclusive results (Kupferman, 1988). This problem is partly illustrated in Table 3.1 by the wide variability of calcium values associated with cork spot by previous researchers.

The primary goal of this study was to test the hypothesis that cork spot is associated with low calcium and high potassium and magnesium concentrations in the fruit. A secondary goal was to compare the value of soil tests, leaf analysis and fruit analysis for diagnosing cork spot.

Materials and Methods

Plant material. Samples were collected from six mature 'd'Anjou' pear trees in the side hill block at Mid-Columbia Experiment Station in Oregon. Three of the trees, designated E5, F20 and K13 had histories of producing normal or marketable fruit with a very low incidence of cork spot. The other three trees, K6, P6 and P7, had documented histories of producing fruit with a high incidence of the disorder. In 1988, tree K6 produced a relatively normal harvest and was reclassified as a normal tree for that year.

Soil tests. Soil was collected from a depth of 5.0 to 15.0 cm at three locations along the drip line of a tree. These three subsamples were mixed together to form one composite sample per tree. Samples were gathered on Nov. 23, 1987, and Sept. 2, 1988, and analyzed in the soil testing laboratory at Oregon State University.

Leaf analysis. A total of 48 leaves was collected from each tree. Leaves were sampled from different sides and elevations within the tree. Each leaf was selected from the middle of a shoot of current season's growth. Only leaves free of disease or other damage were selected. The petiole (or stem) was left on the leaf. Leaf samples were collected on Aug. 18, 1987, and Aug. 15, 1988, and submitted to the plant analysis laboratory at Oregon State University for analysis.

Fruit analysis. During the 1987 cropping season, fruit samples were prepared for analysis at the time of harvest (Aug. 26, 1987), and after 144, 151, 158 and 165 days in cold storage (-0.5° C $\pm$ 0.5° C air storage). During the 1988 season, samples were prepared at 107, 85, 52 and 24 days before harvest, at the time of harvest (Sept. 7,

1988), and after 56, 86, and 138 days in cold storage. The procedure was to select six fruit from the same tree to form one sample. The fruit were rinsed with distilled water and wiped dry. The peel of the lower half of the fruit was removed with a paring knife, chopped into 4.0 mm square pieces, and immediately covered with a cloth wetted with .5% (w/v) sodium metabisulfite solution to minimize oxidation. The tissue was frozen at -20°C for 12 hours and then freeze-dried for six days. Fresh and dry weights were recorded for each sample. The sample was rendered into powder and stored in recloseable plastic bags in a freezer at -20°C. The samples were analyzed for mineral nutrients at the plant analysis laboratory at Oregon State University. Total nitrogen was measured by the micro-Kjeldahl method and P, K, Ca, Mg, S, Al, B, Cu, Fe, and Zn were determined by an inductively-coupled argon plasma (ICAP) apparatus. The 1988 postharvest samples were analyzed at the Mid-Columbia Experiment Station. Samples were placed in porcelain crucibles, oven dried for two days at 80°C, and ashed at 500°C for six hours. The samples were brought to 100 ml with repeated washings of 10% (v/v) HCL. $SrCl_2$ (35 $g/1^{-1}$) in 0.1 M HCl was added (3 ml/100 ml sample) prior to bringing to final volume. Calcium, magnesium, and potassium were measured with a Perkin Elmer 2280 atomic absorption spectrophotometer.

Results and Discussion

Summaries of mineral analysis means for 1987 and 1988 are presented in Tables 3.2, 3.3, 3.4 and 3.5. Unpaired t tests were used to analyze for significant differences between normal and cork spot nutrient levels. For both years, no significant differences were noted in the nutrient levels of soil samples from normal trees and trees prone to cork spot. Similarly, no significant differences were noted in leaf nutrient levels of normal trees and trees prone to cork spot. These findings are evidence that soil tests and leaf analysis have little value in diagnosing cork spot. These results confirm the work of Raese (1984).

In 1987, fruit peel analysis at harvest revealed no differences between normal and disordered fruit (Table 3.2). One-hundred-forty-four days after harvest cork spotted fruit had higher levels of phosphorus and potassium than normal fruit. In 1988, fruit peel analysis at harvest showed that fruit affected by cork spot were higher in their concentrations of potassium, calcium, and magnesium than normal fruit (Table 3.3). One-hundred-thirty-eight days after harvest only potassium content was higher in affected fruit than normal fruit.

Our results did not confirm the findings of earlier researchers that cork spot is the result of calcium deficency in the fruit. On the contrary, our results indicate fruit calcium values are too variable to have any relationship with the disorder (Table 3.4). In January of the 1987 crop year cork spotted fruit had significantly lower (5% level) calcium content than normal fruit. In September of

the 1988 crop year disordered fruit at harvest had significantly higher (5% level) calcium concentration than normal fruit. This flip-flop in the calcium values of affected fruit and our inability to detect measureable differences (5% level) between cork spotted and normal fruit during other months in the 1987 and 1988 crop years (Table 3.4) indicate calcium levels do not appear related to cork spot.

Potassium concentration in the fruit peel yielded the greatest measureable differences between cork spotted and normal fruit. In January and February of the 1987 crop year and in September, November, and January of the 1988 crop year potassium levels were significantly higher in disordered fruit than normal fruit (Table 3.5).

In conclusion, the results of soil tests and leaf analyses cannot be related to cork spot (Tables 3.2 and 3.3). Fruit analysis does provide measureable differences between normal and disordered fruit, but the results are prone to wide variation (Tables 3.4 and 3.5). Based on our observations in 1987 and 1988 and contrary to the findings of previous researchers (Table 3.1) cork spot could not be consistently linked to a deficiency of calcium in the fruit (Table 3.4). We found cork spot to be most closely associated with high levels of potassium in the fruit (Table 3.5), and secondly, to high levels of magnesium in the fruit (Table 3.5). Earlier reports conflict on the relationship of potassium and magnesium to cork spot. Woodbridge (1971) reported cork spotted fruit had higher potassium and lower magnesium levels than normal fruit. Vaz (1984) found disordered fruit had higher levels of potassium and magnesium than normal fruit.

Raese (1988) found affected fruit had lower magnesium levels than normal fruit. Other studies revealed no differences in potassium and magnesium levels between disordered and normal fruit (Mason and Welsh, 1970); Raese, 1980; 1984; Richardson and Lombard, 1982).

Richardson and Al-Ani (1982) have suggested ratios of nutrients provide a stronger relationship with cork spot than absolute levels of individual nutrients. This was not the case in the present study. No ratio of nutrients was found to be closely linked to cork spot. For example, the ratios nitrogen/calcium and calcium/potassium and magnesium are presented in Table 3.6.

Our hypothesis that cork spot is associated with low calcium and high potassium and magnesium concentrations in the fruit was partly supported by our results. Though calcium levels were not uniformly low (Table 3.4) for the two years, potassium and magnesium levels tended to be high for 1987 and 1988 (Table 3.5). Based on the evidence generated by our two year study, we conclude cork spot is most closely related to high concentrations of potassium in the fruit, but that no element or elements were consistently associated with the disorder.

Table 3.1. Calcium concentrations expressed as ppm dry weight in normal 'd'Anjou' pear fruit and fruit with cork spot. Values drawn from review of literature.

			Fruit Calcium			
			Normal		Cork spot	
Location	Year	Tissue	Mean	Threshold	Mean	Reference
British Columbia	1969	flesh	319	---	219	Mason & Welsh, 1970
Washington	----	peel	720	---	398	Woodbridge, 1971
	----	flesh	292	---	212	Woodbridge, 1971
Oregon	1973-75	segment	---	333*	---	Richardson & Lombard, 1979
Oregon	----	segment	---	460*	---	Richardson & Al-Ani, 1982
Oregon	1981	peel	1686	---	1426	Raese, 1984
		flesh	571	---	492	Raese, 1984
Oregon	1982	peel	1388	---	932	Raese, 1984
		flesh	451	---	348	Raese, 1984; 1986
Oregon	1983	peel	1239	---	691	Raese, 1984
		flesh	440	---	264	Raese, 1984

* Recalculated value assuming dry weight is 15% of fresh weight.

Table 3.2. Summary of mineral analysis means and standard errors from 1987 season. Each value is the average of three measurements.

	Soil test					
Tree type	OM %	P ppm	K ppm	Ca meq/100g	Mg meq/100g	B ppm
Normal	2.74 ± .22	80 ± 10	286 ± 47	5.9 ± .8	1.4 ± .2	.68 ± .1
Cork spot	2.16 ± .18	107 ± 11	278 ± 13	5.3 ± .5	1.5 ± .1	.54 ± .1

	Leaf analysis					
Tree type	N %	P % dry wt	K % dry wt	Ca % dry wt	Mg % dry wt	B ppm
Normal	2.46 ± .10	.17 ± .01	0.90 ± .40	1.80 ± .01	.33 ± .01	38 ± 7
Cork spot	2.44 ± .05	.18 ± .01	1.68 ± .14	1.71 ± .05	.32 ± .02	45 ± 3

	Fruit analysis (peel) at harvest					
Fruit type	N %	P ppm	K ppm	Ca ppm	Mg ppm	B ppm
Normal	.38 ± .04	629 ± 64	6392 ± 798	416 ± 51	370 ± 28	34 ± 7
Cork spot	.51 ± .02	827 ± 54	8365 ± 428	347 ± 68	468 ± 27	41 ± 3

Table 3.2. Summary of mineral analysis means and standard errors from 1987 season. Easch value is the average of three measurements. (cont.)

	Fruit analysis (peel) 144 days after harvest					
Fruit type	N %	P ppm	K ppm	Ca ppm	Mg ppm	B ppm
Normal	.44 ± .07	642 ± 54	6788 ± 758	594 ± 36	448 ± 34	36 ± 6
Cork spot	.59 ± .03	938 ± 30**	9356 ± 336*	494 ± 107	510 ± 12	43 ± 3

Significant at 5% (*) or 1% (**) levels.

Table 3.3. Summary of mineral analysis means and standard errors from 1988 season. Each value is the average of two to four measurements.

		Soil test				
Tree type	OM %	P ppm	K ppm	Ca meq/100g	Mg meq/100g	B ppm
Normal	n/a	66 ± 7	296 ± 32	5.6 ± .2	1.4 ± .1	.63 ± .1
Cork spot	n/a	85 ± 6	273 ± 31	5.2 ± .3	1.2 ± .1	.50 ± .1

		Leaf analysis				
Tree type	N %	P % dry wt	K % dry wt	Ca % dry wt	Mg % dry wt	B ppm
Normal	2.30 ± .07	.20 ± .01	1.39 ± .1	1.82 ± .08	.40 ± .01	34 ± 4
Cork spot	2.24 ± .02	.18 ± .01	1.47 ± .1	1.92 ± .01	.37 ± .02	34 ± 2

		Fruit analysis (peel) at harvest				
Fruit type	N %	P ppm	K ppm	Ca ppm	Mg ppm	B ppm
Normal	.42 ± .02	668 ± 41	5558 ± 356	565 ± 65	426 ± 23	28 ± 4
Cork spot	.48 ± .01	764 ± 16	6810 ± 82*	850 ± 70*	538 ± 12*	35 ± 0

Table 3.3. Summary of mineral analysis means and standard errors from 1988 season. Each value is the average of three measurements. (cont.)

	Fruit analysis (peel) 138 days after harvest					
Fruit type	N %	P ppm	K ppm	Ca ppm	Mg ppm	B ppm
Normal	n/a	n/a	11755 ± 212	1296 ± 154	595 ± 8	n/a
Cork spot	n/a	n/a	13382 ± 550*	1690 ± 87	699 ± 41	n/a

Significant at 5% (*) level.

Table 3.4. Nitrogen percentage and calcium concentration in 'd'Anjou' pear peel from normal and cork spotted fruit for selected months in 1987 and 1988 crop years.

Crop Yr	Mo	Nitrogen (%)		Calcium (ppm dry wt)	
		N	CS	N	CS
1987	Aug	.38	.51	416	347
1987	Jan	.44	.61*	648	491*
1987	Feb	.48	.59	648	580
1988	May	1.95	1.88	5284	5415
1988	Jun	.98	1.15	3509	4784
1988	Jul	.54	.55	1347	1471
1988	Aug	.54	.50	909	1010
1988	Sep	.42	.48	565	850*
1988	Nov[y]	.95[z]	1.69[z]*	1132	1528
1988	Dec[y]	.77[z]	1.30[z]*	1429	1383
1988	Jan[y]	1.32[z]	1.25[z]	1296	1690

[z] Nitrogen values calculated by linear regression of preharvest protein concn (mg/g fresh wt) against preharvest nitrogen (%) in 1988.

[y] Results for Nov, Dec, and Jan of 1988 are based on a comparison of fruit from two sample trees, whereas the earlier results are based on a comparison of fruit from six trees.

* Significant difference between N and CS at 5% (*) level.

Table 3.5. Potassium and magnesium concentration in 'd'Anjou' pear peel from normal and cork spotted fruit for selected months in 1987 and 1988 crop years.

Crop Yr	Mo	Potassium (ppm dry wt)		Magnesium (ppm dry wt)	
		N	CS	N	CS
1987	Aug	6392	8365	370	468
1987	Jan	6667	8615*	447	495
1987	Feb	6217	8243*	424	556*
1988	May	14704	15260	1583	1679
1988	Jun	11098	11821	1108	1197
1988	Jul	8269	8458	698	716
1988	Aug	7145	6929	522	565
1988	Sep	5558	6810*	426	538*
1988	Nov[z]	8346	10976**	462	615**
1988	Dec[z]	10628	11085	594	575
1988	Jan[z]	11755	13382*	595	699

[z] Results for Nov, Dec, and Jan of 1988 are based on a comparison of fruit from two sample trees, whereas the earlier results are based on a comparison of fruit from six trees.

*,** Significant difference between N and CS at 5% (*) or 1% (**) level

Table 3.6 Ratios of nitrogen/calcium and calcium/potassium and magnesium in 'd'Anjou' pear peel for selected months in 1987 and 1988 crop years.

Crop Yr	Mo	N/Ca x 10,000		CA/K + Mg	
		N	CS	N	CS
1987	Aug	9.26	16.42	.061	.039
1987	Jan	6.89	12.96**	.093	.054
1987	Feb	7.47	10.97*	.099	.064
1988	May	3.69	3.47	.324	.320
1988	Jun	2.79	2.40	.287	.367
1988	Jul	4.01	3.74	.150	.160
1988	Aug	5.94	4.95	.119	.135
1988	Sep	7.71	5.68	.094	.116
1988	Nov[z]	8.92	11.69	.129	.132
1988	Dec[z]	5.47	9.62*	.128	.118
1988	Jan[z]	10.66	7.48	.105	.120

[z] Results for Nov, Dec, and Jan of 1988 are based on a comparison of fruit from two sample trees, whereas the earlier results are based on a comparison of fruit from six trees.

*, ** Significant difference between N and CS at 5% (*) or 1% (**) level.

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Chapter 4

Factors Affecting Pyruvate Kinase Activity in 'd'Anjou[1] Pear Fruit

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Abstract

An assay for pyruvate kinase (PK) was tested as a diagnostic tool for cork spot, the major physiological disorder in pear (Pyrus communis L. cv. d'Anjou) fruit. PK catalyzes the metabolic reaction in glycolysis where phosphoenolpyruvate is converted to pyruvate. Efforts to characterize PK in pear fruit have demonstrated that peel is preferred over flesh for the assay. Assay results were less variable when the enzyme was extracted at 20°C then at 0°C. Enzyme activity remained stable for four hours when extracted at 20°C, whereas activity decreased after four hours when extracted at 0°C. This suggests PK from pear fruit is stable at room temperature. The inclusion of EDTA, a chelating agent, in the extraction buffer increased PK activity and improved the separation of PK activity between normal and disordered fruit. The addition of K^{+} and Mg^{+2} to the assay greatly increased PK activity and of the two cations, Mg^{+2} had the greater impact. Ca^{+2} inhibited PK activity. No relationship was found between PK activity and concentrations of Ca, Mg, and K in

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normal and disordered fruit. Differences in expressing PK activity on a dry or fresh weight basis were minimal. Chemical names used: adenosine diphosphate (ADP), adenosine triphosphate (ATP), ethylene diamine tetraacetate (EDTA).

Introduction

Pyruvate kinase (PK) (ATP: pyruvate phosphotransferase, EC 2.7.1.40) catalyzes the metabolic reaction in glycolysis where phosphoenolpyruvate (PEP) and ADP are converted to pyruvate and ATP (Lehninger, 1982). The reaction occurs in the cytosol. The enzyme requires K^{+} and Mg^{+2} as cofactors and is inhibited by Ca^{+2} (Tomlinson and Turner, 1973; Besford, 1975; Bar-Akiva et al., 1976; Ireland et al., 1980). Published information on PK from higher plants is limited. This is for two reasons. First, the plant enzyme is labile which prevented its complete purification until 1987 (Plaxton, 1988). Second, plant tissues contain a phosphatase which interferes with the measurement of PK (Evans, 1963; Duggleby and Dennis, 1973; Besford and Maw, 1975).

An important theme to the literature on PK is the role of cations on its activity and its potential as an indicator of nutritional status in plants (Evans, 1963; Duggleby and Dennis, 1973; Tomlinson and Turner, 1973; Besford and Maw, 1975; Besford, 1975; 1978; Bar-Akiva et al., 1976; Lavon et al., 1988a; 1988b).

Our interest in PK is in its use as an indicator of cork spot, a serious physiological disorder in 'd'Anjou' pear production in the United States. It is a calcium-related disorder assumed to be similar to bitter pit in apple and blossom end rot in tomato and watermelon. Cork spot is generally associated with low calcium concentration in the fruit (Mason and Welsh, 1970; Woodbridge, 1971; Richardson and Lombard, 1979; Richardson and Al-Ani, 1982; Raese, 1984; 1986; 1987; 1988; Vaz, 1984; Fallahi et al., 1988; Curtis, 1988). Nevertheless,

attempts to use fruit mineral analysis to diagnose the disorder have often given conflicting or inconclusive results (Kupferman, 1988). The search for an alternative tool to diagnose cork spot has led to the present biochemical study. The goal is to lay a foundation for using the PK assay to diagnose cork spot by first investigating some of the factors that influence the enzyme's activity, such as extraction temperature, the chelating agent EDTA, and the cations K^{+}, Mg^{+2}, and CA^{+2}. No previous studies of PK in pear fruit have been reported.

Materials and Methods

Plant material. Fruit were harvested from two mature 'd'Anjou' pear trees at the Mid-Columbia Experiment Station on September 8, 1988 at commercial maturity. One tree was prone to cork spot. In 1987 and 1988, 24% and 23% of the fruit were affected. The second tree had a history of producing normal or marketable fruit. In both 1987 and 1988 less than 1% of the fruit were affected by cork spot.

Sample preparation. After 56 days of storage in air at -0.5°C ($\pm$ 0.5°C) the fruit were sorted into four categories or fruit/tree types: 1. cork spotted fruit from the tree prone to cork spot; 2. normal fruit from the tree prone to cork spot; 3. normal fruit from the normal tree; and 4. cork spotted fruit from a normal tree. The last category was later dropped when it became apparent there were insufficient fruit for sampling. Cork spot is generally found on the lower half of a fruit. Peel was removed from the lower one-half of three fruit and combined to form one sample. Similarly, seven wedges of flesh from the lower one-half of the same three fruit was combined to form a sample. Each category was replicated five times. A 10 g sample of peel or flesh was wrapped in cheese cloth, frozen in liquid nitrogen and lyophilized for biochemical analysis. Another 10 g peel or flesh sample was frozen at -20°C for mineral analysis.

Extraction. One-half gram of lyophilized tissue was homogenized in a mortar and pestle with 0.5 g insoluble polyvinyl-polypyrrolidone (PVPP), 1.5 g white quartz sand, and 10.0 ml of an extraction buffer that consisted of 50 mM imidazole-HCl (pH 7.0), 3 mM dithiothreitol, 5 mM ascorbic acid, 3 mM $Na_2S_2O_5$, 14 mM 2-mercaptoethanol, and 20% (v/v)

glycerol. The extraction buffer was prepared with and without 1 mM EDTA. The macerated tissue was squeezed through two layers of miracloth and centrifuged at 37,044x g for 20 min at 20°C. The supernatant was retained as the crude extract for the enzyme assay. The extraction procedure was performed at either 0° or 20°C.

Assay. PK activity was assayed by the colorimetric measurement of the pyruvate produced from PEP in the presence of ADP (Boyer, 1973; Lavon et al., 1988a; 1988b). The assay was performed in a test tube containing 0.4 ml 50 mM imidazole (pH 7.0) buffer with .625 mM Na_2MoO_4 to inhibit phosphatase activity, 0.1 ml distilled and deionized water, 0.1 ml crude extract, and 0.1 ml 5mM PEP. The assays were conducted with and without 0.1 ml 500 mM KCl and with and without 0.1 ml 50 mM $MgCl_2$. The reaction was started by the addition of 0.1 ml 20 mM adenosine-5'-diphosphate (ADP) and was carried out in a final volume of 1.0 ml. The reaction was incubated for 15 min at 37°C and stopped by the addition of 1.0 ml 0.0125% 2,4-dinitrophenylhydrazine in 2N HCl. The mixture was incubated for an additional 15 min at 37°C and 2.0 ml 2N NaOH added. The mixture was centrifuged at 14,818xg for 5 min to precipitate protein particles. The absorbance of the supernatant was measured at 510 nm.

Protein concentration. Protein levels in the crude extract were assayed by Peterson's modification of the micro-Lowry method (Lowry et al., 1951; Peterson, 1977; Sigma, 1985). The following materials were mixed in a 15.0 ml centrifuge tube: 0.1 ml crude extract, 0.3 ml extraction buffer, 1.6 ml distilled water, and 0.3 ml aqueous solution of sodium deoxycholate (1.5 mg/ml). The mixture was shaken well and

allowed to stand at room temperature for 10 min. 0.3 ml aqueous solution of trichloroacetic acid (72% w/v) was added to the tube and mixed well. The solution was centrifuged for 10 min at 37,044x g at 20°C. The supernatant was discarded, and the pellet blotted dry and dissolved in 1.0 ml Lowry reagent and 1.0 ml distilled water. The solution stood at room temperature for 20 min, then 0.5 ml Folin and Ciocalteu's phenol reagent was rapidly mixed in. Color was allowed to develop for 30 min. The fluid was transferred to a cuvet and percent absorbance read at 700 nm.

Enzyme activity. The activity of PK was expressed as activity (nmol pyruvate/min/ml), specific activity (nmol pyruvate/min/mg protein), or total activity (nmol pyruvate/min/g dry wt or fresh wt).

Mineral analysis. Frozen fruit samples were placed in porelain crucibles, oven dried for two days at 80°C and ashed at 500°C for six hours. The samples were brought to 100 ml with repeated washings of 10% (v/v) HCL. $SrCl_2$ (35 $g/1^{-1}$) in 0.1 M HCL was added (3 ml/100 ml sample) prior to bringing to final volume. Ca, Mg, and K were measured with a Perkin Elmer 2280 atomic absorption spectrophotometer.

Statistical procedure. The effect of EDTA was statistically analyzed by a two by three factorial design. The first factor was extraction without or with EDTA. The second factor was fruit/tree category. The effect of added cations was analyzed by a three by four factorial design. The first factor was fruit/tree category and the second was cation treatment.

Results and Discussion

Buffers. In an earlier study Lavon et al. (1988b) evaluated tris, imidazole, and maleate buffers for the extraction of PK from citrus leaves at pH values of 6.0, 6.5, 7.0, 7.5, and 8.0. The highest PK activity was obtained with an imidazole buffer with a pH of 7.0. These results were verified during the first extractions of PK from pear tissue. All subsequent extractions of PK from pear fruit were done with a 50 mM imidazole buffer at a pH of 7.0.

The extraction of proteins from plant tissue is complicated by the presence of other cellular compounds, such as phenolics, that come into contact with the proteins when the tissue is homogenized (Menendez et al., 1986). When the cell is disrupted the phenolics bind to the proteins which leads to protein denaturation and enzyme inactivation. Phenolic compounds, such as chlorogenic acid, are a potent source of interference in pear tissue. Chlorogenic acid is the primary substrate in the browning of pear fruit by polyphenoloxidase (PPO) activity (Wang & Mellenthin, 1973). The buffer employed in the extraction of PK from pear fruit contains several components--dithiothreitol, ascorbic acid, sodium metabisulfite, and 2-mercaptoethanol-that are included to enhance PK activity by inhibiting PPO. The buffer is a modification of ones used by Lavon et al., (1988a; 1988b) and Menendez et al. (1986). In a preliminary experiment the extraction buffer described above was evaluated with and without glycerol. Duggleby and Dennis (1973) used glycerol to improve PK stability. Our findings verified their results. PK recovery was higher and the enzyme more stable when glycerol was used.

When there was no glycerol in the extraction buffer PK had 68% of the activity of PK extracted with glycerol. In addition, PK activity declined quickly when there was no glycerol. There was a 73% decline in PK activity over 8 hours when there was no glycerol in the buffer. The addition of glycerol to the extraction buffer became standard practice in our lab.

Insoluble PVPP is used in the mortar and pestle to reduce interference by phenolic compounds (Menendez et al., 1986). The proportion of lyophilized sample to extraction buffer and PVPP was important. Best results were obtained using 0.5 g sample, 10.0 ml extraction buffer, and 0.5 g PVPP.

The extracted volume was recorded for each assay. Later the volumes were statistically analyzed and found not to be significant factors in our interpretation. For example, 7.1 ml was the mean extracted volume for both the extractions with and without EDTA.

Assays for PK in other plant tissues are generally complicated by a phosphatase that interferes with the measurement of PK activity (Evans, 1963; Duggleby and Dennis, 1973; Besford and Maw, 1975). This has been only a minor problem in pear tissue, and the addition of a low concentration of molybdate to the assay buffer eliminated phosphatase interference. Four concentrations of molybdate, 0.0625, 0.125, 0.625, and 1.25 mM Na_2MoO_4 were evaluated and 0.625 was found to yield the highest PK activity. The addition of .625 mM Na_2MoO_4 became standard procedure in our assays of PK in pear fruit.

PK activity in peel and flesh. Previous researchers (Woodbridge, 1971; Curtis, 1988) concluded peel was the preferred tissue for

mineral analysis in the study of cork spot because differences in peel calcium were greater between normal and cork spotted fruit than was flesh calcium. A preliminary study (unpublished) indicated that peel was also preferred for the PK assay because it provided more uniform results than flesh. The mean specific activity for peel and flesh was 204 and 456 nmol pyruvate /min/mg protein, respectively. The standard errors were 24 and 99 nmol pyruvate/min/mg protein respectively for peel and flesh. The lower standard error for peel indicated it yielded less variable results. Peel was used in all PK assays for the remainder of this study.

Effect of extraction temperature. The specific activity of PK extracted at 20°C was the same immediately after extraction and four hours later (Table 4.1); whereas the specific activity of PK extracted at 0°C was more variable. This is evidenced by a standard error that was three times that of the 20°C extraction. Furthermore, four hours after extraction the specific activity of 0°C extracted PK had decreased by 12% and the standard error increased to three and one-half times that of the 20°C extraction. These results suggest PK in 'd'Anjou' pear fruit may be a cold labile enzyme. Adams and Rinne (1981) assaying PK in soybean seeds found PK was cold labile. Baysdorfer and Bassham (1984) working with spinach leaves also reported PK was cold labile and best extracted at room temperature. Further results reported in this study were based on a 20°C extraction temperature.

Effect of EDTA. The addition of 1mM EDTA to the extraction buffer lowered protein concentration and increased enzyme activity

(Tables 4.2, 4.3 and 4.4). Furthermore, it improved the separation for the three categories of fruit/tree type. For example, the addition of EDTA lowered protein levels and provided a significant difference in protein concentration between cork spotted and normal fruit. EDTA decreased the protein concentration possibily because of its ability to remove cations, such as Fe^{+2}, Fe^{+3}, Cu^{+2}, Cu^{+3}, Ca^{+2}, and Mg^{+2} from solution. These same multivalent ions form bonds with proteins (Haurowitz, 1950; 1963). Thus, when EDTA sequesters ions it also removes the proteins which are bound to them. It is not clear how EDTA improved the separation in protein concentration between disordered and normal fruit.

Previous researchers working with animal tissue have reported similar increases in enzyme activity when EDTA is added to the extraction buffer. Bailey (et al., 1968) stated that the presence of EDTA gave higher activities. Meli and Bygrave (1972) reported that EDTA was found to stimulate PK activity by relieving the Ca^{+2} inhibition of the enzyme. They explained that because EDTA has a higher affinity for Ca^{+2} than for Mg^{+2} it will preferentially sequester Ca^{+2} in a solution containing both of these ions. Moon (et al., 1980) stated that PK was converted from a less to a more reactive enzyme by including EDTA in the extraction or assay buffer. As Tables 4.2 and 4.3 reveal, the addition of EDTA improved the separation of enzyme activity in certain categories of fruit/tree type. More specifically, with EDTA the specific activity was significantly different for cork spotted fruit from trees prone to the disorder and for normal fruit from normal trees. We suggest that

these differences in enzyme activity between categories were because EDTA altered the ratios of Ca^{+2}, Mg^{+2}, and K^{+} in the crude extract. In turn, the altered ratios increased the activity of PK. In all subsequent extractions the buffer contained 1 mM EDTA. A comparison of Tables 4.2, 4.3 and 4.4 shows minimal differences between dry and fresh weight expressions for protein concentration and PK activity.

Effect of cation cofactors. The addition of the cations, K^{+} and Mg^{+2}, to the PK assay dramatically increased specific activity (Table 4.5). For the purpose of statistical analysis, specific activity was transformed to its natural logarithm. This was done because of a correlation between means and standard deviations. There was no significant interaction between categories of fruit/tree type and cation treatment. There were no significant differences between categories. In other words, each category responded similarly to the addition of cations. There were significant differences between cation treatments. The specific activity of a PK assay without added cations was extremely low (Table 4.5). The addition of either K^{+} or Mg^{+2} to the assay increased specific activity 4 or 22 times, respectively. While an assay including both cations increased specific activity 29 times. It is clear Mg^{+2} has the greater impact on increasing specific activity than K^{+}, but both cations have to be added to obtain maximal activity.

These results are in agreement with earlier observations that PK requires a monovalent cation, such as K^{+}, and a divalent cation, such as Mg^{+2}, for activation (Evans, 1963; Tomlinson and Turner, 1973; Besford, 1975; 1978; Lavon et al., 1988b). The addition of 0.1 ml 500

mM KCl and 0.1 ml 50 mM $MgCl_2$ became standard assay procedure in our lab.

Effect of cation inhibitor. The inhibitory effect of Ca^{+2} on PK activity is clearly demonstrated by the results presented in Table 4.6. Increasing levels of Ca^{+2} decrease the specific activity of PK. For the statistical analysis, specific activity was transformed to its natural logarithm and calcium treatment (concentration) was transformed to its natural logarithm plus one. No significant interaction was found. Multiple regression analysis was used to separate categories of fruit/tree type at the 5% level. The N/N category was significantly higher than the other two categories in its response to calcium treatment. This indicated the N/N category had either more active PK or had a higher quantity per unit protein than the other categories. Multiple regression, ln spec act = 5.92 - .580 x ln Ca concn + fruit/tree type (cs/cs = -.126, N/CS = -.-030, N/N = .156), was used to reveal significant differences, 5% level, between calcium treatments. Our findings confirmed earlier reports that Ca^{+2} strongly inhibits PK activity (Tomlinson and Turner, 1973; Bar-Akiva et al., 1976; Lavon et al., 1988b). Our finding, that PK in pear fruit was sensitive to Ca^{+2} levels in its ionic environment, suggests that an assay for PK, which is influenced by soluble or physiologically active calcium, may serve as a diagnostic tool for cork spot in pear fruit.

Mineral concentrations. Concentrations of total Ca, Mg, and K were determined for each category of fruit/tree type (Table 4.7). Ca was significantly lower in CS/CS than in N/CS, but CS/CS was not

significantly different from N/N. Cork spotted fruit had significantly lower levels of Ca than normal fruit from the same tree, but the cork spotted fruit had the same Ca levels as normal fruit from a normal tree. Mg and K shared a similar pattern. CS/CS was not significantly different from N/CS, but both categories had higher concentrations of Mg and K than N/N. Fruit from a tree prone to cork spot, regardless whether the fruit was cork spotted or normal, had higher levels of Mg and K than normal fruit from a normal tree. The results of the mineral analysis (Table 4.7) underscore the principal problems of using fruit Ca concentration to diagnose cork spot, namely the variable nature of the results and the inability to set a threshold or absolute level below which fruit develop cork spot. These problems inspired the present study. By identifying the important factors in the activity of PK in 'd'Anjou' pear fruit we have laid the foundation for testing the PK assay's ability to diagnose cork spot.

Table 4.1. Protein concentration and specific activity of pyruvate kinase in 'd'Anjou' pear peel extracted at warm (20°C) or cold (0°C) temperature. Pyruvate kinase was assayed immediately after extraction and again 4 hours later.

	Extraction temp (C)	
	20°	0°
Protein concn (mg/g dry wt ± SE)	7.36 ± .103	7.81 ± .374
Specific activity after extraction [z]	272 ± 4.33	276 ± 12.8
Specific activity 4 hrs after extraction[z]	273 ± 4.80	245 ± 16.6

[z] nmol pyruvate/min/mg protein ± SE.

Table 4.2. Protein levels and pyruvate kinase activity in 'd'Anjou' pear peel extracted without EDTA and with 1 mM EDTA and expressed on a dry weight basis.

Treatment	Fruit type[z]	Tree type[y]	Protein concn (mg/ml)	Total protein (mg/g dry wt)	Enzyme Activity: Activity (nmol Pyruvate/ min/ml)	Enzyme Activity: Specific (nmol pyruvate/ min/mg protein)
0mM EDTA	CS	CS	.663a[x]	8.93 a	158 ab	239 a[w]
	N	CS	.632 a	9.00 a	151 a	240 a
	N	N	.650 a	9.71 a	151 a	233 a
1mM EDTA	CS	CS	.616 a	8.82 a	182 c	306 a
	N	CS	.467 b	6.68 b	167 bc	370 ab
	N	N	.400 b	5.54 b	169 bc	436 b

[z] Fruit type: CS = cork spot, N = normal.

[y] Tree type: CS = 23% 1988 harvest affected by cork spot, N = <1% 1988 harvest affected by cork spot.

[x] Mean separation within columns by Fisher's protected LSD, 5% level.

[w] The means of specific activity were only separated within EDTA treatments because of the dissimilarity of variances between EDTA treatments.

Table 4.3. Total protein concentration and total pyruvate kinase activity in 'd'Anjou' pear peel extracted without EDTA and with 1 mM EDTA and expressed on a fresh weight basis.

Treatment	Fruit type[z]	Tree type[y]	Total protein (mg/g fresh wt)
	CS	CS	2.50 a[x]
0mM EDTA	N	CS	2.51 a
	N	N	2.58 a
	CS	CS	2.46 a
1mM EDTA	N	CS	1.86 b
	N	N	1.47 b

[z] Fruit type: CS = cork spot, N = normal.

[y] Tree type: CS = 23% 1988 harvest affected by cork spot, N = <1% 1988 harvest affected by cork spot.

[x] Mean separation within columns by Fisher's protected LSD, 5% level.

Table 4.4. Total pyruvate kinase activity in 'd'Anjou° pear peel extracted without EDTA and with 1mM EDTA.

	Total activity (nmol pyruvate/min/g)	
Treatment	dry wt	fresh wt
0mM EDTA	2180	599
1mM EDTA	2450**	674**

** ANOVA revealed a highly significant difference between treatments at the 1% level (**).

There were no differences between fruit/tree categories.

Table 4.5. Effect of adding cations, K^+ and Mg^{+2}, to the assay on the specific activity of pyruvate kinase in 'd'Anjou' pear peel.

Fruit type[z]	Tree type[y]	Cation treatment				
		No cations	K^+	Mg^{+2}	K^+ and Mg^{+2}	Avg
CS	CS	16[xw]	45	233	306	150 a
N	CS	13	53	283	370	180 a
N	N	10	54	336	436	209 a
	Avg	13 a	51 b	284 c	371 d	180

[z] Fruit type: CS = cork spot, N = normal.

[y] Tree type: CS = 23% 1988 harvest affected by cork spot, N = <1% 1988 harvest affected by cork spot.

[x] Fisher's protected LSD was used to separate means, 5% level.

[w] nmol pyruvate/min/mg protein.

Table 4.6. Effect of adding calcium to the assay on the specific activity of pyruvate kinase in 'd'Anjou' pear peel.

Fruit type[z]	Tree type[y]	Calcium concentration (mM $CaCl_2$) 0	2.5	5.0	10.0	Avg
CS	CS	306[xw]	166	119	82	168 a
N	CS	370	177	130	87	191 a
N	N	436	209	156	106	227 b

[z] Fruit type: CS = cork spot, N = normal.

[y] Tree type: CS = 23% 1988 harvest affected by cork spot, N = <1% 1988 harvest affected by cork spot.

[x] Fisher's protected LSD was used to separate means of fruit/tree types, 5% level. Multiple regression identified significant differences between calcium treatments, 5% level.

[w] nmol pyruvate/min/mg protein.

Table 4.7. Specific activity of pyruvate kinase and mineral concentrations in 'd'Anjou' pear peel.

Fruit type[z]	Tree type[y]	Specific activity PK (nmol pyruvate/min/ mg protein)	Mineral concn (ppm dry wt)		
			Ca	Mg	K
CS	CS	306 a[x]	1528 a	615 a	10976 a
N	CS	370 ab	2087 b	654 a	10284 a
N	N	436 b	1132 a	462 b	8346 b

[z] Fruit type: CS = cork spot, N = normal.

[y] Tree type: CS = 23% 1988 harvest affected by cork spot, N = <1% 1988 harvest affected by cork spot.

[x] Mean separation within columns by Fisher's protected LSD, 5% level.

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CHAPTER 5.

PYRUVATE KINASE ACTIVITY AND PROTEIN CONCENTRATION IN 'D'ANJOU' PEAR FRUIT WITH CORK SPOT

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Abstract

An assay for pyruvate kinase (PK) was tested as a diagnostic tool for cork spot, the major physiological disorder in pear (Pyrus communis L. cv. d'Anjou) fruit. PK activity and protein concentration was measured in peel of normal and affected fruit during selected months in 1987 and 1988. The results showed protein concentration was more closely associated with cork spot than PK activity. Furthermore, the results indicated the PK assay was a poor diagnostic tool for cork spot.

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Introduction

Cork spot is a serious physiological disorder in pear (Pyrus communis L. cv. d'Anjou) production in the United States. The disorder is recognized by a dimpled or bumpy surface on the calyx end of the fruit. Beneath the dimples, which are sometimes yellowish in color, is found brown, necrotic tissue. Fruit affected by cork spot are not marketable.

A review of the literature in Chapter 2 indicated cork spot is generally linked with low calcium concentration in the fruit. The ratio of nitrogen to calcium in the fruit has also been correlated with the disorder. The review further showed that the results of fruit mineral analysis can vary widely and cannot be reliably used to diagnose the problem. This variability in results led to a search for alternative methods of identifying cork spot. Chapter 4 employed a biochemical approach and laid the foundation for evaluating an assay for the enzyme pyruvate kinase (PK) as a diagnostic total for cork spot.

Chapter 4 also closely examined PK activity in peel of normal and cork spotted fruit at one point in time, i.e. 56 days after the 1988 harvest. The results showed the PK assay provided measureable differences between normal and disordered fruit, whereas calcium analysis did not.

The present chapter more broadly follows PK activity over a two year period in normal and disordered fruit. The chapter compares the results of measuring PK activity and protein concentration. The goal was to see which measurement was most closely associated with cork spot

and, thus, might possess the greater value as a diagnostic tool for the disorder.

Materials and Methods

Plant material. Samples were collected from six mature 'd'Anjou' pear trees in the side hill block at Mid-Columbia Agricultural Research and Extension Center in Oregon. Three of the trees, designated E5, F20 and K13 had histories of producing normal or marketable fruit. The other three trees, K6, P6 and P7, had documented histories of producing a high incidence of cork spotted fruit. In 1988, tree K6 produced a relatively normal harvest and was reclassified as a normal tree for that year.

Sample preparation. During the 1987 cropping season, fruit samples were prepared for analysis at the time of harvest (Aug. 26, 1987), and after 144, 151, 158 and 165 days in cold storage (-0.5° C +/- 0.5° C air storage). During the 1988 season, samples were prepared at 107, 85, 52, and 24 days before harvest, at the time of harvest (Sept. 7, 1988), and after 57, 86, and 138 days in cold storage. The procedure for the 1987 samples and the 1988 preharvest and harvest samples was to select six fruit from the same tree to form one sample. The fruit were rinsed with distilled water and wiped dry. The peel of the lower half of the fruit was removed with a paring knife, chopped into 4.0 mm square pieces, and immediately covered with a cloth wetted with .5% (w/v) sodium metabisulfite solution to minimize oxidation. The tissue was frozen at -20°C for 12 hours and then freeze-dried for six days. Fresh and dry weights were recorded for each sample. The sample was rendered into powder and stored in reclosable plastic bags in a freezer at -20°C.

The post harvest samples from the 1988 growing season were

prepared slightly differently. Only two trees were sampled; a normal tree, E5, and a tree prone to cork spot, P7. Peel was removed from the lower one-half of three fruit and combined to form one sample. A 10.0 g sample of peel was wrapped in cheese cloth, frozen in liquid nitrogen, lyophilized, and stored in a freezer at -20°C.

Extraction. One-half gram of lyophilized tissue was homogenized in a mortar and pestle with 0.5 g insoluble polyvinyl polypyrrolidone (PVPP), 1.5 g white quartz sand, and 10.0 ml of an extraction buffer that consisted of 50 mM imidazole-HCl (pH 7.0), 3 mM dithiothreitol, 5 mM ascorbic acid, 3 mM $Na_2S_2O_5$, 14 mM 2-mercaptoethanol, 1 mM EDTA and 20% (v/v) glycerol. The macerated tissue was squeezed through two layers of miracloth and centrifuged at 37,044x g for 20 min at 20°C. The supernatant was retained as the crude extract for the enzyme assay. The extraction procedure was performed at 20°C.

Assay. PK activity was assayed by the colorimetric measurement of the pyruvate produced from PEP in the presence of adenosine-5'-diphosphate (ADP) (Boyer, 1973; Lavon et al., 1988a; 1988b). The assay was performed in a test tube containing 0.4 ml 50 mM imidazole (pH 7.0) buffer with .625 mM Na_2MoO_4 to inhibit phosphatase activity, 0.1 ml distilled and deionized water, 0.1 ml crude extract, 0.1 ml 5mM PEP, 0.1 ml 500 mM KCl, and 0.1 ml 50 mM $MgCl_2$. The reaction was started by the addition of 0.1 ml 20 mM ADP and was carried out in a final volume of 1.0 ml. The reaction was incubated for 15 min at 37°C and stopped by the addition of 1.0 ml 0.0125% 2,4-dinitrophenylhdrazine in 2N CHl. The mixture was incubated for an additional 15 min at 37°C and 2.0 ml 2N NaOH added. The mixture was centrifuged at 14,818x g for 5 min to

precipitate protein particles. The absorbance of the supernatant was measured at 510 nm.

Protein concentration. Soluble protein levels in the crude extract were assayed by Peterson's modification of the micro-Lowry method with precipitation (Lowry et al., 1951; Peterson, 1977; Sigma, 1985). The results were expressed as mg/g dry weight (Table 5.3), mg/g fresh weight (Table 5.3), and mg/ml (Table 5.2).

Enzyme activity. The PK assay results were expressed as specific activity (nmol pyruvate/min/mg protein) (Table 5.2) or total activity (nmol pyruvate/min/g dry weight or fresh weight) (Table 5.1).

Statistical procedure. Assay results were analyzed by month. Monthly means for normal fruit and cork spotted fruit were calculated from three to 12 measurements. A t-test (Sokal and Rohlf, 1981) was used to test for significant differences between the means for normal and cork spotted fruit.

RESULTS

Total activity of PK was measureably different between normal and cork spotted fruit in the months of June and November of the 1988 cropping season, but not in any of the other months in which the enzyme was assayed during the 1987 and 1988 cropping seasons (Table 5.1). Whether total activity was expressed on a dry weight or fresh weight basis was of little importance. Total activity appears to be a weak candidate for diagnosing cork spot.

Specific activity of PK was significantly higher in affected fruit than normal fruit in August of the 1987 cropping season, but was significantly lower in disordered fruit than normal fruit in the months of September, November, and December of the 1988 season (Table 5.2). It is important to note the reversal from 1987 to 1988 in the relationship between normal and cork spotted fruit. This reversal precludes the use of specific activity to diagnose cork spot. Protein concentration provided the greatest measureable difference between normal and cork spotted fruit for the most months in the 1987 and 1988 crop years (Tables 5.2 and 5.3). Regardless of the units of measurement (mg/ml, mg/g dry wt, or mg/g fresh wt), protein concentration was higher in disordered fruit than normal fruit in the months of June, July, August, September, November, and December of the 1988 crop year. Protein concentration expressed as mg/ml was also higher in affected fruit than normal fruit in August of 1987.

In summary, this analysis by month indicated the assay for protein concentration provided more consistent differences between normal and cork spotted fruit than does the assay for PK.

Discussion

This study was not able to associate the activity of PK with the incidence of cork spot. This result stands in contrast of the results of previous researchers and preliminary results by the present authors (Chapter 4). An important theme to previous research on PK was its value as an indicator of nutritional status in plants (Evans, 1963; Duggleby and Dennis, 1973; Tomlinson and Turner, 1973; Besford and Maw, 1975; Besford, 1975; 1978; Bar-Akiva et al., 1976; Lavon et al., 1988a; 1988b). The value of the PK assay as an indicator of physiological disorders in fruit crops due to imbalances of potassium, magnesium, and calcium was discovered by Bar-Akiva (et al., 1976). He suggested that the enzyme possessed promise as a diagnositc tool for calcium-related disorders, such as cork spot.

The sensitivity of PK in pear peel to the interactions of calcium, potassium, and magnesium was demonstrated by the present authors in an earlier study (Chapter 4). The study was conducted in November of 1988 and at that time the assay for PK provided a significant difference in total activity between normal and cork spotted fruit peel (Table 5.1). In the present study the PK assay was applied to samples of peel from selected months of 1987 and 1988. In the 12 months peel was assayed, a significant difference in total activity was noted in only two of the months (Table 5.1). It is not clear why the activity of PK could not be more closely correlated with cork spot, perhaps the analytical techniques need further refinement or the number of sample trees increased. The preliminary conclusion of this study is the PK assay appears to have little value as a

diagnostic tool for cork spot.

An important finding in this study was to learn that cork spotted fruit had higher protein concentrations than normal fruit. It was a pattern generally free of the wide variability, reversals, or lack of measureable differences often associated with fruit calcium analysis or assays for PK. High protein content has also been reported for apple fruit with bitter pit and other calcium-related disorders, such as internal breakdown (Faust and Shear, 1968; 1969).

The symptoms of cork spot first became apparent in our sample trees 21 days before harvest in the 1988 crop year. Yet the protein assay detected differences between fruit from normal trees and fruit from trees prone to cork spot as early as June or three months before harvest (Table 5.3). Faust and Shear (1968) reported that apple tissue with corking disorders had higher protein content than normal tissue and that the increase had occurred by the time the first visible signs of the disorder developed. Combined together, the present and previous results indicate that protein concentration merits further study for its potential to diagnose or predict cork spot early in the growing season.

The reason for higher protein concentration in cork spotted fruit has not been established. The authors advance the following explanation as speculation and a guide for future investigations. Cork spot is probably the result of several stressful factors, such as an imbalance of nutrients, and drought and high temperatures in the weeks or months preceeding harvest. These factors may combine to alter protein synthesis in pear fruit. The result was higher levels

of protein in fruit affected by the disorder.

The response of plants to stress with emphasis on the analysis of gene expression is a relatively new area of study in plant science (Sachs and Ho, 1986). It is based on the premise that stress influences gene expression which in turn controls protein synthesis. It is believed plants can respond to a wide variety of stress situations by synthesizing 'stress' proteins. For example, corn synthesized heat-shock proteins in response to high temperature stress (Cooper and Ho, 1983). Other plants synthesized different sets of protein during distinct stress conditions such as flooding, high salt concentrations, or intense ultra-violet light exposure (Sachs and Ho, 1986). It is felt that these stress-induced proteins allow plants to make biochemical and structural adjustments that enable them to cope with the stress conditions. This area of research is of interest not only because it expands our understanding of coordinate gene expression (Sachs and Ho, 1986), but it may also provide the means to develop trees resistant to cork spot. Studies on the stress-induced proteins and the genes encoding them may lead to the engineering of crop plants more resistant to normally encountered stress conditions, such as the ones that may favor cork spot. For this reason further work is recommended to elucidate the protein patterns in 'd'Anjou' pear fruit affected by cork spot.

Table 5.1. Total activity of pyruvate kinase in 'd'Anjou' pear peel expressed on a dry weight and fresh weight basis for selected months in 1987 and 1988 crop years.

Crop Yr	Mo	Total activity (nmol pyruvate/min/g dry wt)		Total activity (nmol pyruvate/min/g fresh wt)	
		N	CS	N	CS
1987	Aug	182	548	29	96
1987	Jan	448	421	81	78
1987	Feb	764	664	139	123
1987	May	413	576	78	108
1988	May	3284	3332	645	690
1988	Jun	1383	2068*	319	485**
1988	Jul	1004	1394	214	294
1988	Aug	1256	1473	254	303
1988	Sep	1228	1035	239	208
1988	Nov[z]	2338	2621*	622	734*
1988	Dec[z]	2335	2112	635	581
1988	Jan[z]	2056	1913	549	529

*,** Significant difference between N and CS at 5% (*) or 1% (**) level.

[z] Results for Nov, Dec, and Jan of 1988 are based on a comparison of fruit from two sample trees, whereas the earlier results are based on a comparison of fruit from six trees.

Table 5.2. Protein concentration and specific activity of pyruvate kinase in 'd'Anjou' pear peel for selected months in 1987 and 1988 crop years.

Crop Yr	Mo	Protein concn (mg/ml)		Specific activity (nmol pyruvate/min/ mg protein)	
		N	CS	N	CS
1987	Aug	.327	.432*	38	92*
1987	Jan	.387	.527	168	119
1987	Feb	.440	.388	248	255
1987	May	.317	.388	91	109
1988	May	.926	.982	258	246
1988	Jun	.308	.456*	312	321
1988	Jul	.225	.390**	296	242
1988	Aug	.312	.422*	293	245
1988	Sep	.325	.414**	260	175*
1988	Nov[z]	.400	.616*	436	306*
1988	Dec[z]	.344	.503**	515	301**
1988	Jan[z]	.525	.491	281	284

*,** Significant difference between N and CS at 5% (*) or 1% (**) level.

[z] Results for Nov, Dec, and Jan of 1988 are based on a comparison of fruit from two sample trees, whereas the earlier results are based on a comparison of fruit from six trees.

Table 5.3. Protein (total) concentration in 'd'Anjou' pear peel expressed on a dry weight and fresh weight basis for selected months in 1987 and 1988 crop years.

Crop Yr	Mo	Protein concn (mg/g dry wt)		Protein concn (mg/g fresh wt)	
		N	CS	N	CS
1987	Aug	4.69	5.92	0.76	1.03
1987	Jan	2.71	3.69	0.49	0.68
1987	Feb	3.08	2.71	0.56	0.50
1987	May	4.44	5.43	0.83	1.02
1988	May	13.14	13.77	2.56	2.84
1988	Jun	4.43	6.42**	1.02	1.51*
1988	Jul	3.33	5.68**	0.68	1.20**
1988	Aug	4.46	5.97**	0.88	1.23**
1988	Sep	4.88	5.92**	0.94	1.18**
1988	Nov[z]	5.54	8.82**	1.47	2.46**
1988	Dec[z]	4.56	7.04**	1.24	1.94**
1988	Jan[z]	7.35	6.78	1.96	1.87

*,** Significant difference between N and CS at 5% (*) or 1% (**) level.

[z] Results for Nov, Dec, and Jan of 1988 are based on a comparison of fruit from two sample trees, whereas the earlier results are based on a comparison of fruit from six trees.

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CHAPTER 6.

Summary

Cork spot is the principal physiological disorder in 'd'Anjou' pear production in the United States. The review of the literature showed the disorder to be generally associated with low fruit calcium concentration (Table 3.1). The review also showed that the results of fruit calcium analysis (Tables 3.1 and 3.4) are so variable that they cannot be reliably used to diagnose cork spot. This variability led to the present search for an alternative method for diagnosing cork spot. The present study evaluated an assay for the enzyme PK as a diagnostic tool.

The study tested three hypotheses. The first was that cork spot is associated with low calcium and high potassium and magnesium concentrations in the fruit. These elements were thought to have roles in the development of the disorder (Bar-Akiva et al., 1976), and this study demonstrated the great influence they exert on the activity of PK (Tables 4.5 and 4.6). The results of fruit analysis for 1987 and 1988 partly supported the hypothesis. Potassium and magnesium levels tended to be higher in cork spotted fruit than in normal fruit for both years (Table 3.5). However, calcium levels varied widely and did not lend themselves to a pattern (Table 3.4).

The second hypothesis is that cork spot is associated with high PK activity. PK activity was closely examined in peel of normal and cork spotted fruit at one point in time, i.e. 56 days after 1988 harvest (Chapter 4). The results (Table 4.2) showed cork spotted

fruit had significantly higher PK activity than normal fruit. The PK assay was then more broadly applied to peel of normal and disordered fruit for selected months of 1987 and 1988 (Chapter 5). Total activity, expressed on a fresh weight basis, was significantly higher in cork spotted fruit than normal fruit in only two of the 12 months sampled (Table 5.1). Specific activity was highly variable (Table 5.2). The results of the PK assays provided very weak support for the second hypothesis.

The third hypothesis is that cork spot is associated with unique proteins or polypeptides. Assays in 1987 and 1988 indicated that fruit affected by cork spot have higher protein concentrations than normal fruit (Tables 5.2 and 5.3). Preliminary results of gel electrophoresis of cork spotted and normal fruit conducted in Israel revealed enhanced levels of a polypeptide with a molecular weight of 31 kD in cork spotted fruit (Figures B.1 and B.2). No unique polypeptides were noted. Thus, the third hypothesis was not supported.

Several questions were raised during this research project which deserve to be addressed by future researchers. 1. Is cork spot a major or a minor problem for the pear industry in the Pacific Northwest? Interviews of industry representatives by the author gave a muddled picture of the disorder's impact. For this reason, in this study all discussions of cork spot incidence were based on the measured yields of the six sample trees monitored during the study (Table A.2). The normal trees in the study averaged 1, 4, and 4% cork spotted fruit at harvest for 1987, 1988, and 1989, respectively. The

trees prone to cork spot averaged 28, 19, and 31% disordered fruit at harvest, respectively, for the same three years. But it is not clear that these figures, drawn from small research plots, can be applied to the industry as a whole. 2. Does cork spot only emerge in the three weeks prior to harvest, as observed in this study, or does it continue to develop in cold storage? Previous researchers (Richardson and Al-Ani, 1982) reported that cork spot is primarily a storage disorder. For example, Richardson and Lombard (1979) stated that 10-25% of disordered fruit are detectable at harvest, the remainder appearing during storage. Cursory inspections of fruit held in cold storage during this study suggest the disorder does not develop in storage. For example, at harvest in 1987 66 normal fruit were collected from each of the three normal trees in our study. The fruit were packed in cardboard boxes and stored at -0.5° C ($\pm$ 0.5° C) for 118 days. The fruit were than individually inspected. No examples of cork spot were found among the 198 fruit. This was not a replicated, scientific study, but the results suggest cork spot may not be a storage disorder. The subject merits further study. 3. Is cork spot associated with large fruit and light crop loads? This pattern is apparent for bitter pit in apple (Van der Boon et al., 1970; Sharples, 1974), but it is debateable whether it applies to cork spot of pear. Fruit size or fruit weight, in particular, can vary greatly in its relationship to cork spot (Table A.7). Raese (1988) reported a trend for cork spot to be related to large fruit and light crop loads, though in an earlier study (1984) he found fruit size can vary considerably each year. The relationship of cork spot to fruit size

and crop load is worthy of further investigation.

An important finding of this study was to learn that cork spotted fruit have higher protein concentrations than normal fruit. It is a pattern generally free of the wide variability, reversals, or lack of measureable differences of calcium analysis and PK assays. Assays for protein concentration merit further study for their potential to diagnose cork spot.

In conclusion, the assay for PK possesses the same shortcoming, i.e., wide variability, as fruit mineral analysis in the diagnosis of cork spot. The same factors that work to create the wide variability in calcium levels in fruit are probably also responsible for the variability in PK activity. Therefore, the assay for PK is no better for diagnosing cork spot than the fruit mineral analysis now being employed.

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APPENDICES

Table A. 1. Year of planting and rootstock for sample trees in 'side hill' block at Mid-Columbia Experiment Station.

Tree no.	E5	F20	K13	K6	P6	P7
Yr. planted	1928	1955	1955	1955	1928	1928
Rootstock	*P. ussuriensis*	?	?	?	*P. pyrifolia*	*P. pyrifolia*
Trunk	Anjou	?	?	?	Orel 15	*P. pyrifolia*

? unknown rootstocks may be 'Bartlett' seedlings (Dave Burkhart, personal communication).

Table A. 2. Yield and incidence of cork spot at harvest for sample trees for 1987, 1988, and 1989.

	Tree No.					
	E5	F20	K13	K6	P6	P7
			1987			
Yield (kg)	227	293	259	20	56	58
No. total fruit	970	1320	1110	73	264	253
No. fruit cork spot	9	13	22	26	61	61
Cork spot (%)	1	1	2	36	23	24
			1988			
Yield (kg)	270	117	161	171	294	150
No. total fruit	1317	456	627	687	1542	749
No. fruit cork spot	10	23	25	46	229	172
Cork spot (%)	1	5	4	7	15	23
			1989			
Yield (kg)	215	157	176	160	167	99
No. total fruit	1157	781	925	814	1085	579
No. fruit cork spot	51	47	14	50	227	237
Cork spot (%)	4	6	2	6	21	41

Table A. 3. Mineral analysis results for 1987 season

Soil Test (Nov. 23, 1987)

Tree No.	Type	O.M. %	P ppm	K ppm	Ca meq/100g	Mg meq/100g	B ppm
E5	Normal	2.76	74	378	7.5	1.7	.78
F20	Normal	3.12	65	226	5.4	1.1	.77
K13	Normal	2.34	100	254	4.7	1.5	.48
K6	Cork spot	2.34	86	261	5.6	1.7	.57
P6	Cork spot	2.34	124	304	6.1	1.5	.61
P7	Cork spot	1.8	112	269	4.3	1.3	.44

Leaf Analysis (Aug. 18, 1987)

Tree No.	Type	N %	P % dry wt	K % dry wt	Ca % dry wt	Mg % dry wt	B ppm
E5	Normal	2.49	.17	1.58	1.81	.33	50
F20	Normal	2.62	.17	.93	1.78	.35	27
K13	Normal	2.26	.16	.2	1.81	.32	36
K6	Cork spot	2.4	.19	1.41	1.64	.35	40
P6	Cork spot	2.38	.17	1.78	1.8	.3	47
P7	Cork spot	2.55	.18	1.85	1.69	.31	49

Fruit Analysis at Harvest (Aug. 26, 1987)

Tree No.	Type	N %	P ppm	K ppm	Ca ppm	Mg ppm	B ppm
E5	Normal	.47	700	7675	491	410	44
F20	Normal	.34	502	4929	318	316	21
K13	Normal	.33	686	6573	439	385	36
K6	Cork spot	.53	930	7662	215	427	37
P6	Cork spot	.47	745	8293	437	460	46
P7	Cork spot	.54	805	9139	390	518	41

Table A. 3. Mineral analyses results for 1987 season (cont.).

Fruit Analysis (Peel) after 144 days Cold Storage (Jan. 18, 1988)

Tree No.	Type	N %	P ppm	K ppm	Ca ppm	Mg ppm	B ppm
E5	Normal	.57	742	7893	654	516	44
F20	Normal	.39	558	5338	599	421	25
K13	Normal	.35	626	7134	528	408	38
K6	Cork spot	.53	958	8693	290	493	38
P6	Cork spot	.61	977	9783	652	533	49
P7	Cork spot	.63	880	9593	539	505	42

Fruit Analysis (Peel) after 151 days Cold Storage (Jan. 25, 1988)

Tree No.	Type	N %	P ppm	K ppm	Ca ppm	Mg ppm	B ppm
E5	Normal	.52	794	7584	651	481	41
F20	Normal	.4	664	5170	647	400	25
K13	Normal	.43	960	6884	806	454	36
K6	Cork spot	.45	658	6688	357	436	33
P6	Cork spot	.69	1001	9740	598	540	46
P7	Cork spot	.73	736	7195	510	464	34

Fruit Analysis (Peel) after 158 days Cold Storage (Feb. 1, 1988)

Tree No.	Type	N %	P ppm	K ppm	Ca ppm	Mg ppm	B ppm
E5	Normal	.6	791	6673	657	457	38
F20	Normal	.38	536	4580	539	352	23
K13	Normal	.45	880	6632	780	429	33
K6	Cork spot	.46	722	6485	311	413	34
P6	Cork spot	.64	846	8407	717	588	46
P7	Cork spot	.61	808	8280	636	587	44

Table A. 3. Mineral analyses results for 1987 season (cont.).

Fruit Analysis (Peel) after 165 days Cold Storage (Feb. 8, 1988)

Tree No.	Type	N %	P ppm	K ppm	Ca ppm	Mg ppm	B ppm
E5	Normal	.63	1008	7726	614	500	44
F20	Normal	.48	834	5616	621	419	25
K13	Normal	.33	626	6074	675	388	34
K6	Cork spot	.53	932	7025	361	491	36
P6	Cork spot	.58	868	9410	606	540	48
P7	Cork spot	.7	736	9849	850	714	48

Table A. 4. Mineral analysis results for 1988 season.

Soil Test (Sept. 2, 1988)

Tree No.	Type	pH	P ppm	K ppm	Ca meq/100g	Mg meq/100g	B ppm
E5	Normal	5.8	69	367	5.9	1.3	.65
F20	Normal	5.5	48	211	5.4	1.2	.76
K13	Normal	5.7	83	300	6	1.4	.6
K6	Medium	5.8	65	304	5.3	1.5	.51
P6	Cork spot	5.8	79	304	5.5	1.3	.49
P7	Cork spot	5.4	91	242	4.9	1.1	.51

Leaf Analysis (Aug. 15, 1988)

Tree No.	Type	N %	P % dry wt	K % dry wt	Ca % dry wt	Mg % dry wt	B ppm
E5	Normal	2.51	.2	1.56	2.04	.41	46
F20	Normal	2.24	.18	1.16	1.72	.39	26
K13	Normal	2.23	.2	1.46	1.83	.36	33
K6	Medium	2.2	.22	1.37	1.7	.42	33
P6	Cork spot	2.23	.17	1.57	1.92	.35	36
P7	Cork spot	2.26	.18	1.37	1.91	.39	33

Fruitlet Analysis of Peel (May 23, 1988)

Tree No.	Type	N %	P ppm	K ppm	Ca ppm	Mg ppm	B ppm
E5	Normal	1.95	2659	14704	5284	1583	51
P7	Cork spot	1.88	2293	15260	5415	1679	40

Fruitlet Analysis of Flesh (May 23, 1988)

Tree No.	Type	N %	P ppm	K ppm	Ca ppm	Mg ppm	B ppm
E5	Normal	2.47	3038	17812	2956	1723	51
P7	Cork spot	2.45	2528	19136	2830	1760	37

Table A. 4. Mineral analyses results for 1988 season (cont.).

Fruitlet Analysis of Peel (June 15, 1988)

Tree No.	Type	N %	P ppm	K ppm	Ca ppm	Mg ppm	B ppm
E5	Normal	.98	1500	11098	3509	1108	38
P7	Cork spot	1.15	1471	11821	4784	1197	31

Fruitlet Analysis of Flesh (June 15, 1988)

Tree No.	Type	N %	P ppm	K ppm	Ca ppm	Mg ppm	B ppm
E5	Normal	1.31	1596	15009	2483	1059	38
P7	Cork spot	1.49	1694	15353	2167	1088	30

Fruitlet Analysis of Peel (July 18, 1988)

Tree No.	Type	N %	P ppm	K ppm	Ca ppm	Mg ppm	B ppm
E5	Normal	.54	1021	8269	1347	698	34
P7	Cork spot	.55	1001	8458	1471	716	28

Fruitlet Analysis of Flesh (July 18, 1988)

Tree No.	Type	N %	P ppm	K ppm	Ca ppm	Mg ppm	B ppm
E5	Normal	.64	1005	11143	1072	675	40
P7	Cork spot	.61	1013	11883	976	670	35

Fruitlet Analysis of Peel (August 15, 1899)

Tree No.	Type	N %	P ppm	K ppm	Ca ppm	Mg ppm	B ppm
E5	Normal	.54	896	7145	909	522	39
P7	Cork spot	.50	823	6929	1010	565	30

Table A. 4. Mineral analyses results for 1988 season (cont.).

Fruitlet Analysis of Flesh (August 15, 1988)

Tree No.	Type	N %	P ppm	K ppm	Ca ppm	Mg ppm	B ppm
E5	Normal	.49	792	8116	466	456	40
P7	Cork spor	.44	838	8495	506	455	35

Fruit Analysis of Peel at Harvest (September 7, 1988)

Tree No.	Type	N %	P ppm	K ppm	Ca ppm	Mg ppm	B ppm
E5	Normal	.47	776	6502	744	476	38
F20	Normal	.38	577	4815	437	365	21
K13	Normal	.40	649	5626	558	426	29
K6	Medium	.45	669	5287	520	437	25
P6	Cork spot	.49	780	6893	920	549	35
P7	Cork spot	.47	748	6728	780	526	35

Fruit Analysis of Flesh at Harvest (September 7, 1988)

Tree No.	Type	N %	P ppm	K ppm	Ca ppm	Mg ppm	B ppm
E5	Normal	.49	700	12323	460	294	68
P7	Cork spot	.50	602	12307	431	296	55

Table A. 5. Biochemical analyses results for 1987 season

Tree No.	Extract volume (ml)	Protein concn (mg/ml)	Total protein (mg/g dry wt)	Pyruvate (nmol/ 15 min/ 100 ul)	PK activity (nmol pyruvate/ min/ml)	PK specific activity (pyruvate/ min/mg protein)
Fruit peel at harvest (August 26, 1988)						
E5	7.2	.331	4.77	28	19	58
F20	7.6	.338	5.14	18	126	36
K13	6.7	.311	4.17	9	6	20
K6	7.2	n/a	n/a	n/a	n/a	n/a
P6	6.7	.403	5.40	48	32	79
P7	7.0	.460	6.44	72	48	104
Fruit peel 144 days after harvest (January 18, 1988)						
E5	n/a	.390	n/a	75	50	128
P7	n/a	.600	n/a	68	45	75
Fruit peel 151 days after harvest (January 25, 1988)						
E5	n/a	.460	n/a	117	78	170
F20	n/a	.310	n/a	96	64	206
P6	n/a	.580	n/a	112	75	129
P7	n/a	.400	n/a	91	61	152
Fruit peel 158 days after harvest (February 1, 1988)						
E5	n/a	.520	n/a	222	148	285
F20	n/a	.340	n/a	129	86	253
P6	n/a	.270	n/a	146	97	360
P7	n/a	.410	n/a	97	65	158

Table A. 5. Biochemical analyses results for 1987 season (cont.)

Tree No.	Extract volume (ml)	Protein concn (mg/ml)	Total protein (mg/g dry wt)	Pyruvate (nmol/ 15 min/ 100 ul)	PK activity (nmol pyruvate/ min/ml)	PK specific activity (pyruvate/ min/mg protein)
Fruit peel 165 days after harvest (February 8, 1988)						
E5	n/a	.540	n/a	179	119	221
F20	n/a	.360	n/a	125	83	231
P6	n/a	.350	n/a	134	89	255
P7	n/a	.520	n/a	193	129	247
Fruit peel 260 days after harvest (May 13, 1988)						
E5	7.0	.349	4.89	68	45	130
F20	7.0	.321	4.50	34	23	72
K13	7.0	.282	3.95	30	20	71
K6	7.0	.352	4.93	50	34	95
P6	7.0	.334	4.68	71	48	142
P7	7.0	.478	6.70	64	43	89
Fruit flesh 260 days after harvest (May 13, 1988)						
E5	7.0	.269	3.77	86	57	212
F20	7.0	.174	2.44	41	27	157
K13	7.0	.269	3.77	18	12	45
K6	7.0	.305	4.28	47	31	103
P6	7.0	.263	3.69	56	38	143
P7	7.0	.403	5.65	58	38	95

Table A. 6. Biochemical analyses results for 1988 season.

Tree No.	Extract volume (ml)	Protein concn (mg/ml)	Total protein (mg/g dry wt)	Pyruvate (nmol/ 15 min/ 100 ul)	PK activity (nmol pyruvate/ min/ml)	PK specific activity (pyruvate/ min/mg protein)
Fruitlet peel 107 days before harvest (May 23, 1988)						
E5	7.4	1.258	18.62	335.9	224	178
F20	7.0	.880	12.32	289.3	193	219
K13	7.2	1.054	15.18	398.3	266	252
K6	7.7	.754	11.61	347.8	232	308
P6	6.8	1.029	13.99	347.8	232	225
P7	7.3	1.133	16.54	354.9	237	209
Fruitlet peel 106 days before harvest (May 24, 1988)						
E5	6.9	1.159	15.99	341.0	227	196
F20	7.0	.831	11.63	310.4	207	249
K13	6.5	.912	11.86	389.5	260	285
K6	7.3	.786	11.48	351.9	235	298
P6	7.0	.942	13.19	353.9	236	250
P7	7.0	1.039	14.55	367.2	245	236
Fruitlet peel 105 days before harvest (May 25, 1988)						
E5	7.0	1.166	16.32	344.9	230	197
F20	7.0	.784	10.98	311.4	208	265
K13	7.2	.736	10.60	379.1	253	344
K6	7.0	.794	11.12	363.7	243	306
P6	7.0	.765	10.71	349.1	233	304
P7	6.9	.987	13.62	368.9	246	249

Table A. 6. Biochemical analyses results for 1988 season. (cont.)

Tree No.	Extract volume (ml)	Protein concn (mg/ml)	Total protein (mg/g dry wt)	Pyruvate (nmol/ 15 min/ 100 ul)	PK activity (nmol pyruvate/ min/ml)	PK specific activity (pyruvate/ min/mg protein)
Fruitlet peel 85 days before harvest (June 15, 1988)						
E5	7.3	.474	6.92	231.8	155	326
F20	7.0	.316	4.42	125.4	84	265
K13	7.3	.175	2.56	52.9	35	202
K6	7.1	.291	4.13	134.4	90	308
P6	7.0	.456	6.38	210.0	140	307
P7	7.0	.513	7.18	248.2	166	323
Fruitlet peel 84 days before harvest (June 16, 1988)						
E5	7.8	.431	6.72	195.9	131	303
F20	6.8	.298	4.05	135.4	90	303
K13	7.3	.125	1.82	53.8	36	287
K6	7.2	.338	4.86	150.4	100	297
P6	7.0	.410	5.74	172.2	115	280
P7	7.0	.499	6.99	266.1	178	356
Fruitlet peel 83 days before harvest (June 17, 1988)						
E5	7.3	.577	8.42	260.7	174	301
F20	6.7	.189	2.53	131.0	87	462
K13	7.0	.144	2.02	62.3	42	289
K6	7.0	.333	4.66	199.1	133	399
P6	7.2	.402	5.79	172.8	115	287
P7	7.0	.459	6.43	255.1	170	371

Table A. 6. Biochemical analyses results for 1988 season. (cont.)

Tree No.	Extract volume (ml)	Protein concn (mg/ml)	Total protein (mg/g dry wt)	Pyruvate (nmol/ 15 min/ 100 ul)	PK activity (nmol pyruvate/ min/ml)	PK specific activity (pyruvate/ min/mg protein)
Fruitlet peel 52 days before harvest (July 18, 1988)						
E5	7.3	.356	5.20	195.2	130	366
F20	6.7	.293	3.93	149.0	99	339
K13	7.3	.103	1.50	51.6	34	334
K6	7.4	.318	4.71	156.1	104	327
P6	7.4	.429	6.35	193.5	129	301
P7	7.4	.352	5.21	115.9	77	220
Fruitlet peel 51 days before harvest (July 19, 1988)						
E5	7.0	.285	3.99	160.9	107	377
F20	6.8	.148	2.01	25.9	17	117
K13	7.3	.095	1.39	40.9	27	287
K6	7.1	.236	3.35	74.6	50	211
P6	7.2	.349	5.03	121.1	81	231
P7	7.2	.377	5.43	98.0	65	173
Fruitlet peel 50 days before harvest (July 20, 1988)						
E5	7.0	.369	5.17	209.4	140	378
F20	6.3	.109	1.37	10.0	7	61
K13	7.4	.211	3.12	62.3	42	197
K6	7.0	.303	4.24	136.7	91	301
P6	6.9	.406	5.60	145.2	97	238
P7	7.6	.425	6.46	184.5	123	290

Table A. 6. Biochemical analyses results for 1988 season. (cont.)

Tree No.	Extract volume (ml)	Protein concn (mg/ml)	Total protein (mg/g dry wt)	Pyruvate (nmol/ 15 min/ 100 ul)	PK activity (nmol pyruvate/ min/ml)	PK specific activity (pyruvate/ min/mg protein)
Fruitlet peel 24 days before harvest (August 15, 1988)						
E5	7.0	.409	5.73	215.6	144	352
F20	7.2	.249	3.59	108.9	73	292
K13	6.9	.369	5.09	161.5	108	292
K6	6.8	.319	4.34	146.5	98	306
P6	7.0	.464	6.50	178.6	119	257
P7	7.3	.394	5.75	120.8	81	204
Fruitlet peel 23 days before harvest (August 16, 1988)						
E5	6.5	.347	4.51	170.5	114	328
F20	7.3	.181	2.64	58.2	39	214
K13	7.0	.344	4.82	132.5	88	257
K6	6.9	.327	4.51	148.8	99	304
P6	7.2	.459	6.61	219.5	146	319
P7	7.0	.392	5.49	125.6	84	214
Fruitlet peel 22 days before harvest (August 17, 1988)						
E5	6.8	.469	6.38	179.5	120	255
F20	7.3	.176	2.57	70.8	47	268
K13	7.0	.305	4.27	136.7	91	299
K6	7.5	.338	5.07	95.4	64	188
P6	7.0	.385	5.39	162.7	108	282
P7	7.0	.435	6.09	128.2	86	197

Table A. 6. Biochemical analyses results for 1988 season. (cont.)

Tree No.	Extract volume (ml)	Protein concn (mg/ml)	Total protein (mg/g dry wt)	Pyruvate (nmol/ 15 min/ 100 ul)	PK activity (nmol pyruvate/ min/ml)	PK specific activity (pyruvate/ min/mg protein)
Fruitlet peel 2 days before harvest (September 6, 1988)						
E5	7.0	.351	4.91	150.9	101	287
F20	7.8	.267	4.16	107.7	72	269
K13	7.5	.334	5.01	104.2	70	208
K6	8.0	.331	5.30	48.7	32	98
P6	7.0	.388	5.43	78.5	52	135
P7	7.0	.443	6.20	76.2	51	115
Fruitlet peel 1 day before harvest (September 7, 1988)						
E5	7.3	.351	5.12	144.4	96	274
F20	7.2	.300	4.32	170.0	113	378
K13	7.8	.354	5.52	128.1	85	241
K6	7.8	.360	5.62	89.3	60	165
P6	7.3	.427	6.23	111.0	74.5	173
P7	7.4	.458	6.78	133.0	89	194
Fruitlet peel at harvest (September 8, 1988)						
E5	7.0	.338	4.73	160.2	107	316
F20	7.4	.275	4.07	154.3	103	374
K13	7.0	.354	4.96	150.4	100	283
K6	7.3	.334	4.88	95.4	64	190
P6	6.9	.399	5.51	128.2	86	214
P7	7.3	.369	5.39	123.0	82	222

Table A. 6. Biochemical analyses results for 1988 season. (cont.)

Tree No.	Extract volume (ml)	Protein concn (mg/ml)	Total protein (mg/g dry wt)	Pyruvate (nmol/ 15 min/ 100 ul)	PK activity (nmol pyruvate/ min/ml)	PK specific activity (pyruvate/ min/mg protein)
Peel from cork spotted fruit from tree prone to cork spot, 57 days after harvest (November 3, 1988)						
P7	7.5	.709	10.65	266	177	250
P7	6.5	.794	10.32	278	185	233
P7	7.3	.562	8.20	270	180	320
P7	7.4	.472	6.99	275	183	388
P7	7.3	.543	7.93	278	185	341
Peel from normal fruit from tree prone to cork spot, 57 days after harvest (November 3, 1988)						
P7	7.5	.572	8.589	248	165	288
P7	7.2	.498	7.17	262	175	351
P7	6.7	.498	6.67	235	157	315
P7	7.1	.419	5.95	247	165	394
P7	7.2	.350	5.04	263	175	500
Peel from normal fruit from normal tree, 57 days after harvest (November 3, 1988)						
E5	7.0	.347	4.86	265	177	510
E5	6.7	.447	5.99	229	153	342
E5	7.0	.484	6.78	254	169	349
E5	7.1	.422	5.99	264	176	417
E5	6.8	.300	4.08	253	169	563

Table A. 6. Biochemical analyses results for 1988 season. (cont.)

Tree No.	Extract volume (ml)	Protein concn (mg/ml)	Total protein (mg/g dry wt)	Pyruvate (nmol/ 15 min/ 100 ul)	PK activity (nmol pyruvate/ min/ml)	PK specific activity (pyruvate/ min/mg protein)
Peel from cork spotted fruit from tree prone to cork spot, 86 days after harvest (December 2, 1988)						
P7	7.0	.471	6.59	220	147	311
P7	7.0	.498	6.97	240	160	321
P7	7.0	.541	7.57	219	146	270
Peel from normal fruit from tree prone to cork spot, 86 days after harvest (December 2, 1988)						
P7	7.4	.439	6.50	255	170	387
P7	7.3	.351	5.12	235	157	446
P7	7.0	.434	6.08	225	150	346
Peel from normal fruit from normal tree, 86 days after harvest (December 2, 1988)						
E5	7.0	.343	4.80	268	179	521
E5	7.0	.301	4.21	261	174	578
E5	6.0	.388	4.66	260	173	447
Peel from cork spotted fruit from tree prone to cork spot, 138 days after harvest (January 23, 1989)						
P7	7.0	.536	7.50	206	137	256
P7	7.0	.471	6.59	202	135	286
P7	6.7	.466	6.24	216	144	309

Table A. 6. Biochemical analyses results for 1988 season. (cont.)

Tree No.	Extract volume (ml)	Protein concn (mg/ml)	Total protein (mg/g dry wt)	Pyruvate (nmol/ 15 min/ 100 ul)	PK activity (nmol pyruvate/ min/ml)	PK specific activity (pyruvate/ min/mg protein)
Peel from normal fruit from tree prone to cork spot, 138 days after harvest (January 23, 1989)						
P7	7.0	.525	7.35	231	154	293
P7	7.0	.486	6.80	212	141	291
P7	7.0	.504	7.06	220	147	291
Peel from normal fruit from normal tree, 138 days after harvest (January 23, 1989)						
E5	7.0	.506	7.08	239	159	315
E5	7.0	.593	8.30	223	149	251
E5	7.0	.477	6.68	199	133	278

Table A. 7. Average fruit weight at harvest for sample trees for 1987, 1988, and 1989.

	Tree no.					
	E5	F20	K13	K6	P6	P7
			1987			
Normal fruit	234[z]	222	233	na	na	na
Cork spotted fruit	na	na	na	281	214	229
			1988			
Normal fruit	227	232	240	271	na	na
Cork spotted fruit	na	na	na	na	199	196
			1989			
Normal fruit	183	200	189	195	159	188
Cork spotted fruit	216	213	214	240	137	146

[z] All measurements are in grams.

APPENDIX B

PROTEIN PATTERNS IN 'D'ANJOU' PEAR FRUIT WITH CORK SPOT

Biochemical processes in plants respond dramatically to changes in environmental conditions. Protein synthesis in particular is greatly affected by stress (Cooper and Ho, 1983). The stress may be related to nutrient deficiency, water shortage, heat shock, or other factors and can cause changes in both the types and amounts of proteins synthesized by the plant (Dhindsa et al., 1975a; 1975b).

Nutrient stress in pea root meristem induced the synthesis of a single new polypeptide when overall protein synthesis was declining (Webster, 1980). Water stress in Avena seedlings affected the synthesis of some proteins to a greater extent than the synthesis of others (Dhindsa et al., 1975a). Heat shock in maize seedlings altered the synthesis of normal proteins and triggered the production of unique proteins (Cooper et al., 1983). In Israel, electrophoresis of calcium deficient citrus leaves revealed two polypeptide bands having a molecular weight of 23 and 32 kD that were not present in leaves from trees with normal nutrition (R. Salomon, personal communication). Little is known how stress exerts these effects on protein synthesis, however Sachs and Ho (1986) suggested that stress influenced gene expression which in turn altered protein synthesis. This process acted as a survival mechanism for the plant.

The objective of this preliminary study was to determine the protein patterns associated with cork spot in pear fruit by means of SDS gel electrophoresis (Laemmli, 1970). If unique polypeptide bands can be found associated with cork spot, they may have value as markers

for defining or identifying this disorder.

Plant material. Fruit were harvested from six mature 'd'Anjou' pear trees in the side hill block at Mid-Columbia Agricultural Research and Extension Center in Oregon on August 26, 1987. Three of the trees, designated E5, F20, and K13 had histories of producing primarily normal fruit. The other three trees, K6, P6, and P7, were prone to producing a high percentage of cork spotted fruit. Fruit were stored at -0.5°C ($\pm$ 0.5°C) for 229 days.

Sample preparation. Thin layers of peel tissue were removed from the lower one-half of the fruit with a paring knife and immediately covered with a cloth wetted with .5% (w/v) sodium metabisulfite to minimize oxidation. Peel from six fruit from the same tree were combined to form one sample. The tissue was lyophilized, blended into powder, and sealed in glass jars for transport to the Volcani Center in Israel where protein assays and electrophoresis were performed.

Extraction. 500 mg lyophilized tissue was extracted in a mortar and pestle with 10 ml extraction buffer with 500 mg insoluble PVPP and 1.5 g sand. The extraction buffer was composed of 50 mM Imidazole, 20% glycerol, 3 mM DTT, 5 mM ascorbic acid, 3 mM $Na_2S_2O_5$, and 14 mM mercaptoethanol adjusted to pH 7.0 (modified Menendez et al., 1986). The homogenate was passed through Miracloth and was centrifuged at 37000x g for 20 min. The supernatant was retained for protein assays and electrophoresis. EDTA was not used in the extraction.

Protein assay. Total protein was determined according to Lowry et al. (1951). The determinations were made on protein which had been

precipitated in 10% (w/v) trichloroacetic acid, washed 3 times with absolute ethanol and redissolved in 0.2 M NaOH. BSA was used as a standard.

Electrophoresis. SDS-PAGE was performed at 4°C according to Laemmli (1970). Aliquots equivalent to .015 mg protein were loaded into separating gels that contained a gradient of 5% to 15% polyacrylamide. Polypeptide bands were visualized by staining with Coomassie brilliant blue R.

Results. SDS gel electrophoresis of peel from normal and cork spotted fruit 229 days after harvest revealed an increase in a polypeptide band with a molecular weight of 31 kD in the cork spotted peel (Figure B.1). No unique polypeptide bands were found associated with cork spot with this technique. Assays for soluble protein in the same sample material did not indicate a significant difference between normal and cork spotted fruit (Table B.1). This is in contrast to the data shown in Chapter 4. This is probably due to the fact that EDTA was not used in the extraction buffer. Protein levels were only different (Table 4.2) when EDTA was used in the extraction buffer.

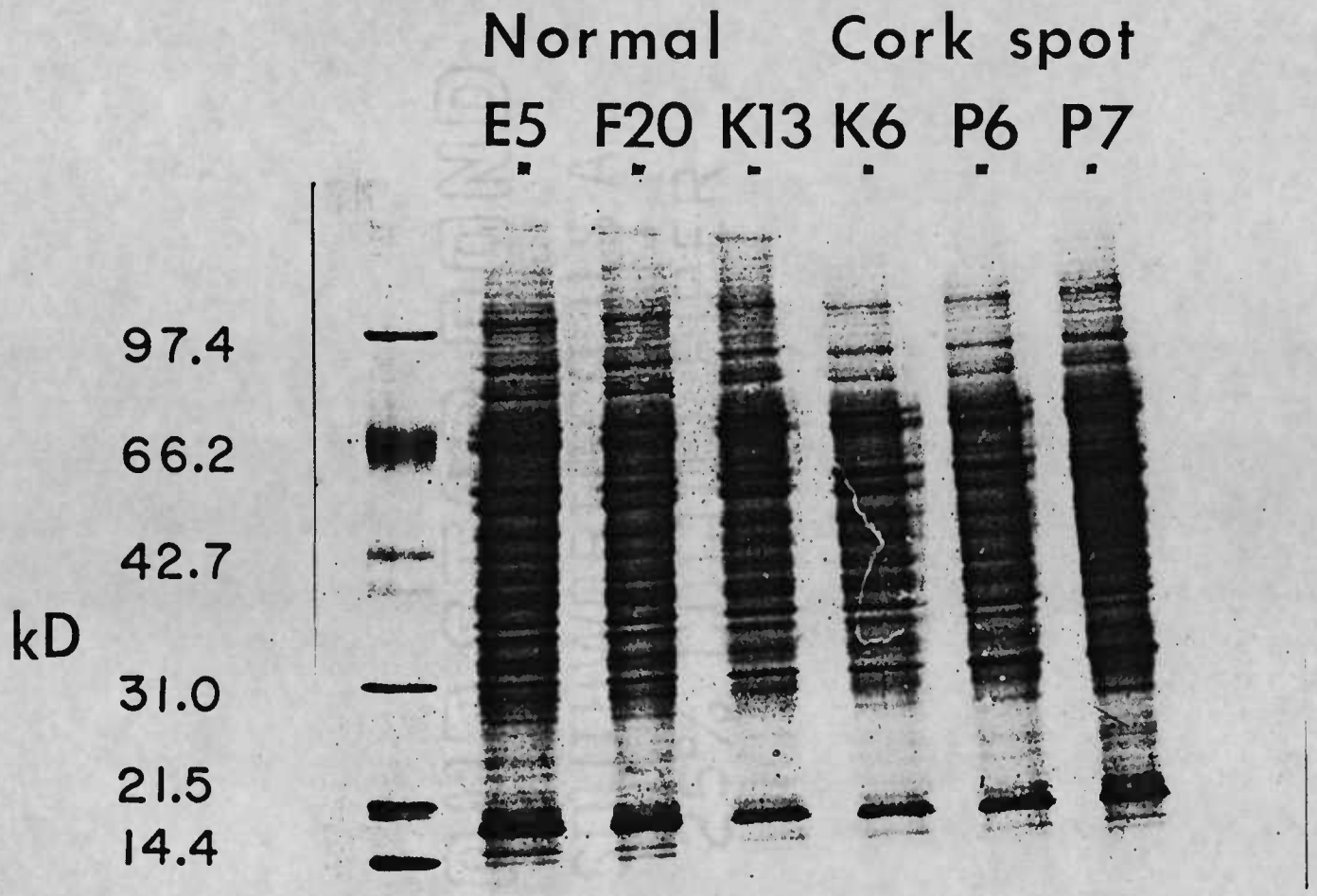

Fig. 8.1. Electrophoresis revealed the polypeptide profile of 'd'Anjou' pear peel from normal and cork spotted fruit.

Table B.1. Protein (total) concentration in 'd'Anjou' pear peel 229 days after harvest.

	NORMAL			CORK SPOT		
Tree No.	E5	F20	K13	K6	P6	P7
Protein concn (mg/g dry wt)	4.89 NS	4.50	3.95	4.93	4.68	6.70

NS There was not a significant difference (5% level) between normal and cork spotted fruit.

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