ETHYLENE-INDUCED TISSUE BREAKDOWN IN FRUIT OF WATERMELON
[Citrullus lanatus (Thunb.) Matsum and Nakai]

BY

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Although watermelon [Citrullus lanatus (Thunb.) Matsum and Nakai] fruit have been classified as climacteric, there are reports that they exhibit cellular breakdown when exposed to ethylene gas. This study comprises an investigation of the postharvest behavior of watermelon fruit under normal conditions and in response to ethylene or propylene. Parameters measured included the effects of ethylene on ripening and respiratory activity, electrolyte leakage, cell wall ultrastructure, and cell wall hydrolases. All experiments included a comparison of the changes which occurred during both ethylene-induced breakdown and natural ripening of fruit.

Respiratory rates in harvested melons showed little change throughout ripening. Respiratory activity was enhanced in the presence of ethylene but returned to normal levels upon removal of the gas. Endogenous ethylene production was not initiated by exposure of fruit to propylene and was detected only in fruit exhibiting symptoms

of decay. The results supported the hypothesis that watermelon fruit was nonclimacteric.

Ethylene-treated tissue exhibited extensive cell separation, high activity of D-galacturonase, and enhanced electrolyte leakage. These ethylene responses did not appear to represent merely an acceleration of normal ripening since they were not observed in fruit not exposed to ethylene.

The sequence of events which resulted in watermelon tissue breakdown emphasized the predominant role of the cell wall. The increase in D-galacturonase activity observed after the first day of ethylene treatment coincided with the development of a distinct zone of separation in the middle lamella of the cell wall. Following longer periods of ethylene treatment, extensive pectin degradation was apparent from the increased quantities of polymers fractionating on Ultrogel AcA 34. Ultrastructural studies revealed that cell walls started to show signs of deterioration after the first day of ethylene treatment.

The membrane system did not seem to play a major role in initiating watermelon tissue breakdown, since no change in electrolyte leakage was noted during the first 3 days of ethylene treatment. The increase in electrolyte leakage observed after the 3rd day of ethylene treatment was apparently due to cell wall degradation and weakening which led to the rupture of the membrane.

CHAPTER I INTRODUCTION

Watermelons [Citrullus lanatus (Thunb) Mats. and Nak.] are among the most important vegetable crops grown in Florida. A total area of 64,000 acres was planted during the 1983-84 crop year. Production was placed at 10 million cwt., and the total value of the crop was estimated at \$62.1 million (Florida Agric. Stat., 1984).

Early in the season, watermelons were shipped in mixed loads with other commodities that produce ethylene or stored in warehouses near ethylene-producing products. Exposure of watermelons to ethylene emanations adversely affected their quality and rendered them unfit for consumption. This observation prompted Risse and Hatton (1982) to investigate the postharvest response of watermelons to ethylene. They found that exposure of watermelons to various ethylene concentrations for 3 or 7 days accelerated their deterioration. Ethylene-treated watermelons had thinner rinds and were softer than untreated fruit. The melons were spongy, watery, and had developed off-flavors. Ethylene treatment had a harmful effect on preripe watermelons as well. Earlier, Shimokawa (1973) observed that watermelon tissue exhibited maceration when exposed to ethylene emanations evolved by 'Prince' melons. Enzymic studies of macerated tissue revealed higher activities of cell wall hydrolytic enzymes as compared to healthy tissues. These results are seemingly contradictory to the data presented by Mizuno and Pratt (1973), who reported that watermelons

produced ethylene in a manner characteristic of climacteric fruits. It has been well established (McGlasson et al., 1978; Rhodes, 1980a; Biale and Young, 1981) that ethylene treatment of preripe climacteric fruits will shorten the time required for the induction of the climacteric. Ethylene treatment will also trigger autocatalytic ethylene production and enhance normal fruit ripening to aesthetically acceptable quality. On the other hand, a ripe climacteric fruit which is in the climacteric or postclimacteric phases is insensitive to further applications of ethylene (Biale and Young, 1981; Solomos, 1983). Hence, the fact that watermelons are deleteriously affected by ethylene treatment casts doubt on their climacteric categorization.

The objectives of this research are

- 1) to study the effects of ethylene treatment on respiration and endogenous ethylene production in watermelon fruit at various stages of ripening;
- 2) to determine the nature of the ethylene-induced breakdown in watermelon tissue: is it due to cell wall breakdown, increased membrane permeability, or both?
- 3) to study the effect, if any, of ethylene on the structural polymers of the cell wall, and in particular the pectins, a) examining tissue for presence of D-galacturonase, and b) examining tissue for evidence of pectin degradation;
- 4) to carry out ultrastructural studies of ethylene-treated and control tissues.

CHAPTER II REVIEW OF LITERATURE

Respiration and Ethylene Production of Fruits in Response to Exogenous Ethylene or Propylene

Introduction

Studies of the respiratory activity of fruits indicate that the climacteric pattern of respiration is not shown by all fruits. The respiration rate of some fruits was found to follow a slow downward drift after detachment. Hence, Biale (1960) classified fruits as climacteric or nonclimacteric on the basis of their respiratory patterns. Examples of climacteric fruits are tomatoes, mangoes, bananas, apples, and avocados. Nonclimacteric fruits are typically represented by citrus fruit, grapes, cherries and others (Biale, 1960). The ripening of climacteric fruits was found to be accompanied by rapid compositional changes that include hydrolysis of storage polysaccharides, breakdown of cell wall materials and softening, changes in organic acids, increases in aroma, increased ethylene production, loss of astringency, and changes in color. When climacteric fruits are picked at relatively immature stages, they will undergo the same physiological changes as mature fruits, but with an inferior quality (McGlasson et al., 1978). Nonclimacteric fruits, however, are characterized by a slow utilization of soluble sugars rather than the hydrolysis of polysaccharides. The capacity of many

nonclimacteric fruits to ripen after picking is very limited (Rhodes, 1980b).

One of the most important criteria used to differentiate between climacteric and nonclimacteric fruits is their specific response to ethylene. Highly sensitive techniques have revealed that ethylene is produced throughout the growth and development of both climacteric and nonclimacteric fruits. Nonclimacteric fruits produce ethylene at a very low rate throughout their growth and maturation and the attainment of full maturity is not associated with increased ethylene production. However, the rate of ethylene production increases dramatically during the ripening of climacteric fruits (McGlasson et al., 1978; Rhodes, 1980a).

Fruit Response to Applied Ethylene

Climacteric and nonclimacteric fruits behave quite differently in their response to exogenously applied ethylene. A treatment of 0.1 to 1.0 ppm ethylene for one day is sufficient to initiate ripening in most climacteric fruit. The respiration rate of the fruit increases to a peak value, the magnitude of which is independent of the concentration of ethylene applied. Once ripening is initiated, removal of exogenous ethylene does not affect the progress of ripening and the fruit becomes insensitive to further applications of ethylene (Biale and Young, 1981; Solomos, 1983). Ethylene treatment shortens the period after harvest required for the induction of the climacteric rise without affecting the respiratory peak (Rhodes, 1980a). Treatment of climacteric fruits with ethylene at concentrations higher

than a threshold level of 0.1 ppm will, depending on fruit maturation, induce autocatalytic ethylene production (McGlasson et al., 1978).

The stage of fruit maturity affects the time lag to the induction of the respiratory climacteric. Wang and Hansen (1970) treated immature pears with 500 ppm ethylene for 24 hrs and reported a decrease in flesh firmness, an increase in soluble pectins, and the fruits attained full ripeness without a concomitant change in the respiratory activity. However, in fully mature, ethylene-treated pears, the climacteric rise in respiration developed together with ripening. The induction of the climacteric rise in respiration of immature fruits required at least 48 hrs of exposure to ethylene. Kosiyachinda and Young (1975) reported that ethylene was not the single control factor in the induction of the climacteric. The effect of ethylene on respiration seemed to be independent from its effect on ripening. Wang et al. (1972) studied the effects of ethylene treatment on the respiratory rate and chemical composition of pears harvested at different stages of maturation. Respiration rate of immature fruits varied according to ethylene concentration. Ethylene production in response to exogenously applied ethylene occurred only in fully mature fruits during the development of the respiratory climacteric. Ethylene-induced softening started before the climacteric rise in respiration. It was suggested that ripening as measured by softening was not dependent on the development of the respiratory climacteric. Akamine and Goo (1979) studied respiration and ethylene production in fruits of species and cultivars of Psidium and Eugenia. Fruits of Psidium species exhibited typical climacteric

type respiration with the increase in ethylene production preceding the respiratory rise by one day. In contrast, the respiration rate of ethylene-treated fruits of Eugenia species increased and then decreased to the control level upon removal of the gas. Those fruits showed a response typical of nonclimacteric fruits to ethylene. The climacteric rise in respiration was shown to occur even when the fruits were still attached to the tree, except avocado fruits which do not ripen on the tree (Biale and Young, 1981).

Gazit and Blumenfeld (1970) reported that unpicked avocado fruits did not respond to 50 ppm ethylene for 48 hrs. Ethylene treatments immediately after harvest also resulted in no response. Fruits responded to ethylene only when the treatment was initiated at least 25 hrs after harvest. The lack of response to ethylene was attributed to the presence of endogenous inhibitory substances (Gazit and Blumenfeld, 1970). Paull (1982), working with soursop fruits, concluded that fruit response to ethylene was dependent upon the sensitivity of the tissue to the initiation of ripening brought about by an increase in ethylene receptor sites, a decrease in inhibitors, or an increase in activators.

Treatment of nonclimacteric fruits with ethylene causes a respiratory rise, the peak value of which is proportional to the logarithm of the ethylene concentration applied (Rhodes, 1980b). Removal of the exogenous ethylene leads to a fall in the rate of respiration to its value before the onset of treatment. The enhanced respiration can be repeatedly stimulated by successive ethylene treatments (McGlasson et al., 1978; Rhodes, 1980b). Application of

ethylene to nonclimacteric fruits has no effect on the normal low rate of basal ethylene evolution. Burg and Burg (1962) earlier suggested that the essential difference between climacteric and nonclimacteric fruits lies in their relative abilities to produce ethylene autocatalytically in response to the accumulation of threshold levels of ethylene within their tissues.

Ethylene has been used for many years for citrus degreening (Grierson and Newhall, 1960), and is known to increase respiration rate as well as stem end rot (Kusunose and Sawamura, 1980). Vines et al. (1965) reported that exposure of 'Valencia' oranges to 50 ppm ethylene for 24 hrs caused a 400% increase in respiration rate. After withdrawal of ethylene, respiration rate declined but did not return to the control levels within 7 days. The relationship between climacteric and nonclimacteric fruits was further clarified by studies of mutant strains of tomato. Herner and Sink (1973) showed that rin tomato mutant fruits had low rates of respiration and ethylene production and showed very limited degree of ripening. Exogenous ethylene treatments caused a rise in respiration but without autocatalytic ethylene production or ripening. The continuous presence of ethylene was necessary for a sustained rise in respiration rate and the fruit was capable of repeated stimulation by successive ethylene treatments (Herner and Sink, 1973). Frenkel and Garrison (1976) reported on the initiation of lycopene synthesis in rin tomato mutant fruits. Detached rin fruits synthesized no lycopene in the absence of ethylene. However, lycopene synthesis was initiated at 10 ppm ethylene and elevated levels of oxygen.

Internal ethylene levels in nonclimacteric fruits usually range from 0.02 to 0.2 ppm (Burg and Burg, 1962). Studies on postharvest respiration and ethylene production in jujube fruits (Kader et al., 1982) showed that internal ethylene production rate ranged from 0.05 to 0.25 l/kg-hr. Their data indicated that Chinese jujube had a nonclimacteric pattern of respiration and did not exhibit accelerated ethylene production during ripening.

Initiation of a respiratory rise in response to ethylene treatment is not restricted to fruits. Reid and Pratt (1972) compared the respiratory response of potato tubers to ethylene treatment with that of oranges. Ethylene treatment increased the respiration rate of potato tubers 5 to 10 times that of the control. Ethylene concentrations of 2, 20, and 90 ppm resulted in the same respiratory response. Tubers' response to applied ethylene was similar to that of nonclimacteric fruits such as oranges. Reid and Pratt (1972) proposed that the respiratory rise in climacteric fruits and that of wounded plant tissue was induced by a rise in endogenous ethylene concentration.

Fruit Response to Applied Propylene

Experiments demonstrating autocatalytic ethylene production by climacteric fruits in response to exogenously applied ethylene were made possible by the use of propylene (McMurchie et al., 1972). Propylene is a biologically active analogue of ethylene and although it is not as active as ethylene, it also initiates fruit ripening. The equivalent concentration of propylene required to give a

comparable response was found to be 130 times that of ethylene (Burg and Burg, 1967). By applying propylene to fruits it is possible to measure endogenous ethylene evolution accurately, since propylene and ethylene are readily separable by gas chromatography. McMurchie et al. (1972) treated climacteric bananas and nonclimacteric oranges and lemons with 500 ppm propylene. In bananas, propylene initiated a typical climacteric pattern of respiration, ripening, and autocatalytic production of ethylene. Propylene-treated lemons and oranges had increased respiratory rates without being accompanied by autocatalytic ethylene production. The authors proposed that two systems were involved in the responses of fruits to ethylene. In system I, ethylene biogenesis was initiated by factors involved in the regulation of aging processes. This brought the concentration of ethylene to a sufficient level to induce system II which led to the respiratory climacteric, ripening changes, and further ethylene production. Hence, nonclimacteric fruits lacked system II but possessed system I. The effect of propylene treatment on the stage of fruit maturation was investigated by Sfakiotakis and Dille (1973) in apples. The ability of propylene to stimulate ethylene production increased progressively with fruit maturation. The lag time to the onset of autocatalytic ethylene production decreased with increased maturation, thereby reflecting increased tissue sensitivity. McGlasson et al. (1975) found that continuous application of 300 to 1000 ppm propylene advanced ripening in normal tomato fruits of all maturation stages by at least 50%. Exposure to propylene stimulated respiration in non-ripening mutant fruits but without a change in

endogenous ethylene production. Eaks (1980) and Brecht and Kader (1984a, 1984b) also reported on the relationships between propylene concentration and stage of fruit maturation. Immature fruits usually responded by showing an increased respiration rate. Upon termination of the treatment, the respiration rate fell to the untreated control. Propylene did not induce ethylene production in immature fruits, but did so in mature ones. Propylene concentrations of 1250 to 12,500 ppm advanced the onset of ethylene production, stimulated ripening, and enhanced softening of slow-ripening nectarine fruits (Brecht and Kader, 1984b). Propylene-treated avocado fruits (Eaks, 1980) had a progressively shorter time to the climacteric and to softening as they matured.

Respiration and Ethylene Production of Fruits as Affected by Microorganisms

Few studies have been carried out on the contribution of microbial activity to respiration and ethylene production of fruits. In some cases, many fruits which have been classified as climacteric were found to be decayed and owed much of their high respiration rates and ethylene production to microorganisms (Oslund and Davenport, 1983). An increase in respiratory activity was found in lemons and oranges naturally infected with Penicillium digitatum, Diplodia natalensis, and Phomopsis citri (Vines et al., 1965), and in lemons artificially infected with Penicillium digitatum (Eaks, 1955). Studies on respiration and ethylene production of carambola fruits revealed that exogenously applied ethylene had no significant effects on either respiration or autocatalytic ethylene production (Oslund and

Davenport, 1983). Higher levels of CO₂ and ethylene produced by fully ripe fruits were mainly attributed to microbial activity. The respiratory and ripening patterns of healthy fruits were typical of those for nonclimacteric fruits. Barkai-Golan and Kopeliovitch (1983) reported on ethylene evolution and respiration of Rhizopus-infected rin and nor tomato mutant fruits. Rhizopus stolonifer infection stimulated ethylene and CO₂ production by fruits of the two non-ripening tomato mutants, and caused the climacteric-like patterns of respiration typical of normal fruits. The rates of respiration and ethylene production of uninfected mutant fruits remained low and constant. When normal fruits were inoculated in the postclimacteric (red) stage, new climacteric peaks with rates higher than those of uninfected fruits followed fungal infection. Similarly, Zauberman and Barkai-Golan (1975) studied changes in respiration and ethylene evolution induced by Diplodia natalensis in orange fruit. The respiration rate and ethylene evolution were higher in infected than in healthy fruits. In uninfected fruits, however, very low and constant levels of ethylene evolution were recorded. Accelerations in respiration and ethylene evolution were also induced in avocado fruits inoculated with Fusarium solani (Zauberman and Schiffmann-Nadel, 1974). Increases in respiration and ethylene production rates started earlier than in healthy fruits, but their pattern and intensity were the same. In vitro studies revealed that when the fungus was cultured on potato dextrose agar under optimal growth conditions, it evolved no ethylene during 14 days of growth. Early studies by Biale and Shepherd (1941) and Biale (1948) showed that citrus fruits inoculated

with P. digitatum evolved considerable amounts of ethylene and the authors suggested that the fungus, by producing ethylene, induced the increased fruit respiration. However, D. natalensis did not seem to produce ethylene (Zaubermann and Barkai-Golan, 1975) and therefore it appeared that ethylene evolution in infected fruits was a direct response of the fruit to fungal attack. Studies by Barkai-Golan and Kopeliovitch (1983) on tomato fruits revealed that the highest rates of ethylene production occurred in the healthy tissue at the margin of the rot and little ethylene was produced by rotted tissues with actively growing fungus. Hence, ethylene appeared to be a product of the host rather than the fungus.

Membrane Permeability Changes During Ripening and Senescence

The semipermeable nature of biological membranes is of crucial importance in maintaining compartmentation. Early investigators (Sacher, 1966; Simon, 1977a) have suggested that the respiratory rise during ripening of certain fruits was due to membrane permeability changes. They postulated that the loss of permeability barriers could lead to changes in protoplasmic compartmentation, leading to access between enzymes and substrates, and result in the metabolic changes associated with the climacteric.

Effect of Ethylene on Membrane Permeability

Ethylene has been shown by many workers (Hanson and Kende, 1975; Kende and Hanson, 1976; Suttle and Kende, 1978, 1980; Ferguson and Watkins, 1981; Borochoy and Faragher, 1983) to be involved in the

regulation of senescence in a wide variety of plant organs.

Senescence in plant tissues is known to be accompanied by changes in membrane permeability (Sacher, 1973). Leakage of electrolytes, sugars, or pigments is a commonly used method to assay membrane permeability in plant tissues. Ethylene treatment prematurely induced senescence and induced endogenous ethylene production in flowers of Ipomoea tricolor (Kende and Baumgartner, 1974). The authors proposed a model to explain the mechanism by which ethylene can induce ethylene synthesis or autocatalysis. They suggested that low, system I levels of ethylene caused loss of cellular compartmentation which caused intermixing of previously sequestered components of the ethylene-generating system. Hanson and Kende (1975) reported that exogenous ethylene treatment induced senescence and enhanced efflux of solutes from the cells of morning glory flower tissue. Electrolyte leakage was a consequence of increased permeability of either the tonoplast or the plasmalemma or both. The view that ethylene increases the permeability of the tonoplast is consistent with observations made by Suttle and Kende (1978, 1980). Working with isolated petals of Tradescantia, they showed that exogenous ethylene treatment hastened the onset as well as the increase in anthocyanin and electrolyte leakage from the petals. The sensitivity to applied ethylene increased as the petals matured. They supported the hypothesis put forward by Kende and Baumgartner (1974) to explain autocatalytic ethylene production.

The loss of tonoplast integrity would allow the mixing of vacuolar hydrolases with their cytoplasmic substrates resulting in

autocatalytic ethylene production. It was concluded that ethylene regulated senescence by determining the rate of loss of compartmentation which played a central role in the deteriorative changes occurring during senescence.

Ferguson and Watkins (1981) studied cation leakage from apple fruits during development and ripening. Leakage of K^+ exceeded that of Ca^{2+} and Mg^{2+} and was not affected by the external presence of divalent cations. Leakage of Ca^{2+} and Mg^{2+} was markedly increased by the external presence of either ion. Whereas K^+ and Mg^{2+} leakage increased with fruit senescence, Ca^{2+} leakage decreased. Ion leakage was found to increase during the climacteric rise in apple fruit. A close relationship had been reported (Berard and Loughheed, 1982) between an increase in membrane permeability and a rising production of ethylene in apples. Apple fruits held under low pressure storage had reduced membrane permeability due to delayed ethylene production. Similar results were reported by Kobayashi et al. (1981) with water-stressed plum leaves.

Changes in Membranes During Senescence

Beutelmann and Kende (1977) correlated changes in membrane lipid content with aging symptoms in morning glory flowers. They found that the level of phospholipid had already started to decline before visible signs of senescence were evident. The rate of phospholipid loss accelerated sharply with ethylene production whereas synthesis of new phospholipids fell by 40%. Exogenously applied ethylene accelerated phospholipid loss and senescence of flower tissues whereas

benzyladenine retarded both of these processes. Likewise, Suttle and Kende (1980) found that the increase in membrane permeability of Tradescantia flowers was accompanied by a massive loss of phospholipids. Their results suggested that the observed increase in membrane permeability was a direct result of an increased phospholipid degradation presumably caused by an increase in the activity of pre-existing phospholipases.

The compositional changes that take place in membranes during senescence play a significant role in their permeability. It has been shown that sterol to phospholipid ratio increased with senescence and this increase was proportional to the decrease in membrane fluidity (Borochoy et al., 1978, 1982; Borochoy and Faragher, 1983; Borochoy and Weinberg, 1984; Thompson et al., 1982). Borochoy et al. (1978) studied senescence in rose petals and reported that the microviscosity of the plasmalemma increased during senescence due to an increase in sterol to phospholipid ratio. The increased ratio was presumably brought about by a decrease in the amount of phospholipids caused by the action of endogenous phospholipases. Further studies (Thompson et al., 1982) indicated that lipid microviscosity of membranes from senescing carnation flowers increased with advancing senescence and coincided with the climacteric-like rise in ethylene production. Exogenous ethylene treatment of the flowers increased the microviscosity and accelerated rigidification of membranes. Membrane rigidification was accompanied by an increased sterol to phospholipid ratio that reflected selective phospholipid loss with senescence. Studies by Borochoy et al. (1982) on rose petals revealed that there

was no quantitative change in the level of free sterols. The content of phospholipids decreased without any significant change in their composition. They concluded that the fluidity of rose petal membranes decreased with age as a result of a decrease in phospholipid content brought about by both reduced synthesis and enhanced degradation.

Although it has been accepted that membrane lipid phases are dominated by the liquid crystalline phase, recent evidence (Legge et al., 1982a) indicates the presence of a gel phase at physiological temperatures in senescent tissues. The presence of lipids in the gel phase in biological membranes is known to induce an increase in membrane permeability (Borochov and Weinberg, 1984). Studies with rose flowers (Legge et al., 1982b) revealed that microsomal membranes from the tight bud stage contained liquid crystalline lipid and only traces of gel phase lipid. With advancing senescence, however, the proportion of gel phase lipid increased.

It has also been observed that changes that occur in the fatty acid chain saturation of membrane phospholipids could affect membrane permeability (Wade and Bishop, 1978; Wade et al., 1980). Generally, an increase in saturation would make the lipids less fluid at any given temperature. Wade and Bishop (1978) studied changes in lipid composition of ripening banana fruits. They found that the relative proportions of the different lipids remained constant but the fatty acid composition changed during ripening. The change was confined to the phospholipid fraction in which there was an increase in total unsaturation of the fatty acids. In contrast to the information presented above, the authors suggested that increased lipid

unsaturation resulted in increased membrane fluidity which consequently resulted in increased membrane permeability. Further studies by Wade et al. (1980) suggested that increased passive permeability of ripening banana fruit was due to increased polyunsaturation of membranes. They concluded that the increased electrolyte leakage observed in banana fruit tissue treated with propylene could be correlated either with changes in membrane lipid composition or with changes in tissue water potential.

Lipid peroxidation might also contribute to the modification processes in membranes, presumably through generation of a gel phase domain in membrane lipids. The presence of such domains might cause an increase in membrane permeability (Borochov and Weinberg, 1984). Data presented by Dhindsa et al. (1981) showed a close relationship between increased solute leakage and increased level of lipid peroxidation in senescent tobacco leaves.

Electrolyte leakage from fruit tissues may, however, depend upon factors other than membrane permeability. Of particular importance, once other ripening changes such as soluble solids accumulation have begun, the gradient in water potential between fruit tissues and the bathing solution may result in measurement artifacts (Sacher, 1973; Simon, 1977a). Incubation of tissues in hypotonic media can lead to membrane damage particularly in the relatively fragile cells of ripening fruit (Wade et al., 1980). Simon (1977b) studied electrolyte leakage from apple fruits and found that when the tissues were placed in water, 90% of their electrolytes and soluble carbohydrates leaked out in 2-3 hours. Leakage was greatly reduced when tissues were

placed in 1 M glycerol or 0.5 M KCl. It was suggested that apple fruit cells burst in water but not in isotonic media. Leakage from tissues of other soft fruits followed similar trends and became more extensive with advancing maturation.

The Role of Calcium in Delaying Senescence

Calcium has long been known to play a significant role in numerous physiological processes in plant tissues. The maintenance of relatively high calcium concentration in fruit tissues delayed ripening in tomatoes (Wills et al., 1977; Wills and Tirmazi, 1979; Buescher and Hobson, 1982), and avocados (Tingwa and Young, 1974); reduced CO₂ and ethylene production (Tingwa and Young, 1974; Wills and Tirmazi, 1982); maintained firmness of apple fruits (Sams and Conway, 1984); and inhibited abscission (Poovaiah and Leopold, 1973a). Ripening of green tomatoes (Wills and Tirmazi, 1979) was inhibited when Ca content of the fruit was raised to greater than 40 mg/100 g fresh weight. The fruits showed no signs of ripening even after 6 weeks' storage at 20°C, and the application of 1000 ppm ethylene for 3 weeks had no effect. Inhibition of ripening was not restricted to Ca as other divalent ions such as Mn, Co, and Mg were also effective. Monovalent metal ions were less effective than Ca.

The breakdown of pectic substances in the middle lamella and cell wall may result in loss of wall integrity in ripening fruits (Sams and Conway, 1984; Ferguson, 1984). Free carboxyl groups on D-galacturonase polymers play an important role in stabilizing and maintaining wall integrity through the cooperative binding of Ca ions

(Demarty et al., 1984). Sams and Conway (1984) reported a negative relationship between soluble polyuronide content and Ca concentration in apple fruits. The binding of Ca is crucial for strengthening fruit tissue and making it more resistant to hydrolytic enzyme attack (Buescher and Hobson, 1982). Sawamura et al. (1978) reported that the presence of Ca decreased hydrolytic enzyme activity in tomato fruits, and Ca was found to inhibit the natural decline in resistance to D-galacturonase activity in the cell wall.

The nonenzymic mechanism of fruit softening has been explained on the basis of the important role played by Ca in pectin stabilization. Removal of Ca destabilizes the pectic matrix resulting in the weakening of the cell wall (Huber, 1983b).

The role of Ca in membrane structure and function is well established (Rousseau et al., 1972; Poovaiah and Leopold, 1973b; Lieberman and Wang, 1982; Ferguson, 1984). The ability of Ca to delay senescence is a direct result of its role in maintaining membrane integrity and hence cellular compartmentation (Hecht-Buchholz, 1979; Poovaiah, 1979; Legge et al., 1982a). During senescence, the most obvious indication of membrane change is an increase in electrolyte leakage which indicates the loss of selective permeability. Poovaiah (1979) demonstrated that Ca decreased membrane permeability during tomato fruit senescence. Levels of bound Ca in mutant fruits were high at advanced stages of maturation whereas in normal fruits bound Ca decreased about 3-fold during maturation. Changes associated with senescence were suppressed by Ca as a consequence of its role in maintaining cell wall structure and membrane integrity. Lieberman and

Wang (1982) reported that protein leakage from apple tissue slices was significantly reduced when the incubation medium contained 100 mM Ca plus Mg. Their results indicated a membrane preservation effect of Ca, Mg, or Ca plus Mg during rapid aging of apple fruit tissues.

Calcium reduces membrane permeability presumably through tighter packing of lipids which is brought about by binding of Ca to negatively charged phospholipids resulting in aggregation (Ferguson, 1984). Studies by Legge et al. (1982a) revealed that Ca rigidified and stabilized membranes mainly at their surfaces, whereas its effect was less pronounced deeper within the lipid bilayer. The ability of Ca to tightly pack lipid domains with a subsequent reduction in membrane permeability also results in reduced membrane fluidity. Borochoy et al. (1978) showed that the presence of 5 to 25 mM Ca ions in the incubation medium of rose petals increased the microviscosity of the plasmalemma.

The inhibition of senescence by Ca may also involve protection of membranes from free radical or peroxidative attack. It was found that apple fruit tissues incubated in 100 mM Ca or Mg showed considerably less lipid peroxidation as compared to the untreated control (Lieberman and Wang, 1982).

One of the main evidences supporting the role of Ca in maintaining membrane integrity is made possible by the use of electron microscopy. Ultrastructural studies (Hecht-Buchholz, 1979) of Ca-deficient potato sprout cells revealed extensive disintegration of the plasmalemma and the tonoplast.

Activity of Hydrolytic Enzymes During Fruit Ripening

Introduction

The cell wall of fruits is basically composed of cellulose, hemicelluloses, polyuronides, and associated glycoproteins and enzymes. These major components are extensively associated to form strong yet flexible matrices. The cell wall also includes a morphologically distinct layer known as the middle lamella, which is located between the primary cell walls of adjoining cells and forms a continuous intercellular matrix. Since this layer is particularly rich in pectic polysaccharides, it is believed to be the region of the cell wall most affected during fruit softening (Huber, 1983b).

Softening of many fruits during ripening has long been attributed to enzymic action that results in the solubilization of cell wall polysaccharides (Hobson, 1964, 1965; Pressey and Avants, 1971, 1973a, 1973b, 1975; Zauberman and Schiffmann-Nadel, 1972; Buescher and Tigchelaar, 1975; Paull and Chen, 1983). Enzymes reported to be involved in fruit softening are the D-galacturonases, cellulases, and pectinmethylesterases (Rexova-Benkova and Markovic, 1976; Pressey, 1977; Huber, 1983b). In this context, discussion will focus on the D-galacturonases (the polygalacturonases) and cellulases only, since they are the major carbohydrases associated with fruit cell wall.

D-Galacturonases

Rexova-Benkova and Markovic (1976) suggested that pectolytic enzymes be referred to as D-galacturonases on the basis of their

preferred substrates--the D-galacturonans. D-Galacturonases were first detected in ripe tomato fruit (Hobson, 1964, 1965; Buescher and Tigchelaar, 1975). These enzymes were found in a wide range of fruits and are generally associated with fruit ripening and softening.

Usually there is little or no D-galacturonase activity in immature fruits and activity increases during ripening (Hobson, 1964; Sawamura et al., 1978; Ahmed and Labavitch, 1980). Hobson (1964) studied blotchy ripening in tomato fruits and found low D-galacturonase activity in the apparently normal red area and a severe decrease in activity in the abnormal, non-red areas. He concluded that blotchy ripening was due to a failure in D-galacturonase synthesis. Buescher and Tigchelaar (1975) compared D-galacturonase activity in normal and mutant tomato fruits and found that D-galacturonase activity increased during ripening of normal fruits. However, in mutant fruits, D-galacturonase activity was not detected and the authors concluded that the lack of softening of mutant fruits was due to the lack of D-galacturonase. Hobson (1965) concluded that softening of tomato fruits during ripening was directly associated with the activity of D-galacturonase. Studies on D-galacturonase synthesis (Tucker and Grierson, 1982) could not detect D-galacturonase activity in green tomatoes. D-galacturonase appeared on the onset of ripening and was one of the major cell-wall-bound proteins in ripe fruit. The data of Tucker and Grierson indicated that during tomato ripening, D-galacturonase was synthesized de novo.

Pressey et al. (1971) studied D-galacturonase activity, water-soluble pectin, molecular weight distribution of water-soluble pectin,

and fruit firmness during ripening of freestone peaches. D-galacturonase activity was absent in unripe fruits but it developed during ripening and was accompanied by increasing amounts of water-soluble pectins and a loss of firmness. Gel-filtration studies revealed that the molecular weights of pectins decreased progressively during ripening, indicating a role for D-galacturonase in pectin solubilization.

D-galacturonases are classified on the basis of their mode of action as endo-D-galacturonases (E.C. 3.2.1.15) and exo-D-galacturonases (E.C. 3.2.1.67) to designate a random or terminal action pattern, respectively (Rexova-Benkova and Markovic, 1976; Huber, 1983b).

Many research workers have reported on the occurrence of one or more forms of D-galacturonases (Pressey and Avants, 1971, 1973a, 1973b, 1975; Hunter and Elkan, 1974; Tucker et al., 1980; Huber, 1983a). Pressey and Avants (1973a) separated two D-galacturonases from extracts of ripe tomatoes and referred to them as PG I and PG II. In all of the samples examined, PG II was the predominant enzyme. The enzymes differed in stability with respect to both temperature and pH, and their molecular weights were 84,000 and 44,000 for PG I and PG II, respectively. Polygalacturonase II was found to be more effective than PG I in reducing the viscosity of pectic acid (endo-D-galacturonase). Similarly, Pressey and Avants (1973b) separated two D-galacturonases from ripe peach fruits and named them PG I and PG II. Polygalacturonase I hydrolyzed polygalacturonic acid from the nonreducing ends of the molecules and released galacturonic acid as

the product. It required Ca^{2+} for activity, functioned optimally at pH 5.5 and hydrolyzed low molecular substrates more rapidly (exo-D-galacturonase). In contrast, PG II cleaved substrate molecules at random, with a pH optimum of 4.0, and was most reactive with intermediate molecular weight substrates--all characteristics of endo-D-galacturonases.

Pressey and Avants (1978) investigated the textural differences between clingstone and freestone peach fruits by comparing their pectolytic activities and pectin solubility. Preripe fruits from both types of peaches had very low levels of water soluble pectins and no detectable D-galacturonase activity. Whereas ripe clingstone peaches had exo-D-galacturonase and insoluble pectin, ripe freestone peaches had both exo and endo-D-galacturonases and high levels of water-soluble pectin. The textural differences between the two peach types were basically due to enzyme composition rather than levels of total D-galacturonase.

Hunter and Elkan (1974) identified only endo-D-galacturonase from ripe tomatoes possibly due to less sensitive extraction techniques. In contrast, Pressey and Avants (1975) were successful in resolving only an exo-D-galacturonase from cucumber fruits. They reported that cucumber exoenzyme was similar to peach exoenzyme (Pressey and Avants, 1973a) in pH optimum, cation activation, molecular weight, and in its stepwise removal of monomer units from the non-reducing ends of the substrate molecules. The participation of exo-D-galacturonase in cucumber softening was not through random cleavage of pectin but rather through specific hydrolysis of terminal linkages.

Huber (1983a) examined polyuronides from cell wall preparations of ripening tomato fruits using gel-filtration chromatography. The increase in the quantity of polymers that fractionated on the gel indicated that polyuronides were extensively degraded during ripening. Low molecular weight polyuronides were first observed in fruit harvested at the turning stage and continued to increase with the advancement of ripening. There was a close relationship between the appearance of degraded polyuronides and the activity of endo-D-galacturonase which seemed to be solely responsible for the degradation of the wall polymers.

Research workers could not agree as to whether it was D-galacturonase or ethylene that led to the initiation of ripening in fruits. Tigchelaar et al. (1978) suggested that D-galacturonase played a key role in fruit ripening. They found that ethylene was not evolved unless D-galacturonase activity increased and ethylene had no effect on D-galacturonase activity. It had also been suggested (Tigchelaar and McGlasson, 1977) that the rise in the activity of D-galacturonase was an early or even the primary event in fruit ripening. These suggestions are in disagreement with other findings (Babbitt et al., 1973; Poenicke et al., 1977; Awad and Young, 1979; Paull and Chen, 1983). The data presented by Sawamura et al. (1978) indicated that exposure of tomato fruits to different ethylene concentrations resulted in an increased D-galacturonase activity. The activity of the enzyme was found to increase rapidly at considerably higher ethylene concentrations. Saltveit and McFeeters (1980) reported increased D-galacturonase activity in cucumber fruits after a

burst of ethylene production. D-galacturonase activity in harvested mature or immature cucumber fruits could be induced by exogenously applied ethylene. A close relationship was found to exist between D-galacturonase activity and the rise in respiration and ethylene production (Babbitt et al., 1973; Awad and Young, 1979). Babbitt et al. (1973) studied the effects of growth regulators on pectolytic enzymes and ripening of tomato fruits. Growth regulators markedly influenced the rate of the normal sequence of changes during ripening. Softening, color development, and D-galacturonase activity were accelerated by ethephon and delayed by gibberellic and indole acetic acids.

Paull and Chen (1983) studied the relationship between hydrolytic enzymes and ethylene production during the ripening of papaya fruits. D-galacturonase was not detectable in the preclimacteric stage but it increased during the climacteric rise and declined during the postclimacteric stage. The increase in D-galacturonase activity occurred simultaneously with increased ethylene production and the peak of D-galacturonase activity was correlated with maximum ethylene production. No D-galacturonase activity was detected prior to the rise in ethylene production indicating a possible role of ethylene in initiating D-galacturonase activity.

Emanations from ethylene-producing fruits could be effective in initiating hydrolytic enzymes activity in other fruits, not only in the same storage room, but even in the field. Shimokawa (1973) observed that watermelon tissue was macerated by emanations evolved by 'Prince' melons. The presence of ethylene in the emanations was

demonstrated by gas chromatography. Enzymic studies of macerated tissue revealed higher activities of pectinase as compared to healthy tissue not exposed to ethylene.

One of the recent tools used to demonstrate the crucial role of D-galacturonase in pectin degradation is isolated cell wall material (Gross and Wallner, 1979; Themmen et al., 1982). Cell wall preparations from green normal and mutant tomato fruits were equally degraded in vitro by a cell wall-bound protein extract from ripe normal tomatoes. Similar cell wall-bound protein extracted from mutant fruits was not effective in in vitro cell wall degradation because it lacked D-galacturonase. The fact that purified D-galacturonase was capable of in vitro degradation of cell wall material indicated that D-galacturonase was the major enzyme in cell wall-bound protein extracts responsible for wall degradation.

Controlled atmosphere storage has been shown to influence pectolytic activity remarkably. Goodenough et al. (1982) reported that the appearance of D-galacturonase and red pigmentation in mature green tomato fruits was prevented by storage conditions of 5% O₂, 5% CO₂, and 90% N₂. The authors speculated that the reduced respiration rate under these conditions might have supplied less ATP to the fruit biosynthetic systems resulting in the retardation of D-galacturonase synthesis. Upon removal of fruits to ambient conditions, D-galacturonase was synthesized and fruits changed color and ripened normally.

There are causes of cell wall modifications during ripening other than enzymic mechanisms. Huber (1984) reported on the roles of

polyuronides and hemicelluloses in strawberry fruit softening. He found that soluble polyuronides were unchanged during early development of strawberry fruits but increased markedly throughout ripening. Polyuronide solubility was not accompanied by molecular weight changes indicative of enzymic action. This observation was supported by the fact that D-galacturonase activity was not detected in strawberry fruits. The author suggested that increased levels of soluble polyuronides were due to the synthesis of more freely soluble forms during ripening. Hemicelluloses were also found to be altered, showing an increased proportion of low-molecular weight polymers during ripening.

Cellulase

The degradation of cell wall components other than the pectic substances may also contribute to softening. Considerable evidence indicates that changes in cellulose may occur during ripening in some fruit. Hall (1964) and Dickinson and McCollum (1964) were among the first workers to report on cellulase in fleshy fruits. They observed an increase in cellulase activity during tomato ripening and suggested that the enzyme might be involved in the softening process. A close relationship exists between cellulase activity, the climacteric rise in respiration, ethylene production, and fruit softening (Sobotka and Watada, 1971; Pesis et al., 1978; Awad and Young, 1979). In contrast, Paull and Chen (1983) detected significant cellulase activity in papaya fruit at harvest. Cellulase has been suggested to play a role during fruit growth (Hall, 1964). Cellulase facilitates cell

enlargement through breaking cellulose fibrils and allowing cell wall expansion.

Hinton and Pressey (1974) found low cellulase activity in immature peach fruits but it increased during ripening. The greatest increase in cellulase activity occurred before a significant change in fruit firmness. Since cellulase was observed to appear before fruit softening, it might be involved in the initiation of the processes leading to tissue softening and disintegration. The large quantity of cellulase found in avocado fruit mesocarp and the changes in its activity during ripening suggests that it plays a critical role in the ripening process (Awad and Young, 1979; Awad and Lewis, 1980). In avocado fruits, it appeared that the initial phase of softening was correlated with cellulase activity whereas D-galacturonase was involved in the later stages of ripening (Awad and Young, 1979). Sobotka and Watada (1971) reported a close association between increased cellulase activity and softening of ripening tomato fruits. Tomato lines with firmer fruit exhibited lower cellulase activity than those with softer fruit. In contrast to these findings, Hobson (1968) reported that cellulase activity was not correlated with fruit firmness. His data showed that cultivars with firmer fruit had higher cellulase activity than those with softer fruit. Likewise, Buescher and Tigchelaar (1975) compared cellulase activity in normal and mutant tomato fruits. Cellulase activity increased during ripening and softening in normal fruits, whereas in mutant fruits, cellulase activity increased during "ripening" yet the firmness of the fruit was not affected. The authors concluded that the lack of softening in

mutant fruits was associated with the lack of D-galacturonase activity.

The difference in opinion concerning the role of cellulase in fruit softening can be partly explained by the lack of detailed information regarding the different types of cellulases and their specific roles in fruit ripening. The difficulty in detecting cellulolytic enzymes in fruits is attributed to the insolubility of cellulose. Researchers have circumvented this problem by using carboxymethylcellulose (CMC), which is a soluble derivative of cellulose, as a substrate. The high viscosity of aqueous solutions of CMC makes it ideal for viscometric methods used for the determination of cellulase activity. Most of the cellulases reported in ripening fruits have been of the C_x or carboxymethylcellulase types, which alone have little capacity to degrade insoluble forms of cellulose (Huber, 1983b). Microorganisms have been reported to produce at least four different types of enzymes which have distinctly different roles in the degradation of cellulose (Li et al., 1965). Few reports have provided information on the multiple enzymic nature of the cellulase complex of higher plants (Lewis and Varner, 1970; Pharr and Dickinson, 1973; Sobotka and Stelzig, 1974; Pesis et al., 1978). Pharr and Dickinson (1973) extracted two enzymes from ripening tomatoes. One enzyme reduced the viscosity of CMC and generated reducing groups, oligosaccharides, and glucose. The other enzyme hydrolyzed cellobiose rapidly but the rate of cleavage decreased with increased substrate chain length. No evidence was found for the presence of exocellulase which attacked insoluble cellulose. Lewis and Varner (1970)

demonstrated the existence of two forms of C_x cellulase in abscission zones of aging bean explants. Awad and Lewis (1980) established the optimum conditions for extraction and purification of avocado cellulase. The enzyme was purified to a single protein with a molecular weight of 49,000. Studies of cellulase activity in peaches (Hinton and Pressey, 1974) revealed low activity in immature peaches, but it increased considerably during ripening. Peach cellulase degraded CMC and was therefore a C_x cellulase. Sobotka and Stelzig (1974) separated four enzyme-containing fractions by $(NH_4)_2SO_4$ fractionation from postbreaker tomatoes. Two enzymes were endocellulases that degraded both CMC and insoluble cellulose. The third enzyme was identified as a nonspecific β -glucosidase and the fourth was an $\text{exo-}\beta$ -1, 4-glucanase. Collectively, the enzymes were capable of completely degrading insoluble cellulose to short chain cellodextrins and glucose.

Cellulase is believed to be involved in cell wall breakdown in abscission zones in a wide range of plant material (Lewis and Varner, 1970; Lewis and Koehler, 1979; Sexton et al., 1980). It has also been demonstrated that ethylene treatment increases cellulase activity in abscission zones. Lewis and Koehler (1979) reported that ethylene treatment promoted leaf abscission and enhanced cellulase activity in kidney bean seedlings. Sexton et al. (1980) induced abscission of bean leaves by treating them with 50 ppm ethylene. Total cellulase in the abscission zones increased dramatically after 18 hours and the force necessary to break the abscission zone declined. Abscission

zones treated with cellulase antiserum failed to break, confirming the central role played by cellulase in the abscission process.

Ethylene has also been shown to induce cellulase activity in fleshy fruits. Ethylene treatment of mutant tomato fruits increased cellulase activity (Poovaiah and Nukaya, 1979). Pesis et al. (1978) reported that ethylene treatment of avocado fruits induced cellulase activity and the response increased with degree of maturation. The enzyme appeared to be an endocellulase, the activity of which seemed to be controlled by ethylene. Babbitt et al. (1973) studied the effects of growth regulators on cell wall hydrolases, respiration, and ripening of tomatoes. They found that growth regulators significantly influenced cellulase activity. Ethephon markedly accelerated cellulase activity whereas gibberellic and indole acetic acids delayed it. The authors suggested that softening was initiated by cellulolytic enzymes and that pectolytic enzymes were involved in subsequent changes in texture.

Ultrastructural Changes in Fruit Cells During Ripening

Fleshy fruits are usually composed of parenchymatous cells of very large size (150 to 700 μm dia.), with large air spaces between them. Ultrastructural studies of fruit cells during ripening have proven difficult due to their large size and fragile nature. The high water content of fruit cells dilutes the fixatives and hinders the penetration of embedding resins. The development of suitable fixation procedures to preserve the fine structure of the cell organelles and the improvement of the embedding resins for better penetration are

vital for minimization of artifacts in sectioning. Moreover, the choice of a suitable staining procedure to increase electron density and achieve sufficient contrast is equally important. The distribution of chemical components within a cell can be achieved by the development of appropriate specific stains. Albersheim and Killias (1963) reported that the treatment of onion root tip tissue with alkaline hydroxylamine prior to staining with ferric chloride increased electron density throughout the cell wall and particularly the region of the middle lamella. The distribution of iron indicated that pectin existed throughout the primary wall as well as in the middle lamella.

The ripening process of fruits had been considered to be a senescence phenomenon involving loss of membrane integrity and resulting in the breakdown of compartmentation and death (Bain and Mercer, 1964). However, later studies (Simpson et al., 1976) employing improved fixation procedures revealed no obvious degenerative changes in all non-plastid cytoplasmic structures in cells of ripe tomato fruits. Of all the cellular organelles, mitochondria retain their ultrastructure throughout the ripening period and until late in senescence.

Most of the research work on fruit ultrastructural changes during ripening was done on avocado. Pesis et al. (1978) studied cellulase activity and softening in avocado fruits and reported that the middle lamella of the cell wall of soft fruits was dissolute and sparse as compared to the orderly tight fibrils evident in hard fruits. The authors suggested that cellulase, in addition to the pectic enzymes, played an important role in avocado fruit softening.

Platt-Aloia and Thomson (1981) studied the mesocarp tissue of ripening avocado fruits using thin section electron microscopy. When ripening began, one of the most obvious changes was a loosening and eventual breakdown of the cell wall. In post-climacteric soft fruit, all of the organelles and membranes appeared whole and intact, but the cell wall had almost completely disappeared. Although ripening in avocados involved the degradation of the cell walls, it did not involve the loss of compartmentation.

Extensive ultrastructural studies were carried out at different ripening stages of avocado fruits (Platt-Aloia et al., 1980). Ultrastructural changes in the cell wall were correlated with changes in the activity of wall hydrolytic enzymes. Initial wall breakdown involved degradation of pectins in the matrix and in the middle lamella and corresponded with increased polygalacturonase activity. The loss of fibrillar components of the cell wall indicated a possible role for cellulase in fruit softening. No correlation existed between location of wall degradation and the presence of plasmodesmata.

Ben-Arie et al. (1979) followed the ultrastructural changes in the cell walls of ripening apple and pear fruits. Structural alterations in the cell wall of apple fruits became evident at advanced stages of softening, and involved dissolution of the middle lamella. Likewise, softening in pears was associated with the dissolution of the middle lamella, accompanied by a gradual disintegration of fibrillar material throughout the cell wall. Application of polygalacturonase and cellulase enzymes to tissue discs from firm pear fruit led to ultrastructural changes observed in

naturally ripening pears. In apple fruits, polygalacturonase alone was sufficient to dissolve the middle lamella of the cell wall. In contrast to the findings of Platt-Aloia et al. (1980), these workers found that the cell wall areas containing plasmodesmata maintained their structural integrity throughout the ripening process in both apple and pear fruits.

Electron microscopic studies on non-climacteric fruits did not show cell wall degradation. Ultrastructural studies on the epicarp of ripening oranges (Thomson, 1969) did not show any significant changes in the fine structure of the cells that could be correlated with ripening, except for the transformation of chloroplasts to chromoplasts.

Simpson et al. (1976) conducted comparative ultrastructural studies of fruits of mutant and normal tomatoes to determine whether possible ultrastructural differences could be related to the lack of normal ripening in the mutants. They did not note any unique differences between normal and mutant tomato fruits. However, recently Crookes and Grierson (1983) studied ultrastructural changes in the pericarp of normal tomato fruits during ripening. They found that changes in cytoplasmic ultrastructure were not consistent with the speculation that ripening was a senescence phenomenon. In ripe fruit, a high degree of ultrastructural organization of the mitochondria, chromoplasts, and rough endoplasmic reticulum was retained. The most striking changes were noted in the structure of the cell wall, which started with the dissolution of the middle lamella and ended with the eventual disruption of the primary cell wall. Changes in the cell

wall were correlated with the appearance of polygalacturonase isoenzymes. Those changes were duplicated by the application of purified tomato polygalacturonase isoenzymes to mature green fruit tissue.

Most work on fruit abscission has generally dealt with chemically-induced fruit drop. Few authors have reported on anatomical and ultrastructural studies of abscission-promoting regions of fruit (Nelson et al., 1984; Jerie, 1976; Reed and Hartmann, 1976). Nelson and co-workers (1984) reported that the application of the ethylene releasing compound, CGA 15281, enhanced the formation of abscissional zones in peach fruits. In treated fruits, the formation of abscissional zones preceded that in controls by 3 days. The development of discrete separation layers at the base of fruits was attributed to enzymic action resulting in cell wall degradation.

CHAPTER III
RESPIRATION AND ETHYLENE PRODUCTION IN WATERMELON
FRUIT EXPOSED TO ETHYLENE OR PROPYLENE

Introduction

Fruits differ greatly in their response to applied ethylene (Biale and Young, 1981). Treatment of climacteric fruits with ethylene promotes ripening as evidenced by the initiation of the respiratory climacteric and autocatalytic ethylene production. Once ripening is initiated, removal of exogenous ethylene does not affect the progress of ripening and the fruit is apparently insensitive to further ethylene applications (Wang and Hansen, 1970; Akamine and Goo, 1979; Solomos, 1983). The response with nonclimacteric fruit is variable but generally does not include ripening initiation. Ethylene applied to nonclimacteric fruit increases the respiration rate, and upon removal of the gas, respiration rate returns to basal levels (McGlasson et al., 1978; Rhodes, 1980a). Respiration in nonclimacteric fruit can be repeatedly stimulated by ethylene treatments administered in succession (Herner and Sink, 1973). Application of ethylene to nonclimacteric fruit has no effect on endogenous ethylene production (Burg and Burg, 1962; Rhodes, 1980b).

Propylene is an active analogue of ethylene and can also initiate fruit ripening. The equivalent concentration of propylene required to give a comparable response is 130 times that of ethylene (Burg and Burg, 1967). Propylene has been employed to demonstrate that

autocatalytic ethylene production by climacteric fruit can indeed occur in response to exogenously applied ethylene (McMurchie et al., 1972; Sfakiotakis and Dilley, 1973; Eaks, 1980; Brecht and Kader, 1984a, 1984b). Propylene facilitates accurate measurement of endogenous ethylene production since they are readily separated by gas chromatography (McGlasson, 1975; Eaks, 1980).

Microorganisms can significantly contribute to increased respiratory activity and ethylene production in decayed or otherwise wounded fruits (Eaks, 1955; Vines et al., 1965). High rates of CO_2 and ethylene evolution were reported for decayed lemons (Eaks, 1955), lemons and oranges (Vines et al., 1965), tomatoes (Barkai-Golan and Kopeliovitch, 1983), and avocados (Zauberman and Schiffman-Nadel, 1974). Microbial infection caused climacteric-like patterns of respiration and ethylene production in nonclimacteric fruits (Biale, 1948; Biale and Shepherd, 1941). Oslund and Davenport (1983) reported that lychee fruit, reportedly climacteric (Vines and Grierson, 1966), were nonclimacteric and showed enhanced CO_2 and ethylene only when infected by microorganisms.

On the basis of postharvest CO_2 and ethylene production, Mizuno and Pratt (1973) classified the watermelon as a climacteric fruit. Shimokawa (1973) reported that ethylene enhanced the respiration rate of watermelon fruit, but upon withdrawal of ethylene, respiration rate returned to control levels. He also reported that applied ethylene did not trigger autocatalytic ethylene production. Recently, Risse and Hatton (1982) showed that exposure of watermelon fruit to various concentrations of ethylene accelerated tissue deterioration.

Placental tissue in ethylene-treated fruits was soft, watersoaked, and unacceptable in taste. Ethylene treatments had an adverse effect on preripe fruits as well. Shimokawa (1973) also observed that watermelon tissue was macerated by exogenously applied ethylene. It is evident from numerous studies that ethylene treatment of fruit exhibiting an orthodox climacteric ripening pattern does not induce adverse effects beyond those normally observed during the latter stages of ripening. The more general response is ripening initiation (McGlasson et al., 1978; Rhodes, 1980b; Biale and Young, 1981). The data considered together present an unsolved dilemma. The objective of this study is to determine the postharvest respiratory and ethylene production characteristics in watermelon fruit in response to exogenously applied ethylene or propylene.

Materials and Methods

Plant Material

'Charleston Gray' watermelons [*Citrullus lanatus* (Thunb.) Matsum and Nakai] were grown at the Horticultural Unit near Gainesville, Florida. Fruits were harvested immature (white flesh), preripe (pink), and ripe (red) in June-July, 1983. During the 1984 season, watermelon fruits were harvested at the ripe stage only. Criteria used as harvesting indices included yellowing of the ground spot, withering of the tendril adjacent to the stem, and the audible response of tapping the fruit with knuckles. Some fruits were sacrificed to verify uniformity of development. Fruits in all treatments were washed with sodium hypochlorite solution (150 ppm) and

then dipped for 30 sec. in an aqueous suspension of Botran (2,6-dichloro-4-nitroaniline, Upjohn) at the rate of 0.8 g/liter for postharvest decay control. Individual fruits were placed in air-tight 24 liter plastic containers at 18°C and ventilated with a constant flow of air or the desired gas mixture. At appropriate intervals during the experiments, watermelon samples were cut longitudinally and rind and placental tissue characteristics noted. The firmness of the rind and the placental tissue was determined using an Instron universal testing instrument (Model 1132, Instron Corporation, 100 Royall Street, Canton, Mass. 02021). Instron probe size was 1 cm in diameter, and tissue sample size was 5 x 5 x 2 cm.

Gas Treatments

Endogenous ethylene production by ethylene-treated fruit could not be determined separately because it could not be differentiated from that used in the treatment. Therefore, propylene, which is an active analogue of ethylene, was employed to trigger endogenous ethylene production (if any) by watermelon fruit.

Air, air and ethylene, or air and propylene combinations were mixed at the desired concentrations by means of a flow through system equipped with flow meters and regulating valves (Gull, 1981). A constant flow rate of the different gas treatments was maintained at 12 liters.hr⁻¹.container⁻¹. Ethylene or propylene gas mixtures were administered to the individual fruits at concentrations of 50 and 6500 1.liter⁻¹, respectively. Relative humidity ranged between 80% and 90%. For ethylene and CO₂ measurements, the containers were sealed

for 4 hrs. and gas samples withdrawn through rubber septa using gastight syringes. Levels of ethylene and propylene were determined with a gas chromatograph equipped with an activated alumina column and flame ionization detector. Levels of CO_2 were determined with an infrared gas analyzer (Beckman 215 A).

Results

Effect of Ethylene Treatment and Stage of Maturation on Respiration

The respiratory patterns of immature, preripe, and ripe watermelon fruit are shown in Fig. 3-1 (A-C). Immature fruit (A) exhibited higher respiratory rates as compared to fruit from the other two maturation stages (B and C) which exhibited comparable respiration rates and respiratory drifts which decreased during storage. Ethylene induced a climacteric-like rise in the respiration rates of fruits from all maturation stages (Fig. 3-1, A-C). Respiration rates of ethylene-treated watermelon fruit from all maturation stages decreased during storage and then within several days increased again. This increase was associated with ethylene-induced tissue damage and subsequent pathogen proliferation. Respiration rates of untreated fruit slowly declined with time. Untreated fruit did not produce detectable amounts of ethylene (Fig. 3-1, A-C). When ethylene-treated fruits of all maturation stages were examined internally, they appeared leaky and soft (Table 3-1), and had off odors and an unacceptable taste. Ethylene-induced breakdown of watermelon fruit tissue was visible on the third day of treatment, and on the sixth day the rind became very soft (Table 3-1) and decay symptoms appeared.

Fig. 3-1. Respiration and ethylene production by harvested 'Charleston Gray' watermelon fruit. Fruit were harvested and treated with air ($\circ\Delta$) or air + 50 ppm ethylene ($\bullet$) as described in Materials and Methods. ($\bullet\circ$), respiration (CO_2 , $\text{kg}^{-1}\cdot\text{hr}^{-1}$). (Δ), ethylene production ($\mu\text{l}\cdot\text{kg}^{-1}\cdot\text{hr}^{-1}$). Watermelons harvested immature (A), preripe (B), and ripe (C). Vertical bars represent standard error of the mean of five replications.

Table 3-1. Firmness of ripe watermelon fruit exposed to 0 or 50 ppm ethylene during 0, 3, 6, and 9 days' storage at 18°C.^z

Days	Treated		Control	
	Rind	Center	Rind	Center
Firmness (Newton)				
0	144.0 ± 4.7	6.3 ± 0.6	144.9 ± 5.8	7.0 ± 0.7
3	127.2 ± 8.4	5.8 ± 0.2	158.1 ± 2.1	9.3 ± 0.9
6	123.2 ± 3.1	3.1 ± 0.5	156.6 ± 8.3	9.7 ± 0.9
9	78.7 ± 5.5	1.1 ± 0.1	150.3 ± 5.1	6.7 ± 0.5

^zEach value is the mean of 5 replications plus or minus the standard error of the mean.

The respiratory response of ripe watermelon fruit to intermittent ethylene treatment is shown in Fig. 3-2. Ethylene twice stimulated the respiration of fruit, and upon removal of the gas, respiration rate returned to control levels.

Effect of Propylene Treatment on Respiration and Ethylene Production

Propylene treatment of ripe watermelon fruit stimulated respiration in a manner similar to that caused by ethylene (Fig. 3-3). Respiration rate of propylene-treated watermelon fruit declined close to control level upon removal of the gas and then increased steadily after the 10th day. Respiration rate of untreated fruit slowly declined and was typical of trends observed in other experiments (Fig. 3-1). Propylene treatment at $6500 \mu\text{l.liter}^{-1}$ did not trigger ethylene production during the first 10 days of treatment. The rise in ethylene production observed after the 10th day of propylene treatment was always accompanied by the widespread appearance of decay on the surface of fruits. This rise in ethylene production during fruit decay was also accompanied by a rise in CO_2 production. The bacterium Erwinia herbicola and the fungus species Paecilomyces (identified by J. A. Bartz, Dept. of Plant Pathology, Univ. of Florida) were recovered from decayed watermelon fruit.

Discussion

Ethylene induced a climacteric-like rise in the respiration rate of watermelon fruit of all maturation stages (Fig. 3-1, A-C). Since immature (white) and preripe (pink) fruit also exhibited this response

Fig. 3-2. Respiration of harvested 'Charleston Gray' watermelon fruits in response to exogenous ethylene. Fruit were treated with air (o) or air + intermittent 50 ppm ethylene (●) as described in Materials and Methods. Vertical bars represent standard error of the mean of five replications. Arrows indicate when ethylene was applied or withdrawn.

Fig. 3-3. Respiration and ethylene production by harvested 'Charleston Gray' watermelon fruit in response to propylene. Fruit were treated with air ($\circ\Delta$) or air + 6500 ppm propylene ($\bullet\Delta$). ($\circ\circ$), respiration ($\text{CO}_2 \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$). ($\Delta\Delta$), ethylene production ($\mu\text{l} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$). Vertical bars represent standard error of the mean of five replications. Arrows indicate when propylene was applied or withdrawn.

yet did not ripen during the treatment period, the increase in CO_2 production observed did not represent a climacteric rise in respiration. This is consistent with the findings of Vines et al. (1965) and Herner and Sink (1973) who reported that exposure of oranges and mutant tomato fruits, respectively, to ethylene resulted in increased respiration rates without enhancing ripening.

It has long been known that ethylene treatment of preripe climacteric fruit will shorten the period of time required for the induction of the climacteric (Rhodes, 1970; McGlasson et al., 1978). In this study, ethylene did not induce the respiratory climacteric rise and ripening in watermelon fruit at any of the maturation stages. The rise in the respiration rate of ethylene-treated fruit which started around the 8th day of ethylene treatment was solely attributed to decay of the fruit. Chlorination and fungicide treatments (disinfection) delayed but did not prevent microbial infection in ethylene-treated watermelon fruit. Microorganisms isolated from decayed fruit were identified as the bacterium Erwinia herbicola and the fungus, Paecilomyces. In untreated fruit, however, disinfection prevented microbial infection throughout the duration of the experiment. It appeared that ethylene treatment significantly reduced the resistance of watermelon fruit to microbial attack. The fruits were discarded on the 12th day of the ethylene treatment because of advanced symptoms of decay. The respiration rates of untreated fruit of all maturation stages slowly declined with time, typical of nonclimacteric respiratory drifts (Fig. 3-1, A-C). Untreated preripe (pink) fruit did not continue ripening during storage. Also,

untreated fruit of all stages of maturation did not produce detectable amounts of ethylene. These results support the conclusion that watermelon fruit is nonclimacteric.

Upon internal examination of ethylene-treated fruits, they appeared leaky and soft (Table 3-1), and developed unpleasant odors and an unacceptable flavor. These results are consistent with those reported by Shimokawa (1973) and Risse and Hatton (1982) and provide more evidence in favor of the nonclimacteric nature of the fruit.

Ethylene stimulates respiration of nonclimacteric fruit and upon its removal, the respiration rate declines to its value before treatment (McGlasson et al., 1978). Also, respiration of nonclimacteric fruit can be repeatedly stimulated by successive ethylene treatments (Vines et al., 1965; Rhodes, 1980a). Respiration rate of watermelon fruit was stimulated by two successive ethylene treatments (Fig. 3-2) and upon removal of the gas, respiration rate returned close to control level. These results are typical of nonclimacteric fruit and concur with the findings of Herner and Sink (1973) and Shimokawa (1973).

Propylene treatment at $6500 \mu\text{l.liter}^{-1}$ stimulated respiration in watermelon fruit in a manner similar to that caused by ethylene, but did not trigger ethylene production during the first 10 days of treatment (Fig. 5-3). McMurchie et al. (1972) reported increased respiration rates but not ethylene production in lemons and oranges treated with propylene. After prolonged exposure, propylene-treated fruit developed decay symptoms characteristic of those observed with

ethylene treatment. The rise in ethylene production observed after the 10th day of propylene treatment was associated with decay.

The effect of pathological infection on respiration and ethylene production in the watermelon fruit is similar to that reported by other workers for various pathogenic infections of nonclimacteric fruits (Vines et al. 1965; Zauberman and Barkai-Golan, 1975). Barkai-Golan and Kopeliovitch (1983) reported that Rhizopus infection stimulated both ethylene and CO_2 production and induced the development of climacteric-like respiratory patterns in tomato mutant fruit (rin). Studies on respiration and ethylene production in carambola fruit (Oslund and Davenport, 1983) revealed that the high levels of CO_2 and ethylene produced by the fruits were mainly attributable to fruit decay. Similarly, in this study, the rise in ethylene produced by propylene-treated watermelon fruit was a direct consequence of microbial activity and was not related to autocatalytic ethylene production in response to propylene. Untreated fruits were decay-free and produced no detectable amounts of ethylene throughout the duration of the experiment.

McMurchie et al. (1972) reported that propylene stimulated a typical respiratory climacteric and a rise in ethylene production in bananas. In contrast, lemons and oranges (nonclimacteric fruits) showed only a climacteric-like rise in respiration in response to propylene. Judging by the similarity of the responses of watermelon and citrus fruit to propylene, and from the evidence presented in this context, we conclude that watermelon fruit is nonclimacteric.

CHAPTER IV
ELECTROLYTE LEAKAGE, FIRMNESS, AND SCANNING ELECTRON
MICROSCOPIC STUDIES OF WATERMELON FRUIT TREATED WITH ETHYLENE

Introduction

Exposure of various plant tissues to ethylene has been reported to cause an increase in membrane permeability (Hanson and Kende, 1975; Kende and Hanson, 1976; Suttle and Kende, 1978, 1980; Ferguson and Watkins, 1981). Leakage of electrolytes, sugars, or pigments has been commonly used as an index of membrane permeability in plant tissues (Sacher, 1973; Borochoy and Faragher, 1983). Kende and Baumgartner (1974) and Hanson and Kende (1975) found that ethylene induced senescence and enhanced efflux of solutes from the cells of morning glory petal tissue. Suttle and Kende (1978, 1980) similarly demonstrated that ethylene hastened the onset as well as the rate of leakage of anthocyanin and electrolytes from isolated petals of Tradescantia. A close relationship was reported between increased membrane permeability and ethylene production in apple fruit (Berard and Loughheed, 1982).

Electrolyte leakage from fruit tissues in particular depends upon factors other than membrane permeability. As fruits mature and ripen, they accumulate sugars and acids leading to a high osmotic potential, and at the same time their cell walls are weakened by hydrolytic action and/or other means including calcium fluxes (Simon, 1977a). Incubation of fruit tissues in hypotonic media such as distilled

water or weak buffers may lead to membrane rupture and therefore the potential for overestimation of membrane permeability (Sacher, 1973; Wade et al., 1980).

Calcium is involved in numerous physiological processes in plants. Calcium binds to pectins resulting in stabilization and maintenance of cell wall integrity (Ferguson, 1984; Demarty et al., 1984). Sams and Conway (1984) reported a negative relationship between soluble polyuronide content and Ca concentration in apple fruit. The role of Ca in membrane structure and function stems from its ability to stabilize membranes (Paliyath et al., 1984). Poovaiah (1979) concluded that Ca suppressed changes associated with senescence in tomato fruit through its function in maintaining cell wall structure and membrane integrity. Lieberman and Wang (1982) reported that electrolyte leakage from apple fruit tissue discs was significantly reduced when incubated in a solution containing 100 mM Ca and Mg.

It has been reported that watermelon fruits are adversely affected by exposure to ethylene (Shimokawa, 1973; Risse and Hatton, 1982). Placental tissue of ethylene-treated fruit appear macerated and watersoaked. We have observed that watermelon fruit allowed to overripen (either attached or postharvest) do not show any symptoms of watersoaking (Chapter III). Furthermore, small underdeveloped fruit become watersoaked in response to ethylene. There would thus seem to be a close relationship between ethylene and the disorder, independent of ripening or fruit maturity. Shimokawa (1973) reported that pectinase increased in watermelon fruit exposed to ethylene and this may well contribute to the disorder. However, many fruit types

contain D-galacturonase but do not exhibit watersoaking until perhaps the terminal stages of senescence, long after the period of maximum ethylene production. The objectives of this research are to study the effects of ethylene on electrolyte leakage, firmness, and ultrastructure in watermelon fruit.

Materials and Methods

Plant Material

'Charleston Gray' watermelons [Citrullus lanatus (Thunb) Matsum and Nakai] were grown at the Horticultural Research Farm near Gainesville, Florida. Medium sized (7 to 9 kg) fruits were harvested at the ripe stage. Criteria used as harvesting indices included yellowing of the ground spot, withering of the tendril adjacent to the stem, and the audible response of tapping the fruit with the knuckles. Some melons were sacrificed to verify uniformity of development. Fruits were washed with sodium hypochlorite (150 ppm) and then dipped for 30 seconds in an aqueous suspension of Botran (2,6-dichloro-4-nitroaniline, Upjohn) at the rate of 0.8 g liter⁻¹ for postharvest decay control.

Gas Treatments

Individual fruits were placed in air-tight 24 liter plastic containers at 18°C and ventilated with a constant flow of air or a mixture of air and ethylene. Air and ethylene (50 ppm) combinations were mixed by means of a flow through system and delivered to the fruit at a flow rate of 12 liters hr⁻¹ container⁻¹. Relative humidity

was between 80% and 90%. Fruit were treated with 0 or 50 ppm ethylene for 0, 3, 6, or 9 days.

Electrolyte Leakage

From each treatment, three watermelon fruits were cut longitudinally and visually inspected for symptoms of ethylene damage (watersoaking). Tissue plugs (1.0 cm thick and 1.8 cm in diameter) were prepared from the central part of the placental tissue using a sterilized cork borer. The plugs were briefly rinsed with distilled water and then placed in 10 ml of either distilled water, 0.4 M mannitol, or 0.4 M mannitol containing 2.5 mM CaCl_2 , and shaken gently throughout the experiment. Electrolyte content of the bathing solutions was measured at hourly intervals using a conductivity bridge (YSI model 31, Yellow Springs Instruments Co., Inc., Yellow Springs, Ohio 45387). Firmness of both the rind and the placental tissue was determined using an Instron universal testing instrument (model 1132, Instron Corporation, 100 Royall Street, Canton, Massachusetts 02021).

Scanning Electron Microscopy

Sections of placental tissue (2 x 4 mm) were fixed for 2 hrs in half-strength Karnovsky's fixative (Karnovsky, 1965) at 22°C. The samples were then transferred to 1% OsO_4 (buffered in 0.2 M sodium cacodylate buffer, pH 7.2) for 2 hrs at 22°C, and then washed three times with distilled water. The samples were dehydrated in a standard ethanol series, critical point dried (Balzers Union CPD 010), and sputter-coated with gold (Giko IB-2 Ion Coater, Giko Engineering

Co., Japan). The samples were viewed in the scanning electron microscope (Hitachi S-450).

Results

The effects of storage duration and 50 ppm ethylene treatment on firmness is presented in Table 4-1. Firmness of the rind and the placental tissue decreased with increased duration of exposure to ethylene. However, firmness of untreated tissue remained unchanged throughout the duration of the experiment. Table 4-2 shows the values for electrolyte leakage rates calculated over the first 2 hrs of incubation. Initial rates of leakage increased markedly with increased duration of exposure to ethylene. Rate of leakage was highest in tissues incubated in distilled water, followed by 0.4 M mannitol and least in 0.4 M mannitol containing 2.5 mM CaCl_2 . Preliminary studies showed that 0.4 M mannitol was isotonic with ripe watermelon fruit tissue. The effects of ethylene treatment and duration (averaged over incubation media) on electrolyte leakage is shown in Fig. 4-1 (A). Leakage from all tissues increased with time of incubation. Exposure of watermelon fruit to ethylene for 6 and 9 days resulted in significantly higher electrolyte leakage as compared to the untreated controls. The effect of incubation medium on electrolyte leakage is shown in Fig. 4-1 (B-D). Leakage in all incubation media followed a trend similar to that observed in Fig. 4-1 (A) and described in Table 4-2. Electrolyte leakage in 0.4 M mannitol and 0.4 M mannitol containing 2.5 mM CaCl_2 was 65.3 and 38.5%, respectively, of leakage in distilled water (Fig. 4-2). Total leakage values are shown in Table 4-3. Leakage values with respect to

Table 4-1. Firmness of ripe watermelon fruit exposed to 0 or 50 $\mu\text{l liter}^{-1}$ ethylene and stored at 18°C.²

Storage period (days)	C_2H_4 treatment	Firmness	
		Rind	Center
		————— (Newton) —————	
0	---	150.1 $\pm$ 3.5	6.4 $\pm$ 0.3
3	-	132.2 $\pm$ 2.5	6.5 $\pm$ 0.2
	+	107.5 $\pm$ 2.5	5.1 $\pm$ 0.2
6	-	130.3 $\pm$ 2.1	7.0 $\pm$ 0.3
	+	86.4 $\pm$ 7.9	3.7 $\pm$ 0.3
9	-	135.0 $\pm$ 3.6	6.8 $\pm$ 0.1
	+	64.4 $\pm$ 4.3	1.0 $\pm$ 0.1

²Each value is the mean of five replications plus or minus the standard error of the mean.

Table 4-2. Rates of electrolyte leakage from watermelon tissue discs prepared from fruit exposed to 0 or 50 $\mu\text{l liter}^{-1}$ ethylene and stored at 18°C.²

Days	Ethylene treatment	Conductance		
		H ₂ O	Mannitol	Mannitol + CaCl ₂
		(mhos)		
0	---	90	18	10
3	-	93	38	13
	+	98	45	15
6	-	105	15	10
	+	163	90	57
9	-	150	55	25
	+	175	120	58

²Values represent the mean initial change in conductance during the first two hrs of incubation.

Fig. 4-1. Electrolyte leakage from the placental tissue of 'Charleston Gray' watermelon fruit. Fruit were treated with air (O_2) or air + 50 ppm ethylene (C_2H_4) as described in Materials and Methods. Durations of treatment were: 0 (○), 3 (◊), 6 (□), and 9 (Δ) days. A, leakage averaged over incubation media; B, tissues incubated in distilled water; C, 0.4 M mannitol; and D, 0.4 M mannitol + 2.5 mM CaCl_2 . Vertical bars represent standard error of the mean of six replications, and when absent, fall under the symbol.

Fig. 4-1.--Continued.

Fig. 4-1.--Continued.

Fig. 4-1.--Continued.

Fig. 4-2. Effect of incubation media on electrolyte leakage from the placental tissue of 'Charleston Gray' watermelon fruit. Fruit were treated as described in Materials and Methods and incubated in distilled water (O), 0.4 mannitol (□), or 0.4 mannitol + 2.5 mM CaCl₂ (Δ). Vertical bars represent standard error of the mean of six replications.

Table 4-3. Total electrolyte leakage values for watermelon tissue discs prepared from fruit exposed to 0 or 50 $\mu\text{l liter}^{-1}$ ethylene and stored at 18°C.²

Days	Ethylene treatment	Conductance		
		H ₂ O	0.4 M Mannitol	0.4 M Mannitol+CaCl ₂
(mhos)				
0	---	496±17	237±21	164±18
3	-	518±40	402±33	175±15
	+	565±19	429±26	345±34
6	-	599±35	168±21	152±14
	+	732±33	551±10	405± 5
9	-	627±33	317±20	185±14
	+	768±35	635± 8	350±31

²Each value is the mean of four replications plus or minus the standard error.

ethylene treatment and incubation media followed trends described earlier (Fig. 4-1, B-D; Table 4-2).

Scanning electron micrographs of placental tissue from fruit exposed to 0 or 50 ppm ethylene for 0, 3, 6, and 9 days are shown in Fig. 4-3. Cell walls of tissues not exposed to ethylene [Fig. 4-3 (A, B, D, F)] appeared upright, rigid, and the cells were well defined. In contrast, cell walls of tissues exposed to ethylene for 3 days exhibited signs of structural weakening and were slightly collapsed [Fig. 4-3 (C)]. Longer exposures to ethylene resulted in essentially complete cellular collapse [Fig. 4-3 (E and G)].

Discussion

The results of these experiments clearly demonstrate that one consequence of exposure of watermelon fruit to ethylene is altered membrane permeability, as indicated by the significant increases in the rate and magnitude of electrolyte leakage. Leakage increased progressively with increased time of exposure to ethylene [Fig. 4-1 (A)]. This enhanced leakage in response to ethylene, particularly in isotonic bathing solution [Fig. 4-1 (C)], suggests an ethylene-induced increase in membrane permeability. These results are consistent with the findings of Hanson and Kende (1975) and Suttle and Kende (1978, 1980) for morning glory and Tradescantia flowers.

Extensive leakage of electrolytes occurred from tissues incubated in distilled water [Fig. 4-1 (B) and Fig. 4-2]. Watermelon fruits have particularly large cells with thin primary walls and relatively high solute contents, which results in high osmotic potential. The (abnormally) high rate of electrolyte loss from tissues incubated in

Fig. 4-3. Scanning electron micrographs of placental tissue from ethylene treated or untreated watermelon fruit. Tissue from freshly-harvested fruit (A), stored for 3 days (B), ethylene treated for 3 days (C), stored for 6 days (D), ethylene treated for 6 days (E), stored for 9 days (F), and ethylene treated for 9 days (G). Magnification was 80X for all plates.

(A)

(B)

Fig. 4-3.--Continued.

(C)

Fig. 4-3.—Continued.

(D)

Fig. 4-3.--Continued.

(E)

Fig. 4-3.--Continued.

(F)

Fig. 4-3.--Continued.

(G)

Fig. 4-3.--Continued.

distilled water was undoubtedly a consequence of cell rupture. Simon (1977b) reported that when apple fruit tissue was incubated in water, 90% of its electrolytes leaked out in 2 hrs, whereas leakage was greatly reduced when tissues were incubated in 1 M glycerol or 0.5 M KCl. The author suggested that apple fruit cells burst in water but not in isotonic media. In this study, when tissues were incubated in 0.4 M mannitol (isotonic), leakage from untreated tissue still occurred but was significantly reduced [Fig. 4-1 (C)].

Leakage was further reduced in all treatments, including the controls when tissues were incubated in 0.4 M mannitol containing 2.5 mM CaCl_2 [Fig. 4-1 (D)]. This was most likely due to the capacity of Ca to stabilize the cell wall and membrane. The cross-linking role of Ca is crucial for stabilization and maintenance of cell wall integrity (Demarty et al., 1984). The ability of Ca to reduce leakage is probably a direct result of tighter packing of membrane lipids made possible by its binding to negatively charged phospholipids (Ferguson, 1984). Poovaiah (1979) demonstrated that Ca suppressed the loss in membrane permeability during tomato fruit senescence. Leakage from apple fruit tissue (Lieberman and Wang, 1982) was significantly lower when the bathing solution contained 100 mM Ca and Mg.

Ultrastructural studies showed that placental tissue of watermelon fruit consisted of large ($\geq 0.5\text{mm}$) parenchyma cells with thin walls. Untreated tissue from all storage durations had well-defined cells with intact, well-preserved cell wall [Fig. 4-3 (A, B, D, and F)]. Although tissues of fruit exposed to ethylene for 3 days showed obvious signs of deterioration, [Fig. 4-3 (C)], however, their electrolyte leakage values did not differ significantly from those of

the control [Fig. 4-1 (A, B, and C)]. The data suggest that ethylene exerts its effect first on the cell wall and then on the membrane system. Cell walls of fruit exposed to ethylene for 6 and 9 days [Fig. 4-3 (E and G)] appeared totally collapsed. This collapse was closely paralleled by a progressive decrease in firmness of fruit as the duration of exposure to ethylene increased (Table 4-1). These results collectively indicate an ethylene-induced weakening and degradation of the cell wall. A close relationship exists between increased electrolyte leakage, reduced tissue firmness, and collapsed cell walls of ethylene-treated tissue. It appears from these results that the increase in electrolyte leakage and reduction in firmness of fruit exposed to ethylene is a consequence of a deteriorative influence of ethylene on both the cell wall and the membranes. Further experiments should be focused towards elucidating the effects of ethylene on the activity of cell wall hydrolytic enzymes and the magnitude of wall degradation.

CHAPTER V
CELL WALL HYDROLASES AND ULTRASTRUCTURE OF
WATERMELON FRUIT AS INFLUENCED BY ETHYLENE

Introduction

Softening of fruits during ripening is mainly attributed to enzymic hydrolysis of cell wall polysaccharides, largely the pectins (Pressey, 1977; Huber, 1983b). Although a number of hydrolases have been implicated in softening, the D-galacturonases (endo-D-galacturonases, E.C. 3.2.1.15, and possibly exo-D-galacturonases, E.C. 3.2.1.67) are believed to be of major importance (Huber, 1983b).

Numerous reports have indicated a role of ethylene in enhancing the appearance of D-galacturonase activity in ripening fruit. Sawamura et al. (1978) reported that exposure of tomato fruit to ethylene resulted in an increase in the activity of D-galacturonase. A close relationship was found to exist between D-galacturonase and ethylene evolution in tomato (Babbitt et al., 1973) and avocado fruit (Awad and Young, 1979). Paull and Chen (1983) showed that D-galacturonase activity was not detected in papaya fruit prior to the rise in ethylene production. They concluded that the development of D-galacturonase activity was initiated by ethylene.

There is some evidence, mostly indirect, that C_x -cellulase (E.C. 3.2.1.4) participates in fruit softening (Hall, 1964; Awad and Young, 1979). Even so, to date there have been no conclusive reports that C_x -cellulase hydrolyses native cell wall cellulose. Ethylene has been

shown to induce C_x -cellulase activity in fleshy fruits (Babbitt et al., 1973; Pesis et al., 1978) and abscission zones (Lewis and Koehler, 1979). Poovaiah and Nukaya (1979) reported that exposure of mutant tomato fruit (rin) to ethylene for 2 days resulted in increased C_x -cellulase activity.

Ultrastructural studies have provided supporting evidence that changes occur in the cell wall during softening (Simpson et al., 1976; Pesis et al., 1978; Ben-Arie et al., 1979; Platt-Aloia and Thomson, 1981). These studies reveal a progressive breakdown and dissolution of the middle lamella and in some cases, a gradual separation of wall fibrils. Initial degradation of the middle lamella is generally correlated with increased D-galacturonase activity (Platt-Aloia et al., 1980; Crookes and Grierson, 1983). This view is supported by the results of Ben-Arie et al. (1979), who duplicated naturally occurring ultrastructural changes in soft apple fruit by exogenous application of D-galacturonase to firm fruit. Another example of the potential role of D-galacturonase in fruit softening may be found in the watermelon fruit. It has been reported that watermelons are adversely affected by exposure to ethylene (Risse and Hatton, 1982). Placental tissue of ethylene-treated fruit became soft and watersoaked. Similarly, Shimokawa (1973) observed that watermelon fruit tissue was macerated by exposure to ethylene. The author reported higher activity of pectinase in ethylene-treated, macerated tissue. We have observed that the ethylene-induced watersoaking syndrome bears little relationship to natural ripening in watermelon fruit. This observation raises questions regarding the precise role of ethylene and wall hydrolases, particularly D-galacturonase, in natural ripening

of watermelon fruit. The objectives of these studies were to compare, ultrastructurally and biochemically, changes occurring during both ethylene-induced breakdown and natural ripening in watermelon fruit.

Materials and Methods

Plant Material

Fruit of 'Charleston Gray' watermelon [*Citrullus lanatus* (Thunb) Matsum and Nakai] were harvested at the ripe stage from plants grown at the Horticultural Research Farm near Gainesville, Florida. Criteria used as harvesting indices included yellowing of the ground spot, withering of the tendril adjacent to the stem, and the audible response of tapping the fruit with knuckles. Some melons were sacrificed to verify uniformity of development. Fruit were disinfected with a solution of sodium hypochlorite (150 ppm) and then dipped for 30 seconds in an aqueous suspension of Botran (2,6-dichloro-4-nitroaniline, Upjohn) at the rate of 0.8 g liter⁻¹ for postharvest decay control.

Ethylene Treatment

Individual fruits were placed in air-tight 24-liter plastic containers at 18°C and ventilated with the desired gas mixture. Air or air and ethylene (50 ppm) combinations were mixed by means of a flow-through system and administered to the containers at a flow rate of 12 liters container⁻¹ hr⁻¹. Relative humidity ranged between 80% and 90%.

Fruit from all treatments were cut longitudinally at 0, 1, 2, 3, 6, and 9 days and visually examined for ethylene damage. Placental tissue samples were collected at the previously mentioned sampling periods and stored in polyethylene bags at -20°C . Samples were also collected from fruit harvested overripe and from ripe fruit stored for 6 months at 15°C .

Preparation of Ethanol Insoluble Solids

Partially-thawed placental tissue (100 g) was homogenized in 400 ml of 100% ethanol for 2 min in a Counter Craft blender. The homogenate was refluxed in a boiling water bath for 20 min to inactivate endogenous enzymes and then stored overnight at -20°C . The suspension was filtered through Miracloth (Biochemical Corp., La Jolla, CA) and washed with 500 ml of 80% acetone followed by 1 liter of 100% acetone. The powder was squeezed to remove excess acetone and air dried at 24°C .

Determination of Total Pectins

Total pectins were measured in the manner described by Ahmed and Labavitch (1977) for cell walls. Samples of 8 mg of alcohol insoluble solids were placed in 30-ml beakers in an ice bath. To these samples, 2.5 ml of chilled concentrated H_2SO_4 were added with continuous stirring. Aliquots of distilled water (0.8 ml) were added twice, each followed by a 5 min interval. After a final 5 min period, 21 ml of distilled water were added. Acid sugar content in the hydrolysates was determined using the procedure described by Blumenkrantz and Asboe-Hansen (1973).

Gel-Filtration Chromatography

Gel chromatography of pectins was carried out according to the procedure described by Huber (1983a). Alcohol insoluble solids (20 mg) were incubated in 2.5 ml of Na-acetate buffer (50 mM, pH 5.0) containing 5 mM Na_2EDTA and incubated at 23°C with stirring, for 6 hr. The suspension was filtered through Miracloth and washed with 1.5 ml of the extraction buffer. The filtrate was centrifuged in a table top clinical centrifuge for 5 to 10 min at maximum speed. The supernatant was filtered through glass filter paper using a light vacuum, and used for gel chromatography.

Gel filtration of pectins was performed on a bed (60 cm high, 1.5 cm wide) of Ultrogel ACA 34 (LKB) packed in Na-acetate buffer (50 mM, pH 5.0) containing 5mM Na_2EDTA and 200 mM NaCl. Approximately 3 mg of pectin in a volume of 2.5 ml buffer were applied to the column and gravity eluted with the acetate-EDTA buffer. Fractions of 2 ml were collected at a flow rate of $10 \text{ ml cm}^{-2} \text{ hr}^{-1}$ and 0.5 ml aliquots of these used for the determination of acid sugars (A_{520}) using the procedure of Blumenkrantz and Asboe-Hansen (1973).

Preparation and Assay of C_x-Cellulase and D-Galacturonase

Partially-thawed placental tissue (100 g) and 6.0 g NaCl were homogenized for 2 min using a Counter Craft blender and the homogenate stored overnight (generally 10 hrs) at 1°C. The homogenate was centrifuged for 20 min at 12,000 g and the supernatant brought to 80% saturation with solid $(\text{NH}_4)_2 \text{SO}_4$. Following overnight storage at 1°C, the salt-saturated supernatant was centrifuged for 20 min at 15,000 g.

The pellet was resuspended in 10 ml of Na-acetate buffer (60 mM, 100 mM NaCl, pH 5.0) and dialysed overnight against 10 mM Na-acetate buffer containing 100 mM NaCl. The enzyme extract was filtered through Miracloth and used for the determination of C_x -cellulase and D-galacturonase activities.

The assay mixture for the determination of C_x -cellulase activity consisted of 0.5 ml (active or boiled) enzyme extract and 3 ml of 1% (w/v) carboxymethylcellulose (CMC) (Hercules Powder Co., type S-710) in Na-acetate buffer (30 mM, pH 5.0) containing 100 mM NaCl and 0.02% Thimerosal. Relative viscosity was calculated from the change in drainage time of the mixture through a calibrated upper portion of a 1.0 ml pipette at 24°C. Total protein content was determined according to the procedure described by Bradford (1976). Bovine serum albumin was used as the protein standard. C_x -cellulase activity was expressed as percent change in viscosity . mg protein⁻¹.hr⁻¹ calculated from readings made over a 6.5-hr period.

Reaction mixtures for the determination of D-galacturonase activity consisted of 0.1 ml of the enzyme extract (active or boiled) and 0.5 ml of 0.1% (w/v) polygalacturonic acid (Sigma) in Na-acetate buffer (30 mM, pH 5.0) containing 100 mM NaCl and 0.02% Thimerosal. The reaction mixture was incubated for 1 hr in a water bath at 34°C. Activity of D-galacturonase was assayed reductometrically using the method described by Milner and Avigad (1967). Total proteins were determined according to the procedure of Bradford (1976). Activity was expressed as g reducing equivalents . mg protein⁻¹.hr⁻¹. Galacturonic acid (Sigma) was used as the standard.

Transmission Electron Microscopy (TEM)

After the desired ethylene treatment periods, watermelon fruit were cut longitudinally. Sections (1 x 2 mm) of placental tissue were dissected from the fruit and immediately fixed in half-strength Karnovsky's fixative (Karnovsky, 1965) for 2 hrs at 22°C. The samples were then transferred to 1% OsO₄ (buffered in 0.2 M Na-cacodylate buffer, pH 7.2) for 2 hrs at 22°C and then washed three times with distilled water. The samples were dehydrated in a standard ethanol series, followed by 100% acetone, and embedded in Spurr's resin (Spurr, 1969). Ultrathin sections were cut with glass knives using an LKB Ultratome III microtome. Sections were stained in succession with 1% uranyl acetate, followed by 0.5% lead citrate, and then 1% BaMnO₄. Sections were examined with a Hitachi HU-11E electron microscope.

Results

Exposure of watermelon fruit to ethylene resulted in a significant reduction in placental tissue firmness (Fig. 5-1). Fruit showed obvious symptoms of watersoaking as time of exposure to ethylene increased.

C_x-cellulase activity in fruit exposed to 0 or 50 ppm ethylene is presented in Table 5-1. Activity was highest in fruit exposed to ethylene for 3 days and lowest in freshly harvested and overripe fruit. Duration of exposure to ethylene had no significant effect on C_x-cellulase activity. Furthermore, activity in freshly harvested fruit (0 days) was not significantly different from that in 9-day ethylene-treated fruit.

Fig. 5-1. Firmness of the placental tissue of 'Charleston Gray' watermelon fruit. Fruit were harvested and treated with air (o) or air + 50 ppm ethylene (●) as described in Materials and Methods. Vertical bars represent standard error of the mean of five replications.

Table 5-1. C_x-cellulase activity in watermelon fruit exposed to 0 or 50 $\mu\text{l liter}^{-1}$ ethylene.

Treatment	Change in viscosity
	(% change . mg protein ⁻¹ .hr ⁻¹)
0 days	7.5 c ^y
1 day (treated)	16.2 ab
2 days (treated)	15.3 ab
3 days (treated)	18.4 a
3 days (control)	11.8 bc
6 days (treated)	16.4 ab
6 days (control)	13.7 abc
9 days (treated)	12.1 abc
Overripe melon	8.6 c
F value ^z	**

^zF values were significant at the 1% level.

^yMean separation by Duncan's multiple range test, 5% level.

Data for D-galacturonase activity are shown in Table 5-2. Activity increased with increased exposure to ethylene and peaked at 6 days. D-galacturonase activity in control or overripe fruit showed no change.

Analysis of alcohol insoluble powders showed that total pectins content did not change in melons not exposed to ethylene, even in fruit stored for periods of up to 180 days (Table 5-3). In contrast, pectin levels decreased markedly in ethylene-treated fruit, showing a 20 and 33% decrease after 2 and 6 day exposure periods, respectively.

Gel-Filtration Chromatography

Gel-filtration profiles of pectins from ripe watermelon fruit exposed to 0 or 50 ppm ethylene are presented in Fig. 5-2. These profiles indicate a significant effect of ethylene on pectin molecular size. Pectins obtained from freshly harvested and untreated fruit [Fig. 5-2 (A); Fig. 5-3] were of apparent high molecular weight and did not fractionate on Ultrogel AcA 34. Even after 180 days of storage [Fig. 5-4 (B)], at which time the melons were by all accounts "overripe", pectin degradation was not nearly as extensive as in fruit treated with ethylene for short periods. Changes were first apparent in pectins as early as 1 day of exposure of fruit to ethylene [Fig. 5-2 (B)], as indicated by the appearance of small quantities of polymers that fractionated on the gel. As time of exposure of fruit to ethylene increased, the molecular weight of pectins progressively decreased, and degradation was quite extensive after 6 and 9 days of ethylene treatment.

Table 5-2. Activity of D-galacturonase extracted from watermelon fruit exposed to 0 or 50 $\mu\text{l liter}^{-1}$ ethylene.

Treatment	Activity
	(g galacturonic acid . mg protein ⁻¹ .hr ⁻¹)
0 days	27.5 ^z
1 day (treated)	35.5
2 days (treated)	70.2
3 days (treated)	68.6
3 days (control)	21.8
6 days (treated)	355.5
6 days (control)	22.7
9 days (treated)	257.9
Overripe melon	44.7

^zValues are means of three replications.

Table 5-3. Total pectin content of watermelon fruit exposed to 0 or 50 $\mu\text{l liter}^{-1}$ ethylene.

Days	Ethylene	
	0 ppm	50 ppm
	% Pectins ^z	
0	30.6 ^y	--
1	28.3	30.0
2	30.6	24.7
3	29.1	22.5
6	27.2	20.0
9	30.3	20.3
20	30.3	--
180	31.3	--

^zData expressed as percentage of alcohol-insoluble solids.

^yValues are means of three replications.

Fig. 5-2. Gel-filtration profiles of polyuronides from 'Charleston Gray' watermelon fruit. Approximately 3 mg of pectin in 2.5 ml of the elution buffer were applied to Ultrogel AcA 34 column and eluted with Na-acetate-EDTA buffer. Fractions were analyzed for acid (A_{520}) sugars. Polyuronides were prepared from freshly harvested fruit (A), ethylene-treated for 1 day (B), 2 (C), 3 (D), 6 (E), and 9 (F). Arrows correspond to elution positions of (left to right) blue dextran 2,000,000, dextran 70,000, dextran 10,500, and glucose.

Fig. 5-3. Gel-filtration profiles of polyuronides from 'Charleston Gray' watermelon fruit not exposed to ethylene. Details as described in Fig. 5-2. Polyuronides were prepared from fruit stored for 1 day (A), and 9 (B). Arrows correspond to elution positions described in Fig. 5-2.

Fig. 5-4. Gel-filtration profiles of polyuronides from overripe 'Charleston Gray' watermelon fruit. Details of preparation were described in Fig. 5-2. Polyuronides were prepared from field-overripe fruit (A), and fruit stored for 180 days at 15°C (B). Arrows correspond to elution positions described in Fig. 5-2.

Ultrastructural Changes in Watermelon Fruit Cell Wall

The placental tissue of ripe watermelon fruit is composed of large, isodiametric, thin-walled parenchyma cells of approximately 500 μ m in diameter. The cells have a large central vacuole and the cytoplasm is confined to a thin region adjacent to the cell wall. Cell walls from freshly harvested and control fruit displayed darkly stained, tightly packed fibrillar material [Fig. 5-5 (A-D)]. The middle lamellar region in most cases was indistinguishable from the primary walls of adjacent cells. Even in overripe fruit [Fig. 5-5 (E)], the cell wall was composed of densely packed fibrils with no apparent evidence of disintegration. The first change in the appearance of the cell wall was noted 1 day after the onset of ethylene treatment [Fig. 5-5 (F)], and was represented by a thin zone of separation along the middle lamella. Progressive dissolution of the middle lamella continued to occur in tissues exposed to ethylene for 2 days [Fig. 5-5 (G)]. Essentially complete cell separation was observed in tissues exposed to ethylene for 3 days [Fig. 5-5 (H)]. At 6 and 9 days of ethylene treatment, the tissues were not able to withstand the fixation procedures.

Discussion

Watermelon fruit exposed to ethylene exhibited significant tissue damage and cellular breakdown. The data indicated that C_x -cellulase activity was not significantly influenced by ethylene, since activity in freshly-harvested fruit was not significantly different from that in 9-day ethylene-treated fruit. The lack of a clear relationship

Fig. 5-5. Transmission electron micrographs of placental tissue from ethylene-treated or untreated watermelon fruit. Fruit treated with air (A-E) or air + 50 ppm ethylene (F-H) were prepared for microscopy as described in Materials and Methods. Sections were prepared from freshly harvested fruit (A), stored for 1 day (B), 2 (C), 3 (D), field overripe (E), ethylene-treated for 1 day (F), 2 (G), and 3 (H). All plates X40,000, except D (X32,000).

(A)

(B)

Fig. 5-5.--Continued.

(C)

Fig. 5-5.--Continued.

(D)

Fig. 5-5.--Continued.

(E)

Fig. 5-5.--Continued.

(F)

Fig. 5-5.--Continued.

(G)

Fig. 5-5.--Continued.

(H)

Fig. 5-5.--Continued.

between C_x -cellulase activity and tissue degradation indicates that C_x -cellulase is not directly involved in the ethylene-induced phenomenon. This is consistent with the view that C_x -cellulase appears to play a minor role in fruit softening (Hobson, 1968; Hatfield and Nevins, 1985).

D-galacturonase activity in placental tissue increased progressively with increased exposure to ethylene (Table 5-2). Increased activity was apparent after 24 hrs and had doubled after 48 hrs of ethylene treatment. This increase was accompanied by visual symptoms of watersoaking and loss of firmness (Fig. 5-1). Total pectin content did not change during the first 24 hrs of ethylene treatment but showed a 20% decrease after 48 hrs (Table 5-3). The decrease in total pectins possibly reflects their extensive depolymerization to products which remain soluble in 80% ethanol. These products would be lost during preparation of the powders. Clearly, the molecular weight of pectins from ethylene-treated fruit decreased progressively with increased duration of ethylene treatment (Fig. 5-2). The appearance of low molecular weight polymers and the decrease in total pectin content followed the increase in D-galacturonase activity. Pectins from untreated fruit showed no evidence of extensive depolymerization. Also, extensive hydrolysis of pectins was not observed in field overripe and in harvested fruit stored for as long as 180 days. These observations indicate that increased D-galacturonase activity is not a characteristic of normal ripening and senescence of watermelon fruit. Enhanced activity of D-galacturonase and extensive depolymerization of pectins were observed only in fruit exposed to ethylene. Saltveit and McFeeters (1980)

showed that D-galacturonase activity in harvested mature or immature cucumber fruit could be induced by exogenously applied ethylene. Shimokawa (1973) observed that watermelon tissue was macerated by exposure to ethylene. He showed higher activity of pectinase in macerated as compared to untreated tissue. In vitro studies by the same author revealed that the maceration symptoms could be reproduced by pectinase but not cellulase. Our results are in agreement with these findings and suggest that watermelon tissue breakdown was attributed solely to the activity of D-galacturonase.

Ultrastructural studies of fruit exposed to ethylene revealed an early disintegration of the middle lamella, followed by separation of the adjacent primary walls [Fig. 5-5 (F-H)]. Cell wall of untreated fruit did not show any sign of disintegration, even in overripe fruit [Fig. 5-5 (A-E)]. The development of a thin line of separation in the middle lamellar region after 24 hrs of ethylene treatment [Fig. 5-5 (F)] coincided with the first notable increase in D-galacturonase activity. The lack of change in total pectin as well as molecular size of pectins during the first 24 hrs of ethylene treatment indicates that extensive degradation of pectins, at least of a magnitude to produce products fractionating on Ultrogel, was not necessary for preliminary cell wall separation to occur. The decrease in total pectins and the appearance of low molecular size polymers were evident 2 and 3 days, respectively, after the first notable increase in D-galacturonase activity. Hence, the evident separation of the primary cell walls after 3 days of ethylene treatment was apparently due to the progressive depolymerization of pectins from the middle lamellar region as a result of D-galacturonase action.

Structural alterations in the cell wall of apple fruit (Ben-Arie et al., 1979) and tomatoes (Crookes and Grierson, 1983) involved the dissolution of the middle lamella which was attributed to increased D-galacturonase activity. The authors further demonstrated that the application of exogenous D-galacturonase to tissue discs from firm fruit led to a dissolution of the middle lamella similar to that occurring in naturally ripening fruit. In this context, it is necessary to differentiate between two systems: in avocado and tomato fruit, D-galacturonase is normally synthesized as an integral part of the ripening process. However, in watermelon fruit, increased D-galacturonase activity is observed only in fruit exposed to ethylene. Furthermore, activity was extremely low and showed no increase in ripe (or overripe) untreated fruit. These results thus indicate that initiation of D-galacturonase activity in fruit treated with ethylene is independent of the normal ripening process.

CHAPTER VI SUMMARY AND CONCLUSIONS

The objectives of this research were to study the effects of ethylene or propylene on respiration and endogenous ethylene production in watermelon fruit of different maturation stages; to determine effects of ethylene on electrolyte leakage, firmness, and ultrastructure; and to compare, ultrastructurally and biochemically, the changes occurring during both ethylene-induced breakdown and natural ripening in watermelon fruit.

Ethylene-treated watermelon fruit became watersoaked and developed unpleasant odors and unacceptable flavor. Exposure of fruit to ethylene or propylene neither enhanced ripening in preripe fruit nor induced a respiratory climacteric. Propylene failed to trigger endogenous ethylene production in watermelon fruit. Increased production of CO_2 or ethylene were observed only in fruit exhibiting obvious symptoms of decay. Respiration rates of untreated fruit followed trends characteristic of nonclimacteric fruit. Additionally, respiration rate of fruit could be repeatedly stimulated by successive ethylene treatments. These results collectively support the conclusion that watermelon fruit is nonclimacteric.

Electrolyte leakage studies were conducted to investigate the influence of ethylene on the membrane system. The high leakage observed from tissues exposed to ethylene as compared to the controls indicated ethylene-induced changes in membrane permeability. Studies on the effect of incubation media showed that the highest leakage

occurred when the tissues were incubated in distilled water due to the bursting of cells. Leakage was significantly reduced when tissues were incubated in isotonic media. Further reduction in leakage occurred when the incubation medium contained CaCl_2 , most likely due to the role of Ca^{2+} in maintaining cell wall and membrane integrity. Ultrastructural studies revealed that cell walls of fruit exposed to ethylene showed obvious signs of damage. The collapse in cell walls and the increase in electrolyte leakage were apparently the outcome of a deleterious effect of ethylene on both the cell wall and the membrane system.

Further studies were conducted to determine the effects of ethylene on cell wall hydrolases, pectin degradation, and cell wall ultrastructure. Enzymic studies indicated that C_x -cellulase was not involved in watermelon tissue maceration. D-galacturonase activity increased by 24 hrs and peaked after 6 days of ethylene treatment. The increase in D-galacturonase activity was followed by the appearance of low molecular weight polymers and a decrease in total pectins. Hence, it was apparent that watermelon tissue breakdown was attributed solely to D-galacturonase activity. Enhanced activity and extensive depolymerization of pectins were observed only in fruit exposed to ethylene. Ultrastructural studies of ethylene-treated fruit revealed an early disintegration of the middle lamella, followed by separation of the adjacent primary walls. Cell wall of untreated fruit did not show any sign of disintegration. The development of a thin line of separation along the middle lamella during the first day of ethylene treatment coincided with the first notable increase in D-

galacturonase activity. The results thus indicated that initiation of D-galacturonase activity in response to ethylene treatment was not a characteristic phenomenon of normal ripening and senescence in watermelon fruit.

The data considered together permit a tentative mechanism to be proposed regarding the sequence of events resulting in watermelon tissue breakdown. Firstly, the data emphasize a predominant role of the cell wall, rather than the membrane, in this breakdown. Although the first notable increase in D-galacturonase activity was observed after the first 24 hrs of ethylene treatment, the appearance of low molecular weight pectic polymers was not observed until after the 3rd day of ethylene treatment. However, ultrastructural studies showed a distinct separation of the middle lamella after 24 hrs of ethylene treatment. Scanning electron microscopic studies revealed that cell walls of fruit exposed to ethylene for 3 days started to show signs of deterioration.

Secondly, the membrane system did not seem to play a major role in watermelon fruit breakdown. No change in electrolyte leakage was noted during the first 3 days of ethylene treatment, but leakage significantly increased with extended exposure to ethylene. The fact that the increase in electrolyte leakage did not occur until after the 3rd day of ethylene treatment indicates that the increase in leakage was due to cell wall degradation and weakening and consequently the rupture of the membranes.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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