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## RIPENING AND POSTHARVEST MANAGEMENT OF PAWPAW FRUIT

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ABSTRACT OF DISSERTATION

Federica Galli

The Graduate School  
University of Kentucky

2007

RIPENING AND POSTHARVEST MANAGEMENT  
OF PAWPAW FRUIT

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ABSTRACT OF DISSERTATION

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A dissertation submitted in partial fulfillment of the  
requirements for the degree of Doctor of Philosophy in the  
College of Agriculture  
at the University of Kentucky

By  
Federica Galli  
Lexington, Kentucky

Director: Dr. Douglas D. Archbold, Professor of Horticulture

University of Kentucky  
2007  
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## ABSTRACT OF DISSERTATION

### RIPENING AND POSTHARVEST MANAGEMENT OF PAWPAW FRUIT

Pawpaw (*Asimina triloba* (L.) Dunal) has significant potential as a new fruit crop. During ripening, loss of firmness is extremely rapid, and this trait may be the biggest obstacle to the development of a broader market as handling without injury is difficult. Cold storage of pawpaw seems limited to 4 weeks at 4 °C. A study of several cultivars with commercial appeal showed that ripening traits such as ethylene production, respiration and loss of firmness were similar in all genotypes, and that no cultivar showed superior responses to cold storage. Cold storage for longer than 4 weeks caused the development of cold injury symptoms such as black discoloration, rapid loss of firmness, impaired respiration, tissue acidification, decrease in antioxidant content, decrease in volatile ester production and development of off-flavor volatile compounds. Overall cold storage injury symptoms observed in pawpaw may be due to oxidative damage linked to the failure of the two major antioxidant systems that could protect against such damage: phenolics and the ascorbate–glutamate system. With the aim of enhancing pawpaw low temperature tolerance and prolonging cold storage length, different techniques such as hot air exposure and hot water dips of fruit prior to beginning cold storage, and intermittent warming periods during cold storage, were evaluated. Despite positive results with these techniques for other commodities, all the strategies failed to appreciably alter fruit ripening, loss of firmness or maintain fruit quality during and/or after cold storage.

KEYWORDS: *Asimina triloba*, aroma volatile, oxidative stress, cold storage, heat treatment

Federica Galli

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03/29/2007

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RIPENING AND POSTHARVEST MANAGEMENT OF PAWPAW FRUIT

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DISSERTATION

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## DEDICATION

To Mete and Roberta who made me smile even when there was nothing to smile about.

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## CHAPTER 1

### LITERATURE REVIEW

#### **Pawpaw: an Old Fruit for New Needs**

The North American pawpaw [*Asimina triloba* (L.) Dunal], sometimes known as the Kentucky, Indian, Hoosier, or Poor Man's Banana, is the largest fruit native to North America. Fossil pawpaw leaves from the Eocene epoch have been recovered in Texas, Colorado, and Wyoming, and the first report on pawpaw was written by Hernando de Soto in 1541 (Pomper and Layne, 2005). Native Americans not only regularly consumed its fruit but also used many other parts of the plant, such as rough material for crafting tools like fishing nets. Following their example, the first American settlers were introduced to the pawpaw fruit, and that may have helped them avoid starvation during their first years as pioneers (Peterson, 1991; Pomper and Layne, 2005). Evidence of the importance of pawpaw in American folklore and rural culture is the high number of rivers, towns and townships named after the fruit during the 19<sup>th</sup> century (Peterson, 1991; Pomper and Layne, 2005). However, despite this early interest, the high perishability of pawpaw fruit prevented the development of a national market, and pawpaw is still mainly consumed in the areas where the trees naturally grow in the forest.

#### **Botanical and Horticultural Overview**

The pawpaw is the only temperate member of the otherwise tropical Annonaceae family (Magnoliales order – Magnoliopsida group); it lists among its members cherimoya (*Annona cherimola* Mill.), custard apple (*Annona reticulata* L.), sugar apple (*Annona squamosa*, L.), atemoya (*Annona squamosa* x *Annona cherimola*) and soursop (*Annona muricata* L.) (Kral, 1960; Pomper and Layne, 2005; Zimmerman, 1941). In addition to the tropical *Annona* relatives, there are eight members of the *Asimina* genus that are native to the southeastern states of Florida and Georgia. These include *A. incarnata* (flag pawpaw), *A. longifolia*, *A. obovata*, *A. parviflora* (dwarf pawpaw), *A. pygmaea*, *A. reticulata*, *A. tetramera* (opossum pawpaw), and *A. X nashii* (Kral, 1960; Pomper and Layne, 2005). In contrast to pawpaw, none of these species produce fruit of commercial

value. However in the last decades they have become interesting alternatives to traditional ornamental trees (Pomper and Layne, 2005). All *Asimina* species are diploid with  $2n = 2x = 18$ , but triploid hybrids of *A. triloba* have been reported as well (Pomper and Layne, 2005).

Pawpaws grow wild in the hardwood forests of 25 states in the eastern United States ranging from northern Florida to southern Ontario (Canada) and as far west as eastern Nebraska (Kral, 1960; Pomper and Layne, 2005). This tree prefers the deep, rich fertile soils of river-bottom lands, and it can be grown successfully in USDA plant hardiness zones 5 (minimum  $-29\text{ }^{\circ}\text{C}$ ) through 8 (minimum  $-7\text{ }^{\circ}\text{C}$ ) (Kral, 1960; Pomper et al., 2003). As listed in Table 1.1, pawpaw trees can grow up to 5 to 10 m, and in sunny locations it naturally assumes a pyramidal habit. It has a life span of almost 20 years, and when grown from seedling it normally loses its juvenile habit during the 5th year (Bratsch et al., 2003; Pomper and Layne, 2005). In grafted trees, scion flowering may be observed by the third year, but fruit production doesn't start before the fourth year. Under natural pollination the average fruit set is greater than 25%, but very low fruit yield may be observed during the first 2 flowering years and when the environment lacks suitable pollinators and/or compatible pollen sources (Bratsch et al., 2003; Pomper and Layne, 2005).

Pawpaw trees are relatively disease-free plants. No substantial, yield-reducing damage to fruit or plants have been caused by known pests including zebra swallowtail (*Eurytides marcellus* (Cramer)) butterfly larvae, leafroller larvae (*Talponia plummeriana* Busk), and Japanese beetle (*Popillia japonica* Newman), or by fungal attack from *Micocentospora asiminae* and *Rhopaloconidium asiminae* which are occasionally observed on leaves and fruit late in the growing season (Peterson, 1991).

Since 1900, pawpaw cultivars have been selected from the wild: 56 clones were selected and named in the first half of the 20<sup>th</sup> century, and other pawpaw cultivars have recently been added to the variety list. Unfortunately, some of the earliest selections have been lost. Today only 40 clones are available to growers. Among them, the most well-known are 'Middletown' (selected in Ohio), 'Mitchell' (selected in Illinois), 'NC-1' (selected in Ontario, Canada), 'Overleese' (selected in Indiana), 'PA Golden' (selected in New York), 'Sunflower' (selected in Kansas), 'Taylor' (selected in Michigan), 'Taytwo'

(selected in Michigan), ‘Wells’ (selected in Indiana), and ‘Wilson’ (selected in Kentucky) (Peterson, 1991).

Although pawpaw trees have been introduced to many temperate countries such as England (1736), Japan (1895), New Zealand and Australia (prior to 1950), India (1983), Italy (1985), Nepal (1989) and Chile (1988), only the Italian cultivar ‘Prima’ has been recently selected outside the United States by Domenico Montanari and Dr. Elvio Bellini (Bellini et al., 2003; D. Montanari, personal communication; Peterson, 1991).

The loss of older cultivars and the destruction of pawpaw patches in the wild by urbanization has caused an erosion of pawpaw genetic diversity (Peterson, 1991; Pomper and Layne, 2005). To avoid further loss of the pawpaw genetic pool, during the past 10 years a collection of almost 1500 accessions of open-pollinated seedlings was assembled at the University of Maryland, and another germplasm collection of more than 1700 plants including more than 40 named cultivars was created at Kentucky State University (Peterson, 1991; Pomper and Layne, 2005).

### **Pawpaw Nutritional Value**

Though a mild allergic response occasionally occurs in those who eat pawpaw, pawpaw is a very interesting new food option for those people who are looking for new, balanced food choices. One hundred grams of pawpaw pulp deliver 80 calories and contain 1.2 g of proteins, 1.2 g of total fat, 18.8 g of carbohydrates, and 2.6 g of dietary fiber (Peterson, 1982). The fat composition of pawpaw fruit consists mainly of unsaturated fatty acids such as palmitoleic (5.8 – 10.2%), oleic (23.2 – 42.0%), linoleic (8.0 – 12.0%) and linolenic (14.0 – 24.4%). The high percentage of linolenic acid in pawpaw pulp make pawpaw-based products highly susceptible to auto-oxidation processes and may be considered the primary cause of the rapid development of rancidity (Peterson, 1982). Palmitic acid is the major saturated fatty acid (21.5 %) in the fruit (Peterson, 1982).

Despite the rancidity problem mentioned above, pawpaw-derived products as carbohydrate-based, fat-reducing agents in baked food formulations have been studied (Wiese and Duffrin, 2003). The use of pureed pawpaw pulp in the preparation of baked foods significantly decreased the trans-fatty acid, total fatty acid, and caloric content, and

increased overall nutritional value. Moreover, customer acceptability of products baked with 25% pawpaw pulp was equal to the acceptability of traditionally-baked products (Wiese and Duffrin, 2003).

Compared to other major fruits such as apple (*Malus domestica*), orange (*Citrus senensis*) and banana (*Musa acuminata*), pawpaw has a higher content of essential minerals and similar levels of vitamins and amino acids (Table 1.2) (Peterson, 1982). In particular pawpaw fruit contains 3-, 22- and 11-fold more magnesium, and 17-, 57- and 104-fold more manganese than banana, apple and orange, respectively (Peterson, 1982). The chemical quality score, an index based on the most limiting essential amino acid present, is 45. This value is linked to the methionine/cysteine content of pawpaw pulp, and it is two to three times higher than that for apple or peach (*Prunus persica*) (Peterson, 1982). Since essential amino acids have to be supplied with the diet since they can not be synthesized *de novo* by the human body, foodstuffs rich in these compounds assure a high degree of protein utilization, and they are important parts of an equilibrated diet. Overall, pawpaw can be considered a potentially valuable food option and an interesting alternative to fruit commonly grown and consumed in the temperate areas of the world.

Pawpaw contains a unique group of compounds, the acetogenins. Acetogenins are long-chain (C-32 or C-37) fatty acid derivatives synthesized by many members of the Annonaceae family such as soursop and pawpaw. More than 250 of these compounds have been isolated from pawpaw twigs and fruits (Orru et al., 2003). These compounds have pesticidal and anti-carcinogenic properties, and show a high potential for use in medications and pesticide products. Acetogenins has been successfully tested *in vitro* against many solid human cancers such as ovarian, cervical, breast and skin cancers (Jaramillo et al., 2000; Pomper and Layne, 2005; Peterson, 1991; Yuan et al., 2003). A patented shampoo made from annonaceous acetogenin is produced by Nature's Sunshine Products, Inc., Utah.

### **Pawpaw Market Interest**

During the 1916 American Genetic Association meeting, pawpaw was listed as one of the most promising crops of the new century (Peterson, 1991). However, almost 100 years later, few commercial-quality pawpaw cultivars are available, and the primary

market for the fruit is still local (Peterson, 1991). Although germplasm selection and breeding have led to more pawpaw cultivars, the two main obstacles to the development of a national market, the rapid postharvest perishability of the fruit and the absence of harvest synchronization within and among trees, have not yet been overcome.

Once ripe, pawpaw fruit are marketable for only 3-4 days when held at room temperature (Archbold and Pomper, 2003). When fully ripe, fruit must be handled with care since their thin skin and extremely soft pulp expose them to bruise and other physical damages (Archbold et al., 2003; Archbold and Pomper, 2003; Peterson, 1991). Pawpaws fail to complete the ripening process if harvested too early (Archbold, personal communication). Recently, it was shown that ripe pawpaw fruit can be cold stored at 4 °C up to 4 weeks with minor changes in fruit quality (Archbold et al., 2003; Archbold and Pomper, 2003). However, comparisons among cultivars and advanced selections for ripening behavior and maximum postharvest storage life have not been reported.

Despite storage and handling problems, pawpaw is becoming a high value alternative crop in the southeastern United States. Thanks to the work done on cultivar development pawpaw growers can offer fruit with consistent quality, and consumer acceptance of pawpaw fruits and fruit-based products is increasing. A consumer acceptance study conducted at a 1999 pawpaw field day at Kentucky State University (KSU), Frankfort, KY, found that pawpaw-based products such as ice cream, cake, and pawpaw/grape juice received high scores from the panelists (Templeton et al., 2003). These results showed that, even though the fresh fruit market may not grow rapidly, there is a potentially significant processing market for pawpaw pulp. Fruit pulp can be extracted, frozen, and used as a flavoring compound in the preparation of juices, ice creams, yogurt and baked goods (Templeton et al., 2003).

### **The Quest for a Ripening Index**

Maturity at harvest is the factor that most influences final fruit quality and fruit storage life. Fruit harvested immature are more subject to shriveling, mechanical damage and, when they do ripen, normally have inferior quality than fruit harvested mature or ripe (Crisosto et al., 1995; Kader, 2002). On the other hand, overripe fruit has a very short shelf life and become too soft and mealy shortly after harvest. Generally fruit



picked too early or too late are more likely to develop physiological disorders and have shorter storage life than fruit harvest and mature.

'Mature' and 'ripe' are two distinct terms that refer to different stages of fruit development (Reid, 2002). To plant physiologists, 'mature' identifies the fruit stage which will ensure proper ripening after harvest. Most postharvest technologists will define 'mature' as a sufficient stage of development that will develop at least the minimum acceptable quality after harvesting and postharvest handling. In contrast, horticultural maturity has been defined as the stage of development at which a plant or plant part possesses the prerequisites for consumption. Based on the characteristics of the product, be it a sprout, vegetative tissue, flower or fruit, a given commodity may be horticulturally mature at any stage of development.

Because the stage of fruit ripening and the time of harvest determine the quality of the marketed fruit, many maturity indices have been developed with the purpose of predicting or identifying the best time of harvest. Despite the fact that fruit that ripened on the tree have the best quality, many fruit are harvested at a physiologically mature but not fully ripe stage to allow long distance distribution. These fruit are firm and can sustain handling with a minimum of damage. Thus, most commonly-used maturity indices are those that compromise between marketing needs and optimum consumer quality (Burdon, 1997; Kader, 2002.).

In fruit production, physical features such as size, abscission force, color, texture, titratable acidity and changes in total soluble solids and physiological features such as respiration and ethylene production have been useful tools for maturity index development (Reid, 2002; Thompson, 2003a). In the pawpaw-related cherimoya, changes in skin color, total soluble solids content, protein content, looseness of seeds, surface traits such as development of a tuberculate surface, and epidermal trichome density are characteristics commonly used for planning the fruit harvest schedule (Merodio and De La Plaza, 1997). Like many fruit, pawpaw undergoes a variety of physical and chemical changes during ripening. As reported by McGrath and Karahadian (1994a), these events and their relationship with aroma development could be useful tools for defining appropriate pawpaw maturity index. However, the lack of a distinctive color change

hinders visual detection of ripening, and harvest requires touching fruit to detect softening and/or pedicel abscission.

### **Fruit Ripening**

Fruit ripening is a complex, genetically-programmed process that culminates in dramatic changes in color, texture, flavor, and aroma of the fruit flesh (Alexander and Grierson, 2002; Brady, 1987; Tucker, 1993). The distinction between ripening and senescence has never been fully defined, and some have stated that ripening is ‘the sum of changes that occur from the latter stages of growth and development through the early stages of senescence and result in characteristic aesthetic and /or food quality’ (Brady, 1987). Even though senescence and ripening share some common features such as chloroplast degradation and increased tissue susceptibility to diseases, ripening includes processes such as softening and pigment accumulation that are not common characteristics of senescence (Brady, 1987).

Fruit can be divided into two groups: climacteric, in which ripening is accompanied by a peak in respiration and a concomitant burst of ethylene, and non-climacteric, in which respiration shows no dramatic change and ethylene production remains at a very low level. In climacteric fruits like tomato (*Lycopersicum esculentum*), apple, melon (*Cucumis melo*) and banana, the ethylene burst is required for and promotes normal fruit ripening (Alexander and Grierson, 2002). Even though the term climacteric was coined by Kidd and West in 1925 to describe the rise in respiration rate that accompanied the maturation phase of apple, it has been shown that autocatalytic ethylene production is invariably associated with increased respiration in climacteric fruit (Watkins, 2002). However, based on the fruit investigated, the respiration rise can precede, coincide with, or follow the ethylene rise.

Diagnostically, climacteric fruit can be separated from non-climacteric fruit by analyzing the response to exogenous ethylene application. In climacteric fruit exposure to ethylene will advance the climacteric peak and autocatalytic production will continue even after the exogenous ethylene source is removed. On the other hand, non-climacteric fruit exposed to ethylene will exhibit a respiration rise independent from the concentration of ethylene applied (Watkins, 2002).

While it has been shown that ethylene affects the transcription and translation of many ripening-related genes, both ethylene-dependent and ethylene-independent gene regulation pathways coordinate ripening processes in climacteric and non-climacteric fruit ripening (Alexander and Grierson, 2002; Watkins, 2002). Wills (1998) showed that when non-climacteric fruit and vegetables were held at ambient temperature (20 °C) and low temperature (0–2.5 °C) and ventilated with air containing ethylene, product storage life of all produce was indirectly correlated to air ethylene concentration. Clearly, in non-climacteric fruit aging and the start of senescence is still under ethylene control.

Tesniere et al. (2004) showed that, even though grape (*Vitis vinifera*) is classified as non-climacteric fruit, fruit ripening involves both ethylene-dependent and ethylene-independent processes. As with many other non-climacteric fruit, grape berries produce a limited amount of ethylene during ripening, and recently it has been shown that, even though their ethylene production is low, grape berry ripening is impaired by 1-methylcyclopropene (1-MCP), a specific inhibitor of the ethylene receptor.

Two different ethylene regulation systems have been detected in plants. In non-climacteric fruit and vegetative tissues, ethylene is controlled by an auto-inhibitory response (System 1). On the other hand, in climacteric fruit and senescent tissues, ethylene production is autocatalytic (System 2) (Alexander and Grierson, 2002; Watkins, 2002). Specifically, System 1 is the ethylene production system operating in vegetative tissues, in non-climacteric fruit and in climacteric fruit until ripening. In tissues with active System 1, ethylene production is low and it can be inhibited by exogenous ethylene. On the other hand, System 2 is the system operating in climacteric fruits and in some flowers. In these organs, ethylene production increases by several hundred times compared to pre-climacteric values and exposure to endogenous or exogenous ethylene induces a large increase in tissue ethylene production that follows a positive feedback response. Autocatalysis can be considered the major characteristic of System 2 and the major feature of climacteric fruit ripening or flower senescence (Brady, 1987; Oetiker and Yang, 1995).

In higher plants ethylene is derived from methionine via *S*-adenosyl-*L*-methionine (AdoMet) (Yang and Hoffman, 1984). The cyclic non-protein amino acid, 1-aminocyclopropane-1-carboxylic acid (ACC), is formed from AdoMet by the action of

ACC synthase (ACS), and the conversion of ACC to ethylene is carried out by ACC oxidase (ACO). In addition to ACC, ACS produces 5'-methylthioadenosine which is utilized for the synthesis of new methionine via a modified methionine cycle. As mentioned above, in climacteric fruit ripening, ethylene biosynthesis is regulated by a positive feedback system (Alexander and Grierson, 2002; Brady, 1987; Watkins, 2002)

The switch from System 1 to System 2 involves increased synthesis of both ACS and ACO and increased tissue sensitivity to ethylene. In tomato it has been shown that the presence of tomato ACS forms LEACS2 and LEACS4 that are associated with fruit ripening and System 1 to System 2 transition. Moreover, it has been shown that maturing apples gradually develop the capacity to produce ACS and ACO during maturation. Thus, exposure to exogenous ethylene results in a large increase in ethylene production due to the induction of ACS and ACO (Alexander and Grierson, 2002; Watkins, 2002). Expression of both ACS and ACO is controlled not only by ethylene, but by various developmental, environmental and hormonal signals (Alexander and Grierson, 2002; Brady, 1987; Watkins, 2002).

Triggered by the ethylene burst, climacteric fruit ripening usually commences in one region of the fruit and spreads to neighboring regions following ethylene diffusion (Alexander and Grierson, 2002). Fruit respiration rises with a parallel increase of ATP levels and glycolytic flux. The increase in respiration is associated with an increase in protein synthesis as well (Brady, 1987). The critical role of ethylene in coordinating the molecular changes characteristic of fruit ripening have been studied via antisense gene repression and/or mutant complementation. Through these techniques it has been shown that expression of many enzymes pertinent to fruit ripening such as HMG-CoA reductase, polygalacturonase (PG), and pectin methylesterase, in addition to ACS and ACO, are directly induced by ethylene (Giovannoni, 2001).

As ripening progresses, changes in skin and/or flesh color linked to chlorophyll degradation and carotenoid and/or anthocyanin accumulation occur. Generally, the change from green to orange, red or purple color during ripening, and the final intensity of color, are related to both carotenoid and anthocyanin accumulation in the fruit (Dhandar and Desai, 1999; Rodriguez-Amaya and Kimura, 2004; Thompson et al., 1999; Thomas-Barberan and Espin, 2001). As photosynthetic membranes are degraded,

chlorophyll is metabolized and carotenoids such as  $\beta$ -carotene and lycopene accumulate. In both tomato and melon, carotenoid biosynthesis in ripening fruit is partially regulated by ethylene (Giovannoni, 2001; Rodriguez-Amaya and Kimura, 2004).

As far as fruit softening is concerned, enzymes such as pectin methylesterase, polygalacturonase,  $\beta$ -glucanase, cellulase and expansin are involved in cell wall disassembly and consequent fruit softening (Brady, 1987; Giovannoni, 2001; Johnston et al., 2002). In apple, experiments with ethylene inhibitors showed that ethylene promoted apple softening and regulated cell wall hydrolyzing enzymes (Johnston et al., 2002). Although it is unlikely that fruit softening has an initiating role in fruit ripening, it has been shown that genotypes that soften rapidly generally ripen rapidly and develop senescence symptoms faster than firmer ones (Brady, 1987).

As summarized by Redgwell and Fischer (2002), polygalacturonase mediated pectin disassembly, one of the major causes of fruit softening, does not contribute significantly to fruit ripening or early fruit softening, but significantly contributes to tissue deterioration in the later stages of fruit ripening. Extensive PG activity can be found in fruit characterized by dramatic losses of firmness during ripening such as peach, tomato and avocado (*Persea americana*). It has been reported that in firmer freestone peach varieties PG activity is lower than in those varieties that reach a softer texture when fully ripe (Redgwell and Fischer 2002).

As ripening proceeds, flavor develops as the sugar–acid balance is altered by starch degradation and soluble sugar accumulation, phenolic compound levels change, and volatile production increases (Alexander and Grierson, 2002; Giovannoni, 2001; Tucker, 1993). Starch occurs as small granules within the cells of immature fruit, and it is converted into sugar as the fruit mature and ripen. In many species, starch breakdown and the consequent rise in glucose, fructose and sucrose concentration are characteristic ripening events (Kader, 2002; Tucker, 1993). Several enzymes such as amylases, starch phosphorylase, and hydrolases are involved in starch metabolism in fruit. Activity of these enzymes usually rises during ripening.  $\alpha$ -(1-4)-Amylase hydrolyzes the  $\alpha$ -(1-4) linkage of amylose producing glucose and maltose. On the other hand,  $\beta$ -amylase attacks only the penultimate C-C bond releasing maltose. Starch phosphorylase attacks the terminal  $\alpha$ -(1-4) linkage to give glucose-1-phosphate which can be converted to glucose-

6-P by glucose phosphate mutase. Maltose as well can be converted to glucose by glucosidase action (Tucker, 1993). Moreover, debranching enzymes able to degrade an  $\alpha$ -(1-6) starch linkage have been identified in several species. In addition to starch metabolism, sucrose can be converted to glucose and fructose by invertase activity (Tucker, 1993). Across species, sugar content of ripe fruit varies from 10 – 25% (fresh weight basis) with sucrose content ranging from a trace in sweet cherries (*Prunus avium*) and grapes to more than 8% in ripe bananas and pineapples (Kader, 2002). The major sugars contributing to the total soluble pool are fructose, sucrose and glucose for most species. Even though, along with ethylene and respiratory bursts, starch hydrolysis is one of the most significant metabolic changes during fruit ripening, it is not directly controlled by ethylene. However, other plant hormones may modulate starch metabolism (Murayama et al., 2002; Oliveira do Nascimento et al., 2006; Purgatto et al., 2002).

Fruit acid content usually decreases during ripening in an ethylene independent manner, due to the utilization of organic acids in the TCA cycle, for respiration and sugar conversion (Kader, 2002; Moretti et al., 2002). Total titratable acidity (TA) varies among species as shown by TA values recorded in tomato at 8 malic acid meq (MA meq)/100g FW (Moretti et al., 2002), cherimoya (2.5 MA meq/100 g FW) (Maldonado et al. 2004), peach at 5 MA meq/100g FW (Fernandez-Trujillo and Artes, 1997), and apple at 5.9 MA meq/100 g FW (Defilippi et al., 2004). Malic and citric acid are the most abundant acids in fruit with the exception of grapes and kiwifruit (*Actinidia deliciosa*) in which tartaric acid and quinic acid, respectively, represent the most abundant organic acids (Kader, 2002).

Organic acids are not the only compounds that contribute to the organoleptic and nutritional qualities of fruit and vegetables. Phenolic compounds such as anthocyanins, catechins, tannins and flavonols, and simple phenols such as chlorogenic and cinnamic acids represent one of the main classes of plant secondary metabolites. In fruit, these compounds are primarily responsible for astringency (Kader, 2002; Amiot et al., 1997). The concentration of phenolics in fruit vary according to cultivar and time of harvest. The total phenolic concentration of common fruit were 330 mg gallic acid equivalents (GAE)/100 g FW in strawberry (*Fragraria x ananassa*), 126 mg GAE/100 g FW in orange, 60 mg GAE/100 g FW in pear (*Pyrus communis*) 48 mg GAE/100 g FW in

apple, 38 mg GAE/100 g FW in peach, 38 mg GAE/100 g FW in banana, and 30 mg GAE/100 g FW in tomato (Proteggente et al., 2002). Oxidation of phenolic compounds via enzymatic browning may modify the quality of fresh fruit and vegetables (Amiot et al., 1997).

Phenolic compounds are normally synthesized in the cell cytoplasm and stored in the cell vacuole. Once the tissues are cut, polyphenoloxidase (PPO) modifies phenolics to create brown, oxidized compounds (Gooding et al., 2001; Thompson, 2003b). During ripening, phenolic polymerization events, changes in molecular size, and hydroxylation reactions change the characteristics of the phenolic pool and cause astringency loss (Kader, 2002). In banana and plantain (*Musa paradisiaca*), astringency loss is mainly linked to tannin polymerization and only partially to the decrease of the total phenolics pools. On the other hand, in both mango (*Mangifera indica*) and guava, polyphenol levels decrease during ripening (Thompson, 2003b).

Even though during ripening, the fruit volatile synthesized at the highest concentration is ethylene, this compound does not impart aroma or flavor. Compounds such as esters, alcohols, acids, aldehydes and ketones, synthesized in very low amounts by the fruit through the fatty acid  $\beta$ -oxidation pathway, the lipoxygenase pathway, the isoprenoid pathways, amino acid metabolism, and the esterification pathway, are the most important components of the aroma profile (Kader, 2002; Thompson, 2003b; Sanz et al., 1997).

Fruit aroma is determined by a large number of volatile compounds, the distribution and biosynthesis of which are dependent on many factors, such as species, cultivar, maturity, and postharvest conditions (Nair et al., 2003; Olias et al., 2002; Shalit et al., 2001; Wang and De Luca, 2005). Consumer assessment of fruit aroma is greatly influenced by the composition of the aroma profile. The specific volatile compounds, their concentration, and the human threshold sensitivity to each one all play a key role in aroma perception (Beekwilder et al., 2004; Defilippi et al., 2005a). Often, the aroma of a particular species or cultivar is characterized by the presence of one or a few characteristic compounds that have a large impact on the final aroma (Wang and De Luca, 2005). In some species like apple, pear, and banana, individual esters are the major components of aroma of ripe fruit (Beekwilder et al., 2004). Compounds such as 3-

methylbutyl acetate in banana, hexyl butanoate in apple (Berger, 1991; Defilippi et al., 2005a) and methyl anthranilate in ‘Concord’ grape (Power and Chestnut, 1921) are considered key compounds in fruit aroma perception of each species and/or cultivar within a species.

At least 350 flavor and aroma compounds have been shown to occur in ripe banana with acetate and butanoate esters accounting for about 70% of the total volatile produced (Thompson, 2003b). On the other hand, more than 400 compounds have been shown to contribute to volatile aroma profile in tomatoes, but no single compounds or simple combinations of these compounds have been recognized as primarily responsible for tomato aroma (Thompson, 2003b). Qualitative and quantitative differences among cultivars have been observed in melon (Shalit et al., 2001). When ripe, fruit of aromatic melon varieties such as ‘Arava’ produced mostly volatile acetates, both aliphatic and aromatic types. In these fruit, ethyl butanoate, ethyl hexanoate, terpenes, medium chain alcohols, and lactones were detected as well (Shalit et al., 2001). On the other hand, non-aromatic fruit such as ‘Rochet’ melon had lower total volatile production, and the dominant compounds were short- and-medium chain alcohols, aliphatic aldehydes and aromatic acetates (Shalit et al., 2001).

Ester compounds synthesized through esterification of alcohol and carboxylic acid represent one of the most important classes of volatile compounds identified in fruit aroma (Sanz et al., 1997). Ethyl acetate, butanoate esters and hexanoate esters are the most important compounds in guava, banana and pear aroma. However, in species such as strawberry, other compounds such as furanones play a major role on fruit aroma final (Sanz et al., 1997; Thompson, 2003a).

Enzymes such as lipoxygenase (LOX), alcohol dehydrogenase (ADH) and alcohol acyltransferase (AAT) are the main enzymes in volatile ester formation. In fact synthesis of ester compounds is related to ripening, and no LOX, ADH and AAT activities are usually found in unripe fruit. Since activity of these enzymes is regulated by ethylene, it is clear that overall aroma formation in fruit is regulated by ethylene in climacteric species (Griffiths et al., 1999; Kader, 2002; Tesniere et al., 2004; Wang and De Luca, 2005).



Recently, it has been shown that ethylene may also play an important role in regulation of some ripening genes in the non-climacteric strawberry (Trainotti et al., 2005), *Citrus* (Alonso et al., 1995) and grape (Chervin et al., 2004; Tesniere et al., 2004). Specifically, Tesniere et al. (2004) reported that *VvADH2* transcription in grape is partially reduced by 1-MCP treatment applied after veraison. The authors concluded that in non-climacteric fruit some aspects of fruit development can be regulated by ethylene through the creation of sub-cellular anaerobic conditions that trigger a small production of ethylene and ADH gene expression. An increase in ethylene signaling and ADH transcription were observed when fruit were exposed to low oxygen treatment.

Similarly, when strawberry fruit progressed from the large green to the white stage, an increased synthesis of ethylene receptors concomitant with the increased synthesis of ethylene in strawberries was observed (Trainotti et al., 2005). Moreover, receptors primarily expressed in ripening strawberries were type-II, those with a higher sensitivity to ethylene. Thus, even the little ethylene produced by ripening strawberries might be sufficient to trigger ripening-related physiological responses.

Volatile compound production normally increases at the onset of increased ethylene production and reaches the maximum concentration following the climacteric peak (Defilippi et al., 2005a; Engel et al., 1990; McGrath and Karahadian, 1994a; Wang and De Luca, 2005). The production of volatile precursors such as aldehydes and alcohols is directly linked to metabolism of long-chain fatty acids through  $\alpha$ - and  $\beta$ -oxidation reactions (Engel et al., 1990). In olive (*Olea europaea*) (Perez et al., 2003) and apple (Dixon and Hewitt, 2000), ester formation followed a multistep pathway that involved LOX (linolenic and linoleic acid  $\rightarrow$  13-hydroperoxide), hydroperoxide lyase (13-hydroperoxide  $\rightarrow$  C6 aldehydes), ADH (C6 aldehydes  $\rightarrow$  C6 alcohols), and AAT (C6 alcohols + acyl CoAs  $\rightarrow$  corresponding esters) activities.

The significant influence of esters on fruit aroma makes AAT and esterase, the enzymes catalyzing the formation and the hydrolysis of ester bonds, the primary enzymes in aroma formation (Dixon and Hewitt, 2000; Olias et al., 2002; Perez et al., 1996; Wyllie and Fellman, 2000). Even though final ester synthesis may be the sum of both ester formation and ester hydrolysis, most agree that the activity of AAT is the key in fruit aroma biochemistry (Olias et al., 2002; Perez et al., 1996; Wyllie and Fellman,

2000). However, since aroma is determined by availability of alcohols, aldehydes, and other minor compounds used in ester formation, the enzymes involved in the synthesis of lactones, aldehydes, and alcohols such as LOX and ADH play important roles (Defilippi, 2005b; Perez, 2003).

Even though  $\beta$ - and  $\alpha$ -oxidation of fatty acids and aldehyde biosynthesis are the primary processes that provide alcohols and acyl CoAs for ester formation, amino acids also represent an important source of fruit aroma volatile compounds (Defilippi et al., 2005a, b; Dixon and Hewett, 2000; Perez et al., 2003; Sanz et al., 1997). During ripening both amino acid biosynthesis and protein degradation increase availability of amino acids such as leucine, valine and phenylalanine (Engel et al., 1990; Wyllie and Fellman, 2000). Once free, these compounds can be used as direct precursors of volatile biosynthesis or may be transformed into new nitrogen or sulfur-containing compounds such as glucosinolates and thiosulfates. The former path is typically found in fruit, whereas the latter is mainly present in vegetables (Sanz et al., 1997). Free amino acids become substrates for amino transferase,  $\alpha$ -keto acid dehydrogenase/decarboxylase and ADH that transform the branched amino acids to acyl-CoAs and alcohol compounds. Through AAT activity, acyl-CoAs and alcohols are transformed to branched and linear chain esters (Engel et al., 1990; Wyllie and Fellman, 2000).

#### *Alcohol acyl transferase (AAT)*

Alcohol acyltransferase is a member of the benzylalcohol acetyl-, anthocyanin-*O*-hydroxy-cinnamoyl-, anthranilate-*N*-hydroxy-cinnamoyl/benzoyl-, deacetylindoline acetyltransferase (BAHD) enzyme superfamily that catalyzes the last reaction of ester formation (Wang and De Luca, 2005). AAT has been purified from and described for many temperate and subtropical fruit such as banana (Harada et al., 1985), melon (Ueda et al., 1997), strawberry (Perez et al., 1993), olive, (Salas, 2004) and apple (Defilippi et al., 2004). In fruit, AAT is localized in the cell cytoplasm, has an optimal temperature of 30–35 °C, optimal pH of 7.5–9, and a molecular mass ranging between 40 and 400 KDa (Harada et al., 1985; Ueda et al., 1997; Perez et al., 1993; Salas, 2004). This enzyme accepts a broad range of acyl and alcohol moieties as substrates, and it can be considered

an ethylene-regulated enzyme (Shalit et al., 2001; Wang and De Luca, 2005), as ethylene induces its expression and resulting activity.

The wide range of alcohols and acyl-CoAs accepted by AAT cannot completely explain the ester composition of fruit like strawberry (Perez et al., 1996), olive (Salas, 2004), and apple (Defilippi et al., 2005b). Two main factors could be involved in determining volatile ester composition in fruits: the inherent properties of the AAT enzyme and the availability of substrates. Olias et al. (2002) showed that AAT isolated from strawberry has varying affinities for different substrates. Generally, AAT substrate affinity decreased with increasing length of the alcohol carbon chain. Banana aroma was mainly correlated to AAT substrate availability and only secondarily linked to AAT substrate affinity (Wyllie and Fellman, 2000). The formation of branched chain compounds such as 3-methylbutyl and 2-methylpropyl esters that characterize the banana volatile profile was mainly linked to the increasing availability of free leucine, alanine and valine during the banana climacteric. These compounds represented the precursors of the branched chain skeleton used by AAT for the production of the branched ester compounds characteristic of banana aroma (Wyllie and Fellman, 2000; Engel et al., 1990).

Wang and De Luca (2005) also showed how slight changes in substrate availability dramatically altered the aroma profile characteristic of ‘Concord’ grape berries. The presence of unique metabolic pathways that supplied both anthranilic acid/anthraniloyl-CoA and methanol precursors explained the unusually high concentration of methyl anthranilate found. Furthermore, the ability of AAT to accept any acyl moiety as substrate seems to indicate that alcohols and not the CoA compounds are rate-limiting factors in ester compounds synthesis (Beekwilder et al., 2004; Perez et al., 1996; Salas, 2004)

AAT traits may be cultivar-related as well. Olias et al. (2002) found that most commercial strawberry varieties presented a very similar pattern of AAT activity for use of straight-chain alcohols. However, heptanol was the preferred substrate for three European varieties, whereas hexanol was the preferred alcohol for the American cultivars. Interestingly, wild strawberry (*Fragaria vesca*) was different; maximum AAT activity was obtained with pentanol, and very high activity was also found using both

butanol and hexanol as substrates. Differences in AAT activity and aroma profile have also been observed among melon cultivars. As a general rule, melon cultivars with high AAT activity showed enhanced ester production and superior aroma when compared with cultivars with low AAT activity (Shalit et al., 2001).

### ***Alcohol dehydrogenase (ADH) and lipoxygenase (LOX)***

Despite the key role played by AAT in fruit aroma compound formation, many studies have demonstrated that the activities of other enzymes such as ADH, LOX and pyruvate decarboxylase could explain not only cultivar differences in volatile production, but also the different susceptibilities of strawberry and apple varieties to off-flavor development during storage (Defilippi et al., 2005b; Ke et al., 1994; Olias et al., 2002). Through LOX activity, fatty acids are transformed to aldehydes, ketones, acids and alcohols. Aldehydes produced by lipid oxidation may be metabolized by ADH to alcohols, an optimal substrate for AAT-mediated esterification (Ke et al., 1994; Defilippi et al., 2005b). On the other hand pyruvate produced through the glycolytic pathway can be transformed to acetaldehyde and ethanol by pyruvate decarboxylase and ADH activity, respectively. Ethanol produced through this pathway can be esterified to ethyl esters by AAT action (Defilippi et al., 2005b; Ke et al., 1994). Thus, sugars can directly contribute to fruit flavor as well as be important substrates for volatile formation via glycolysis.

Since the availability of the alcohol moiety can be limited by the aldehyde-to-alcohol transformation rate, ADH activity may be considered another critical step for ester formation (Defilippi et al., 2005a). The difference in ADH substrate affinity reported for apple and grape could favor the formation of certain alcohols over others and indirectly influence ester formation (Dixon and Hewett, 2000). As ripening progressed, apple ester production increased following ADH activity (Defilippi et al., 2005a). Moreover volatile production observed during bench ripening of apple was at least partially related to the increase of alcohol concentration in the fruit (Defilippi et al., 2005b). Ke et al. (1994) showed that the increase in ethyl acetate and ethyl butanoate production in strawberry cold-stored up to 8 days under normal atmospheres was related to the increase in AAT, ADH and pyruvate decarboxylase activities. However, no

changes in ADH activity were observed during banana fruit ripening, suggesting that the importance of ADH may be species-specific (Wyllie and Fellman, 2000).

The role of LOX in the determination of the final fruit aroma has been shown in tomato (Riley et al., 1996) and olive (Perez et al., 2003), but not in apple (Defilippi et al., 2005b). Riley et al. (1996) observed that an increase in microsomal LOX activity in ripening tomato was consistent with fruit and volatile development during ripening. Similarly, Perez et al. (2003) correlated the reduction in C6 virgin oil aldehyde concentration observed in hot water treated olive with the denaturation of LOX in the pulp. Though LOX may play a role in providing substrates for volatile formation, LOX activity itself may not be the key. Defilippi et al. (2005b) related the increase in (*E*)-2-hexenal observed in apple during ripening to a change in fatty acid availability and not to change in LOX activity. However, even though LOX had highest activities during early ripening stages and ADH activity was higher at the red ripening stage, enzyme activity itself was not a good predictor of the amount of volatiles produced by tomato (Yilmaz et al., 2001)

### **Fruit Ripening in Pawpaw and the Other Annonaceae**

Like many climacteric fruit, pawpaw ripening is characterized by an increase in ethylene production and respiratory activity (Archbold and Pomper, 2003; Koslanund et al., 2005b). Single peaks of each were generally detected 3 days after harvest. Mean ethylene maxima on a fresh weight basis were 4.7 and 7.6  $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  and mean respiratory ( $\text{CO}_2$  production) maxima on a fresh weight basis were 220 and 239  $\text{mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  in 1999 and 2001 harvests, respectively. Ethylene and respiration peak values in pawpaw were similar to those reported for sugar apple and cherimoya, but ethylene values were significantly lower and respiration values significantly higher than the values reported in other climacteric fruit such as apple, which at harvest produces 10-100  $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  and 5-10  $\text{mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  (Kader, 2002). Many *Annona* fruit such as cherimoya, sweetsop and soursop have a diffuse climacteric behavior: upon harvest 2 apparent respiration peaks and one ethylene peak have been recorded (Bruinsma and Paull, 1984; Merodio and De La Plaza, 1997; Taylor, 1993). The first respiratory peak, apparently associated with starch breakdown, was detected 2 days after harvest, while the

second respiratory rise was detected up to four days after harvest. Even though lower in magnitude, the first respiratory peak was accompanied by autocatalytic ethylene production and an increase in the total soluble solids concentration (Merodio and De La Plaza, 1997; Taylor, 1993).

As reported by Koslanund et al. (2005b), ethylene production in pawpaw was associated with increased ACS and ACO activities, an increase in ACC content and a decrease in malonyl-ACC content (MACC). Pawpaw should be considered a low ethylene producer as compared to other species (Kader, 2002).

As pawpaw ripening progresses, a decrease in peel hue angle consistent with the change from green to yellow has been reported (McGrath and Karahadian, 1994a). The intensity of the skin color change has been reported as particularly consistent in some cultivars such as 'PA Golden' (Templeton et al., 2003), though not all cultivars (Archbold, personal observation). Since the color change is only visually evident near the end of ripening, it is not a good harvest index.

Similar to that observed in cherimoya fruit (Merodio and De La Plaza, 1997), there is a dramatic and rapid decline in pawpaw fruit firmness during ripening (Archbold and Pomper, 2003; Koslanund et al., 2005a). McGrath and Karahadian (1994a) reported a significant correlation among the decrease in peel hue angle, increase in volatile production, increase in total soluble sugar (TSS), and decrease in fruit firmness. Fruit softening was not uniformly distributed in the pawpaw pulp, but it appeared to proceed from the surface to the interior (Koslanund et al., 2005a). Softening in pawpaw fruit was related to the activity of hydrolytic enzymes such as polygalacturonase (PG), endo-(1→4)β-D-glucanase (EGase), pectin methylesterase (PME) and endo-β-1,4-mannase (MAN) (Koslanund et al., 2005a). Activity of these enzymes was low in unripe fruit and increased dramatically coincident with and/or following the respiratory and ethylene climacteric peaks. Koslanund et al. (2005a) reported that PME activity reached its maximum at the earliest ripening stage and that the greatest decline in fruit firmness occurred before the peak activities of PG, EGase, and MAN was observed. Thus, PME may first de-methylate polygalacturonic acid and make it an optimal substrate for PG. Once this first step is completed, cell wall degradation may be completed through MAN

and EGase activities. Currently, softening is the only ripening index commonly used to determine pawpaw harvest (Archbold et al., 2003).

In many fruit, starch breakdown via increased amylase activity precedes the increase in ethylene and results in an increase in TSS content. The increases in the major components of the TSS pool, sucrose, fructose and glucose, the degradation of starch during ripening and the formation of mono- and disaccharides not only provides precursors to flavor compound formation but also increases sweetness of the edible fruit (Brady, 1987; Thompson, 2003b). During pawpaw fruit ripening, TSS value increased almost 3-fold reaching a value of 21.9% during the climacteric peak (McGrath and Karahadian, 1994a).

An increase in the concentrations of organic acids was observed during ripening of cherimoya (Maldonado et al., 2004; Merodio and De La Plaza, 1997). At harvest cherimoya contained mostly malate (1 mg/g FW) and citrate (0.5 mg/g FW). An increase in titratable acidity observed in ripening fruit was paralleled by a decline in tissue pH. In custard apple, pawpaw and soursop fruit, the maximum accumulation of total sugars and organic acids not only may provide precursors for volatile synthesis, but they also coincide with the accumulation of total volatile production (McGrath and Karahadian, 1994a; Merodio and De La Plaza, 1997)

The majority of volatile compounds that contribute to pawpaw flavor are short chain methyl and ethyl esters formed through enzymatic degradation of fatty acids during ripening (McGrath and Karahadian, 1994a). Volatile production was generally low in mature but unripe fruit and increased when fruit fully ripened. Similar trends were observed in fruit ripened on the tree versus those bench ripened. As with species such as banana (Engel et al., 1990), melon (Shalit et al., 2001) and mango (McLeod and Snyder, 1985), pawpaw aroma significantly increased during ripening and reached its maximum value in fully ripe fruit. Total volatile production increased by 140-fold when pawpaw fruit went from the unripe to the fully ripe stage (McGrath and Karahadian, 1994a; Shiota, 1991).

Despite its temperate origin, many of the esters found in tropical fruit like banana and mango have been detected in the pawpaw volatile profile, and pawpaw aroma has been described as banana- and mango-like (McGrath and Karahadian, 1994a,b). Ethyl

hexanoate, ethyl octanoate, ethyl butanoate, methyl hexanoate, methyl octanoate and methyl butanoate were the most abundant compounds in the pawpaw fruit headspace. Ethyl hexanoate alone represented more than 50% of total volatile profile of pawpaw fruit at harvest (McGrath and Karahadian, 1994b; Shiota, 1991). High concentrations of methyl and ethyl ester compounds and butanoate compounds such as ethyl butanoate are among the main components of aroma of many tropical fruit. Ethyl butanoate was one of the major components of mango aroma, methyl and ethyl-2-methylbutanoate were among the most predominant compounds in pineapple (*Ananas comosus*) aroma, and 3-methylbutyl butanoate was one of the most important volatiles in the banana volatile profile (Engel et al., 1990; McLeod et al., 1988; Pino and Mesa, 2006; Takeoka et al., 1991; Tokitomo et al., 2005). The similarities between the pawpaw volatile profile and those for mango, pineapple and banana could explain the tropical-like characteristics of pawpaw flavor.

In pawpaw, fruit volatile production followed the ripening trend and reached its maximum a few days after harvest (McGrath and Karahadian, 1994a,b; Shiota, 1991). The dominant compounds of soursop and cherimoya aroma were esters such as methyl hexanoate, and ethyl and methyl butanoate. On the other hand the most common compounds of atemoya flavor were sesquiterpenes such as  $\alpha$ - and  $\beta$ -pinene. In cherimoya, other components of the volatile profile were alcohols such as 1-butanol, 3-methyl-1-butanol, 1-hexanol, esters of butanoates, 3-methylbutyl esters, and acids such as hexanoic and octanoic acid. Moreover, following the first respiratory peak, an increase in ethanol content of the pulp and the production of off-flavour compounds was observed in several *Annona* species (Merodio and De La Plaza, 1997; Taylor, 1993).

Analyzing the pawpaw fatty acid profile, McGrath and Karahadian (1994b) concluded that saturated short and intermediate length fatty acids such as 8:0 and 16:0 fatty acids and unsaturated longer-chain fatty acids such as 18:1, 18:2 and 18:3 fatty acids could be precursors of volatile ester formation. However, even though many of the esters found in pawpaw are produced through fatty acid enzymatic degradation, and octanoic acid is the most abundant fatty acid in ripe pawpaw (Wood and Peterson, 1999; McGrath and Karahadian, 1994b), volatile profile composition may only be partially explained by the distribution of fatty acids in the fruit pulp (McGrath and Karahadian, 1994b).



Besides methyl and ethyl esters, low concentrations of propionic, butanoic, hexanoic, octanoic, decanoic, dodecanoic, tetradecanoic and hexadecanoic acids were detected in pawpaw extracts as well (Shiota, 1991). Moreover low amounts of farnesyl esters such as methyl (E)-(E)-farnesate, (E)-(E)-farnesyl butanoate and (E)-(E)-farnesyl acetate were detected. These compounds are extremely uncommon in the fruit aroma profile, and they may be responsible for the unique flavor of pawpaw fruit. Also, McGrath and Karahadian (1994b) reported small concentrations of lactones that may be responsible for the sweet, creamy, aromatic characteristic of this fruit. No aldehydes or alcohols were detected in pawpaw fruit extracts suggesting that the conversion of these compounds to esters is a rapid process (McGrath and Karahadian, 1994b). Despite these common traits, large variations in fruit aroma compounds were detected among pawpaw cultivars. Concentrations of ethyl butanoate and ethyl octanoate varied up to 10-fold among tree-ripened fruit harvested from different pawpaw varieties (McGrath and Karahadian, 1994b).

### **Postharvest Storage of Fruit**

Harvested fruit are still living organs; hence, even though they are detached from the plant, they continue to exchange gas with and lose water to the environment. Since the connection with the mother plant has been cut, the respiratory substrate and water losses that occur cause permanent changes in fruit composition (Burdon, 1997). Many preharvest and postharvest factors such as genetics, cultural practices, maturity at harvest and postharvest handling techniques influence composition and quality of fruit by the time it reaches the consumer. However, in contrast to preharvest factors, postharvest treatments cannot improve fruit quality above that of a fruit ripened on the plant, but rather only slow down the deterioration rate (Burdon, 1997; Kader, 2002). This is true of both climacteric and nonclimacteric fruit. Generally, the higher the respiration rate of a fruit, the shorter the postharvest shelf life (Kader, 2002). Once harvested, keeping fruit within their optimal range of temperature and relative humidity are the most important factors in maintaining fruit quality and minimizing postharvest loss (Kader, 2002; Crisosto et al., 1995).

## **Effect of Cold Storage on Fruit Quality**

Cold storage is the most common technique used to extend the shelf life of fruit. It has been shown that, above freezing, every 10 °C decrease in storage temperature slows fruit deterioration and overall quality loss by at least 2-fold. Fruit cooling decreases respiration rate and fruit softening, minimizes water loss, inhibits ethylene synthesis, and retards pathogen development and fruit decay (Lurie, 2003; Johnston et al., 2002).

Temperate climate crops such as pome fruit, kiwifruit and stone fruit are best stored near 0 °C. However peaches stored longer than 8 weeks between -1 and 1 °C often develop chilling injury symptoms such as internal browning and wooly texture (Crisosto et al., 1995; Lurie, 2003; Thompson, 2003b). On the other hand, tropical and subtropical fruit should be stored between 6 and 13 °C depending on species and cultivar. For this latter group, storage temperatures below the optimal range can cause chilling injury (Lurie, 2003; Crisosto et al., 1995). Cold injury development depends on the length of exposure, the actual temperature, cultivar, preharvest cultural practices, and the ripening stage of the fruit. It has been shown that ripening generally increases resistance to cold injury (Burdon, 1997).

Pawpaw fruit can be stored for one month at 4 °C with little loss in quality (Archbold et al., 2003). Cold storage delayed the ripening process of both ripe and unripe fruit and significantly delayed the loss of firmness. However, upon removal from cold storage, firmness declined rapidly accompanied by a rise in ethylene production and respiration. Like cherimoya (Merodio and De La Plaza, 1997), cold-stored pawpaw fruit exhibited a higher ethylene maximum than fruit ripened after harvest (Archbold et al., 2003). Preliminary observations indicated that longer storage periods resulted in external and internal black discoloration of the fruit (Koslanund, 2003), possibly symptoms of cold injury similar to other Annonaceae like cherimoya (Martinez-Tellez and Lafuente, 1997). Cherimoya exposed to low temperatures showed decreased sugar and phenolic metabolism (Merodio and De La Plaza, 1997). Ultrastructural analysis indicated that starch degradation, membrane structure, and cell wall disassembly were dramatically impaired.

## **Cold Injury Symptoms in Temperate and Subtropical Fruits**

Despite the fact that low temperature storage is the most effective postharvest approach for prolonging fruit and vegetable shelf life, the negative impact of low temperatures and cold stress have been studied in many crops. External symptoms of cold injury include skin discoloration and desiccation, pitting, internal breakdown, uneven ripening, development of large sunken areas, poor flavor and poor color development (Al-Haq and Sugiyama, 2004; Perez-Tello et al., 2001). Many variables such as species, ripening stage, use of light in the storage rooms, cold storage length, temperature and relative humidity affect the severity of cold stress symptoms. Cells exposed to cold temperatures often present structural abnormalities such as damaged chloroplasts and mitochondria, decreased numbers of starch granules, condensed nuclear chromatin and a high cytoplasmic concentration of reactive oxygen species (ROS) (Kratsch and Wise, 2000). Moreover cold-injured cells present primary membrane lesions, alteration of membrane properties (structure and composition) and increased electrolyte leakage (Salvador et al., 2005a,b; Sabehat et al., 1996; Valdenegro et al., 2005)

Cold temperatures cause metabolic dysfunctions as well. Low ethylene production, impaired photosynthesis, accumulation of acetaldehyde, ethanol and other toxic compounds, activation of proteolytic enzyme cascades, and programmed cell death have been observed in cold sensitive tissues after prolonged cold storage (Kratsch and Wise, 2000; Sabehat et al., 1996; Salvador et al., 2005b; Valdenegro et al., 2005).

Scanning electron microscope (SEM) observation of cold injured longan fruit (*Dimocarpus longan*) showed damage to fibrous tissue, the wax coating and epidermal hairs, and mesocarp and pericarp cells (Jaitrong et al., 2005). These events resulted in a water-soaked appearance and caused oxidative discoloration. Perez-Tello et al. (2001) observed that cold injury symptoms in carambola fruit stored at 2 or 10 °C for one month could be related to a reduction in sucrose content and increase in peroxidase (POD) and polyphenoloxidase (PPO) activity in injured fruit. In peach and nectarine, changes in membrane properties due to the modification of pectin metabolism and cell wall enzymes were responsible for the lack of juiciness and the abnormal tissue fragmentation (wooliness) developed by cold-stored fruit once transported to room temperature (Brummell et al., 2004). The development of wooliness symptoms was accompanied by a reduced respiration rate and ethylene production, decreased depolarization of middle

lamella homogalacturonan, reduced mobilization of polymeric arabinan, and accumulation of Ca-pectate gels in the membrane. Wooliness symptoms have also been observed in plums (*Prunus spp.*) and apricots (*Prunus armeniaca*), and in the most advanced stages the internal flesh shows rupture, discoloration and browning (Zhou et al., 2000; Brummell et al., 2004).

Though pawpaw can be cold stored up to 4 weeks without any major loss in fruit quality (Archbold et al., 2003), longer storage time caused loss of firmness, reduced respiration and ethylene production, and black discoloration (Archbold, personal communication). The discoloration in particular suggests some form of cold injury was occurring with extended storage.

Cherimoya, a close but tropical relative of pawpaw, is extremely sensitive to cold injury. Alique et al. (1994) showed that fruit stored at less than 8 °C for more than 5 days lose their ability to ripen normally and, once moved to 20 °C, develop severe skin browning and off flavor. In particular cold stored fruit had lower ethylene production, less softening and a higher pulp concentration of sucrose and citric acid when compared to fruit stored at higher temperatures (Alique et al., 1994; Maldonado et al., 2004). Munoz et al. (2001) reported that, compared to fruit stored at room temperature, cold-injured cherimoya fruit cells had a lower pH, higher phosphorous concentration, lower phosphorylation potential (ATP/ADP, Pi) and higher total soluble concentration.

The low respiration rate and impaired mitochondrial function often observed in cold-stored fruit suggests that a switch from aerobic to anaerobic metabolism takes place in the tissues (Munoz et al., 2001). Maldonado et al. (2004) showed that cold stored cherimoyas have higher titratable acidity, total malate and malate carboxylation/decarboxylation rate than fruit exposed to high CO<sub>2</sub> levels before being moved to storage temperature, a treatment that increased cold injury tolerance.

SDS-PAGE analysis of cherimoya fruit cold stored for 5 days at 6 °C revealed pulp accumulation of newly-synthesized peptides (21 – 50 kDa) in both early (first 3 days) and late storage phases. Some of these proteins could be responsible for the increase in proteolytic activity and in free amino acid content in chilled fruit (Montero et al., 1995). There were 3 phases according to the metabolic changes observed in cherimoya fruit during storage at cold temperature (Escribano et al., 2001, 2003):

- 1) Phase I. A transitory increase in the amino acid pool, a decrease in polyamine levels and a moderate increase in gamma-aminobutyric acid (GABA) during the first three days of cold storage
- 2) Phase II. A transitory increase in ammonia, phenylalanine ammonia lyase (PAL) activity and a consistent increase in putrescine and lignin content
- 3) Phase III. The development of visible symptoms of cold injury and a sharp rise in GABA.

### ***Fruit browning and phenolic metabolism***

Browning, after mechanical or physiological injury during harvest, processing or cold storage, changes biochemical and nutritional characteristics of fruits. Since the presence of brown pigments is often linked to unpleasant appearance and concomitant off-flavor development, this event affects consumer acceptability and palatability (Concellon et al., 2004). Enzymatic browning is caused by oxidation of natural phenolic compounds and, when uncontrolled, it can cause significant economic loss in both fresh and processed fruit markets (Gooding et al., 2001; Concellon et al., 2004). When molecular oxygen is present, phenolic compounds are oxidized to *o*-diphenols and *o*-quinones. These compounds can later polymerize and form melanins, black, brown or red compounds responsible for tissue browning (Thomas-Barberan and Espin, 2001; Sakihama et al., 2002).

Three enzymes of the phenolic oxidation pathway are considered key factors in browning development: PAL, PPO and POD (Nguyen et al., 2003).

### ***Phenylalanine ammonia lyase (PAL)***

PAL is the first enzyme involved in the phenylpropanoid pathway, and it represents one of the regulatory steps in the biosynthesis of many natural products (Gooding et al., 2001; Maldonado et al., 2001). PAL catalyzes the elimination of ammonia from L-phenylalanine and the consequent production of trans-cinnamate and trans-4-hydroxycinnamate. These compounds can be transformed via the cumarate pathway to chlorogenic acid and caffeic acid derivatives, optimal substrates for POD and PPO reactions (Gooding et al., 2001; Maldonado et al., 2002a; Nguyen et al., 2003).

The ethylene produced by the fruit during long term cold storage and subsequent room temperature ripening may stimulate PAL activity and be indirectly responsible for the accumulation of phenolic compounds in the fruit tissue, as noted in apple (Leja et al., 2003) and mandarin (*Citrus reticulata*) (Martinez-Tellez and Lafuente, 1997). It is not clear whether the increase in PAL activity occurs at the transcriptional, translational or post-translational level. However, in mandarin and banana peel, PAL transcript accumulation and an increase in enzyme activity were concomitant with the development of cold injury symptoms and necrotic spot formation (Lafuente et al., 2005; Nguyen et al., 2003). Temporary increases in PAL activity have been observed in carambola fruit as well during the first 10 days of storage at cold temperature (Perez-Tello et al., 2001).

The changes in PAL activity observed in cold stored fruit suffering from cold storage injury differs not only among species but also among cultivars within species. As an example, scald symptoms in 'Fortune' mandarin were correlated to a continued increase in PAL activity. However, in 'Navelate' mandarin, the rise in PAL activity was not maintained during the whole length of cold storage, and no differences in external symptoms were observed (Lafuente et al., 2005). Nguyen et al. (2003) found that in banana fruit PAL activity increased during cold storage but the rise was greater at 6 °C compared to 10 °C and more in 'Kluai Hom Tong' compared with 'Kluai Khai'.

A strong inverse relationship was found between PAL activity and total free phenolic content in banana peel. This suggests that the rate of phenolic oxidation was more rapid than the rate of phenolic synthesis by PAL (Nguyen et al., 2003). Similarly, Maldonado et al. (2002b) observed that accumulation of lignin in cell walls of cherimoya fruit exposed to cold storage was linked to an increase in PAL activity. In lettuce (*Lactuca sativa*) as well, wound-induced PAL activity led to an increase in soluble phenolic compounds (Thomas-Barberan et al., 1997).

Cherimoya fruit stored for 3 days at 6 °C showed a lower PAL activity than do fruit stored at ambient temperature (Maldonado et al., 2002a). Using this evidence the authors concluded that PAL activity was correlated to the ammonia re-assimilation rate. Since cold-stored fruit have low general metabolism and low ATP values, the ammonia assimilation/detoxification rate in these fruit is low as well causing a suppression of PAL activity.

Despite these differing patterns among species, many studies have concluded that the increase in PAL activity during cold storage may be a useful biochemical marker for onset of cold injury during cold storage (Lafuente et al., 2005; Nguyen et al., 2003).

### ***Polyphenoloxidase (PPO)***

The physiological significance of the PPO enzyme family is yet to be fully understood. Nonetheless, it is known to be involved in pigment formation, oxygen scavenging in chloroplasts, plant defense and tissue browning (Gooding et al., 2001; Ruoyi et al., 2005). The oxidation of phenolic substrates by PPO is believed to be the major cause of the brown discoloration observed in many fruits and vegetables, and it has been shown that potatoes with low PPO gene expression produce tubers that fail to brown during storage (Gooding et al., 2001; Nguyen et al., 2003; Ruoyi et al., 2005).

In banana the decrease of total phenolic concentration and the increase in PPO activity during cold storage were correlated with the development of cold injury and with the temperature during cold storage (Nguyen et al., 2003). Thus, cold injury with induced browning was causally related to the production of complex polyphenol compounds, and the production was catalyzed by PPO. As well, in eggplant (*Solanum melongena*), mango, and pineapple, the PPO activity in chilled tissues was directly correlated to the development of tissue browning (Concellon et al., 2004; Vela et al., 2003; Thé et al., 2001). Moreover, in fruit commonly cold stored for longer periods such as apple, a low PPO activity has been observed, not only when the fruit came out of cold storage, but also after 7 days of bench ripening (Leja et al., 2003).

However in some cold sensitive species, PPO activity was not so strongly affected by low temperatures: in cold-stored mandarin, the temperature effect on enzyme activity was negligible (Martinez-Tellez and Lafuente, 1997) and only a transient increase in PPO activity was observed in carambola fruit stored at cold temperature (Perez-Tello et al., 2001). However fruit used in both studies showed chilling injury symptoms when moved back to room temperature.

Even though the role played by PPO in tissue browning is not clear, the discovery of the enzyme's cellular localization and a close observation of structural modification caused by cold injury clarified many aspects of the enzyme action (Gooding et al., 2001).

In the cell, PPO enzymes have been found in 2 separate areas: associated with the internal thylakoid membranes and inside vesicles located between the plasmalemma and the cell wall. In immature fruit, PPO is predominantly located in plastids and chloroplasts of intact cells. In mature fruit the predominant form is soluble and located on the outside of the plasmalemma (Concellon et al., 2004; Gooding et al., 2001). However, phenolics are synthesized in the cytoplasm and stored inside the cell vacuoles and the cell wall (Gooding et al., 2001). When a tissue is exposed to cold temperature the spatial separation between PPO and its substrates is overcome: phenolic compounds may be deposited on the cell wall as a stress adaptation response and the membrane and organelle damage typically observed in cold injured tissues may bring PPO and phenolics in contact (Concellon et al., 2004; Gooding et al., 2001).

Despite the correlation between PPO activity and tissue browning, Gooding et al. (2001) indicated that fruit discoloration during ripening is not due to new enzyme synthesis but rather to the presence of pre-existing PPO protein. Northern blot analysis performed using PPO cDNA showed that PPO expression reached a peak during the early stage of fruit development and significantly decreased during the latter stages of ripening. This evidence suggested that PPO is produced only by young tissue, which stored the enzyme in the plastid where it gets associated with the internal thylakoid membranes. In this form it remains viable throughout fruit and plant development (Gooding et al., 2001).

### ***Peroxidase (POD)***

Peroxidases are extremely resistant to high temperature and isoenzyme forms have been isolated from both soluble and ionic fractions of apple pulp and peel (Valderama and Clemente, 2004). The POD enzyme family is involved in a great number of oxidative reactions such as color change, chlorophyll degradation, phenol and indole acetic acid oxidation, lignin biosynthesis, flavor development, pulp softening and fruit quality modification (Valderama and Clemente, 2004). Data collected from 'Fuji' apple and lychee fruit showed that POD activity was associated with color development and pericarp anthocyanin accumulation, flavor loss and the production of off-flavor volatiles often observed in cold-stored fruit (Zhang et al., 2003; Qie et al., 2004; Valderama and Clemente, 2004). Moreover, the flesh accumulation of these enzymes is the main cause of



apple senescence (Valderama and Clemente, 2004) and sugar apple fruit browning (Alves et al., 2001).

POD activity is often correlated with ethylene production. Hence, the activity of this enzyme increases during the onset of ripening in many climacteric fruit such as apple and tomato (Masia et al., 1998; Leja et al., 2003). As fruit ripening proceeds, a similar positive trend has been observed in cold-stored pineapple, apple and carambola fruit as well ( Leja et al., 2003; Perez-Tello et al., 2001; Thé et al., 2001). In particular, apple stored for 120 days at 0 °C showed an increased activity of POD not only during cold storage, but also once moved to room temperature (16 °C for 7 days) (Leja et al., 2003). In fruit such as pineapple and carambola the rise in POD activity during cold storage was related to chilling injury symptom development. However, in more resistant species such as apple the rise in POD activity was not related to injury symptoms. On the other hand, in chilled 'Fortune' mandarin fruit, POD activity decreased during cold storage (28 days at 2.5 or 10 °C) exposing the tissue to a high risk of free oxygen radical attack (Martinez-Tellez and Lafuente, 1997).

#### ***Cold storage effect on volatile compounds***

Generally, it has been shown that prolonged cold storage decreases total volatile production. A significant reduction in the volatile produced by peach cold stored for 14 days was found (Lafuente et al., 2005). Senesi et al. (2005) as well observed a general decrease in total volatile production of melon cold stored for a week. Interestingly, the most affected volatiles were esters and alcohols, the most abundant compounds in melon aroma. Similarly, a significant reduction in total volatiles, monoterpenes, sesquiterpenes, hydrocarbons, esters, aldehydes, and norisoprenoids was observed in mango stored below 20 °C up to 28 days. The degree of volatile reduction was positively linked with the intensity of chilling injury symptoms observed in the fruit after removal from cold storage (Nair et al., 2003).

Even in fruit normally cold stored over one month without chilling injury development such as apple and pear, storage eventually caused a decline of total volatile production. Once bench ripened for a few days, apple cold stored for 3 months had enhanced volatile production compared to fruit at harvest, but fruit cold stored for 6

months had reduced total volatile production and ester biosynthesis compared to harvest values (Dixon and Hewett, 2000; Lopez et al., 1998; Fallik et al., 1997). Similarly, pear cold stored for 15 weeks had lower total volatile production when compared with fruit cold stored for shorter periods (Ju et al., 2001).

On the other hand, no clear trends were observed for volatile production of wild strawberry cold stored up to 22 days. Compared to fruit at harvest, fruit cold stored for 22 days at 3 °C had higher methanol, *cis*-hexenol, acetic acid, acetaldehyde, ethanol and ethyl acetate production, but lower ethyl hexanoate, hexanoic acid, hexanol, hexanal, citronellol, 2-heptanone and 2-nonanone. However, no major changes were observed in ethyl butanoate, methyl butanoate, benzyl alcohol and furanone concentrations comparing the beginning to the end of cold storage (Almenar et al., 2006).

Cold storage might alter the aroma profile by effects upon the relevant biosynthetic enzymes. Prolonged storage of chilling-sensitive fruit such as persimmon (Salvador et al., 2005), grapefruit (*Citrus paradisi*) (Sapitnitskaya et al., 2006), strawberry (Perez et al., 1996) mango and avocado (Feygenberg et al., 2005) caused not only the development of cold injury symptoms such as pitting and brown discoloration, but also the accumulation of acetaldehyde, ethanol and ethyl acetate in both pulp and fruit headspace. This accumulation could be related to impaired mitochondrial metabolism and a switch from aerobic to anaerobic metabolism (Almenar et al., 2006; Eaks and Morris, 1956; Perez et al., 1996; Salvador et al., 2005; Sapitnitskaya et al., 2006; Sharom, 1994; Vazquez et al., 2005). As shown by Sharom et al. (1994), membrane electrolyte leakage of pericarp discs obtained from cold-stored tomato fruit increased significantly over the subsequent 4 days of bench ripening that followed cold storage. Moreover, evidence for a pronounced lateral phase separation of lipids within mitochondrial membranes was observed. These events would cause impaired respiration activity in the fruit and the activation of the anaerobic pathway for energy production (Eaks and Morris, 1956)

In contrast with the general increase in fermentative metabolite concentration during cold storage, Almenar et al. (2006) observed a decrease in the concentration of some of the most important compounds of wild strawberry aroma: ethyl hexanoate, 1-hexanol, citronella, benzyl alcohol, hexanal, 2-heptanone and 2-nonanone. In apple cold-stored for up to 6 months, a decrease in hexanal and (E)-2-hexenal concentration was

related to an increase in alcohol and total ester concentration (Muller et al., 2005). However, even though after 6 months fruit were still able to regenerate flavor and total volatile concentration when moved back to room temperature, total volatile production by bench ripened fruit significantly decreased once cold storage was extended to 7 months (Lopez et al., 1998).

Cold storage has had varying effects on the enzymes responsible for volatile production. Perez et al. (1996) observed that cold-stored strawberry had significantly lower AAT activity than fruit stored at room temperature. However, when cold storage exceeded a week, AAT activity slowly increased. The authors hypothesized that upregulation of the esterification pathway observed in cold-stored tissue could be interpreted as a detoxification response. In strawberry, acetaldehyde was transformed by ADH to ethanol which then became an AAT substrate for ethyl butanoate and ethyl acetate production. Since both ethanol and acetaldehyde accumulated in cold-injured tissue and can be volatile precursors, an increase in AAT activity could avoid the accumulation of these toxic compounds in the cell. A similar trend was observed in ADH activity during cold storage of strawberry and grapefruit (Perez et al., 1996; Ke et al., 1994; Sapitnitskaya et al., 2006).

In contrast, no correlation was found between ADH activity and ethanol in pear pulp (Lara et al., 2003). As suggested by the authors, ADH feedback inhibition caused by excess acetaldehyde production could explain the lack of correlation between enzyme activity and ethanol in cold stored pear.

In olive fruit, LOX activity significantly decreased during cold storage causing a reduction in LOX-derived volatile compounds such as C6 alcohol esters and C6 aldehydes derived from linoleic acid (Luaces et al., 2005). Significant effects on LOX activity have been observed in cold-stored versus room temperature-stored kiwifruit (*Actinidia deliciosa*) (WenPing et al., 2003). Low LOX activity and high incidence of tissue necrosis and black spots on the skin were observed in cold-stored guava fruit (Gonzalez-Aguilar et al., 2004). Lara et al. (2003) reported that the development of hypoxic conditions in pear fruit could cause a decrease in LOX activity in pear pulp, possibly leading to shortage of lipid precursors for ester biosynthesis.

### *Cold storage effect on fruit antioxidant systems*

The production of reactive oxygen species (ROS) and/or free radicals is an unavoidable consequence of electron transport in plant tissue. However, many kinds of stress events, such as exposure to pollutants or herbicides, accumulation of phytotoxic metals, drought, ripening, low temperature exposure and senescence, increase the production of free radicals and reactive oxygen compounds in plant tissue (Arora et al., 2002). The generation of reactive oxygen species in a tissue is a non-specific stress response. However, lipid content and composition, membrane fluidity, low adenylate energy charge, and cytoplasmic acidosis favor ROS production and the subsequent peroxidation of lipids (Blokhina, 2000).

Under normal or mild stress conditions, adequate protection against reactive oxygen species such as H<sub>2</sub>O<sub>2</sub> and other destructive ROS reactions is provided by an antioxidant defense system. Major components of this system are POD, glutathione reductase (GR), ascorbate reductase (AR), and antioxidant compounds such as phenolics, carotenoids, ascorbate, and glutathione (Lata et al., 2005; Arora et al., 2002). In particular, ascorbate peroxidase (APX), GR, ascorbate and glutathione are the major players in the most important hydrogen peroxide scavenging system known in living tissue: the Asada–Halliwell pathway or ascorbate–glutathione cycle (Arora et al., 2002).

When stress levels increase above normal or mild levels, the antioxidant defense system may only be moderately upregulated causing an imbalance between oxidative and antioxidative reactions with permanent tissue damage resulting (Arora et al., 2002). Under conditions of severe stress, polyphenol compounds can become a backup defense system and work cooperatively with the ascorbate–dependent pathway. Phenolics can scavenge ROS through direct chemical and enzymatic reactions. During these processes phenoxyl radicals are produced. In healthy tissue phenoxyl radicals can be reduced to their parent compounds by monodehydroascorbate (MDA) radical reductase or by non-enzymatic reactions with ascorbate (Sakihama et al., 2002).

As a general rule, species and/or cultivars with higher antioxidant potential could have better stress resistance and storage characteristics (Lata et al., 2005; Arora et al., 2002). However, when tissues are damaged and radical recovery processes fail, phenoxyl radicals can become prooxidant agents initiating free radical chain reactions in the

membranes and cross-linking with DNA and cell proteins (Sakihama et al., 2002). Moreover, phenolic metabolism may be related to tissue browning events observed in cold injured fruit (Concellon et al., 2004; Sakihama et al., 2002).

Phenolics are not the only compounds in cells that can act both as antioxidant and prooxidant. Fukumoto and Mazza (2000) showed that a phenolic like quercetin and also ascorbic acid exhibited prooxidant activity when added in low concentrations ( $\mu\text{M}$ ) to a linolenic acid emulsion exposed to air. Moreover, prooxidant activity increased when metallic ions were added.

Both the ascorbate-glutathione cycle enzyme activities and tissue phenolic content are increased by stresses such as drought and low temperature (Smirnoff, 1996; Lata et al., 2005). However, when stress conditions are too severe or persistent, tissue antioxidant protection is lost and the concentration of ascorbate, glutathione and phenolics may decrease (Lata et al., 2005; Smirnoff, 1996; Zhang and Kirkham, 1996). Lattanzio et al. (2001) found that apple phenolic levels initially increased during the first 100 days of cold storage, but they decreased when storage was prolonged after this point. The decrease in phenolic content was related to a decline in tissue resistance to infection. Similarly, a decrease in ascorbic acid and glutathione levels in overstored peaches and guava fruit was related to fruit senescence and overall quality deterioration (Gonzalez-Aguillar et al., 2004; Ruoyi et al., 2005; Wang et al., 2006).

### **Prevention of Cold Injury Symptoms**

Due to the importance of cold storage for postharvest handling many different approaches have been tested with the aim of preventing, reducing, or retarding cold injury, increasing crop cold tolerance and prolonging storage and shelf life. In general, prevention of cold injury appears to be linked to the fruits' ability to prevent cell wall degradation processes during storage, slowing the rate of decay and offsetting cellular acidification. When these goals are achieved, normal ripening can proceed properly once the fruits are rewarmed (Zhou et al., 2000; Merodio et al., 1998). Postharvest approaches such as methyl jasmonate infiltration, low  $\text{O}_2$  or high  $\text{CO}_2$  atmospheres, heat treatments and  $\text{Ca}^{2+}$  treatments have been partially successful in decreasing cold injury symptoms in

many crops, but additional research is needed for a better understanding of cold injury physiology and prevention (Lurie, 1998; Merodio et al., 1998; D' Aquino et al., 2005).

### *Alleviating cold storage injury by heat treatment*

Despite the decrease in fruit quality observed after cold storage, consumers still prefer fresh products over the canned or frozen alternatives. For this reason prolonging fruit shelf life and decreasing fruit perishability have been the aim of many studies (Paull and Chen, 2000; Ruoyi et al., 2005). In the previous decades, various approaches such as heat treatments, controlled atmosphere storage, modified atmosphere storage and Ca<sup>2+</sup> treatment have been evaluated with interesting results (Burmeister et al., 1997; Paull and Chen, 2000; Polenta et al., 2006; Ruoyi et al., 2005; Whitaker et al., 1997). Among these options heat treatment seems one of the more promising approaches for delaying fruit ripening, prolonging cold storage and reducing cold storage injury symptoms (Erkan et al., 2005; Lurie, 1998).

Especially after the banning of chemical fungicide and insecticide use after harvest, heat treatments such as hot water baths or high temperature air chambers have become commonly- applied fungal and insect control techniques (Erkan et al., 2005; Lurie, 1998). During research to develop heat treatments for postharvest pest control, it was observed that fruit tolerance to low temperature increased, cold storage injury symptoms decreased, and fruit quality was maintained for longer periods (Paull and Chen, 2000; Erkan et al., 2005; Lurie, 1998). Unfortunately, despite the benefits observed for many species, general guidelines are difficult to draw since different species, different cultivars, and fruit at different ripening stages respond differently to similar temperature/time combinations (Paull and Chen, 2000).

Heat treatments can be applied using hot water, vapor heat or forced hot air. Hot water dips (50–70 °C for 10–60 s) have been effective for fungal pathogen control and insect disinfestation. They can be safely applied directly at the storage/packaging area, and they are commonly applied in the commercial postharvest warehouse (Rodov et al., 1995; Erkan et al., 2005). On the other hand, vapor heat treatment (38–46 °C for 12–96 h) is a quarantine treatment normally applied to fruits and vegetables with the aim of eliminating insect eggs and larvae. In the past the heat was transferred to the fruit using

water condensation techniques. However, commercial facilities currently warm commodities to 40–50 °C by forcing vapor heat (95% RH or above) or moist air (58–90% RH) on the commodities placed in the heating chamber (Lurie, 1998; Thompson, 1996). Similar to the vapor heat treatment, exposure to hot air decreased fungal infection and was effective as a quarantine treatment. Hot air treatments can be considered long term treatments.

Both long duration/moderately high temperature and short duration/high temperature approaches have yielded positive results with many species (Table 1.3), though they have failed with many others (Paull and Chen, 2000). The discovery of the right time/temperature combination and the right methodology is key. Too low a temperature or too brief an exposure can be ineffective, but too high a temperature or too long an exposure will cause heat damage (Erkan et al., 2005; Florissen et al., 1996; Paull and Chen, 2000). Moreover, Erkan et al. (2005) showed that, even though hot air treatment provided the best decay control in ‘Valencia’ orange, the percentage weight loss was higher for these fruit when compared to hot water-dipped fruit.

Despite promising results obtained with many species, and the recent development of a commercially-viable hot water spray machine where fruit are exposed to 50–70 °C water for 10–60 s, the heat treatment mode of action is still not fully understood (Lurie, 1998). However, studies have shown that fruit exposure to high temperatures increased heat shock protein (HSP) transcript levels (Paull and Chen, 2000; Wang et al., 2001), caused fruit weight loss (Erkan et al., 2005), caused permanent or temporary enzyme inactivation due to mRNA temperature damage (Jacobi et al., 2002; Paull and Chen, 2000), caused ripening retardation (Polenta et al., 2006), caused delays in firmness change and softening (Paull and Chen, 2000; Polenta et al., 2006), increased sterol/phospholipid ratio and phospholipid content in cell membranes (Whitaker et al., 1997; Lurie et al., 1995), reduced total acidity due to malic acid metabolism (Polenta et al., 2006), increased fruit respiration (Lurie et al., 1995), increased fruit cell membrane leakage (Lurie et al., 1995), caused cytoplasm coagulation, cytolysis, nuclear changes and altered mitosis (Paull and Chen, 2000), caused reversible ethylene inhibition linked to the temporary loss of ACC oxidase and accumulation of ACC synthase (Paull and Chen,

2000), delayed the climacteric peak, lowered CO<sub>2</sub> and ethylene production once the fruits were moved to ambient temperature (Burmeister et al., 1997; Paull and Chen, 2000), inhibited carotenoid synthesis (Paull and Chen, 2000), caused flavor modification (Paull and Chen, 2000), inactivated cell wall degrading enzymes (Burmeister et al., 1997), caused the temporary inhibition of volatile synthesis and emission (Fallik et al., 1997), and reduced cold injury symptoms (Lurie, 1998; Rodov et al., 1995).

Among these events, HSP synthesis could play a key role in prevention of cold injury and protection of cell integrity. It has been shown that HSP production in response to high temperature exposure prevented irreversible protein denaturation and conferred to the fruit a temporary resistance to sub-lethal temperature. The acquired tissue thermotolerance was lost when HSPs decayed. Several classes of HSPs have been described in plants and named according to their molecular mass: HSP110, HSP90, HSP70, HSP60. A few HSPs with low molecular mass (10–30 kDa) have been isolated as well (Paull and Chen, 2000; Perdue, 1998). It has been shown that HSPs (14–70 kDa) can act as molecular chaperones controlling protein folding, protein transport across membranes and degradation of misfolded proteins (Paull and Chen, 2000; Sabehat et al., 1996). In some species such as papaya (*Carica papaya*), apple, and tomato, HSPs are produced within 30 min after exposure to temperatures in the range of 34–42 °C, and they have been detected in fruit pulp after 21 days at 2 °C (Paull and Chen, 2000; Sabehat et al., 1996).

Using a forced heat chamber system (45 °C) and exposure times of 1, 15, 30, 45 or 60 min, Perdue et al. (1998) showed that, after 12 h of cold storage, a detectable amount of HSP70 could only be found in cherries exposed for more than 1 h to heat. *In vitro* and *in vivo* studies suggest that HSPs induced by heat treatment persist in cold-stored tissues and protect the cells from programmed cell death normally observed under low temperature conditions (Paull and Chen, 2000; Perdue et al., 1998; Wang et al., 2001). However, low and high temperature tolerance changes in cells at different metabolic and cell cycle stages, so the results obtained in one study aren't easily generalized to other cell models or other fruit species (Wang et al., 2001). Generally though, a decrease in malic acid concentration (Polenta et al., 2006) and increase in fruit respiration (Lurie et al., 1995) following heat treatment suggests that heat treatment



enhances basal metabolism and boosts ripening processes. Thus, the heat treatment may 'prime' fruit to store better by an initial acceleration of the ripening process (Lurie et al., 1995).

Although many commodities have positively responded to heat treatment as already mentioned, improper methodologies have caused fruit damage. Excessively high temperature can cause peel browning, pitting, yellowing, abnormal softening, flesh darkening, increased decay and abnormal starch metabolism (Lurie, 1998). As a general guideline, the maximum temperature tolerance for plants can be located in the 42–60 °C range based on the original evolutionary habitat of the species (Paull and Chen, 2000).

Preliminary observations about the beneficial effect of heat treatment in slowing pawpaw ripening were reported by Koslanund (2003). Pawpaw exposed to 42, 46 or 50 °C for 15, 30 and 60 min prior to the beginning of bench ripening presented decreased ethylene production, a lower respiration rate and slower loss of firmness when compared to untreated fruit. Specifically, in heat-treated fruit, the activity of softening enzymes such as pectin methylesterase, endo- $\beta$ -mannanase, cellulase and polygalacturonase was, on average, 40% lower than the enzyme activities measured in control fruit. These preliminary results suggested that heat treatment approaches could be useful for increasing pawpaw fruit tolerance to cold storage and, consequently, extend cold storage length for more than 4 weeks.

#### ***Alleviating cold storage injury by intermittent warming***

Exposing fruit to high temperatures prior to cold storage is just one possible treatment strategy that has decreased cold injury symptoms and prolonged cold storage. Another occasionally successful approach has been the use of intermittent warming (IW) techniques, which consist of exposing fruit to one or more periods of ambient temperature during cold storage (Wang et al., 2003). Beneficial effects of IW were observed for several commodities, such as tomato (Artes et al., 1998a,b), mango (Nyanjage et al., 1998; Zhang et al., 1997), peach (Fernandez-Trujillo and Artes, 1997; Perkins-Veazie et al., 1999; Wang et al., 2003), citrus (Porat et al., 2003), orange (Shirra and Cohen, 1999), apple (Alwan and Watkins, 1999), and eggplant (*Solanum*

*melongena*), okra (*Abelmoschus esculentum*) and sweet pepper (*Capsicum spp.*) (Kluge et al., 1998).

Fernandez-Trujillo and Artes (1997) showed that peach subjected to 1 day at 20 °C for every 6 days at 2 °C, up to 21 days of cold storage, presented faster senescence, higher titratable acidity, lower firmness, higher maturity index (TSS/TA ratio), higher juiciness, less wooliness and better ground and flesh color compared to fruit kept in continuous cold storage. On the other hand, IW treatment prolonged tomato shelf life by at least one week and, when panel-tested, IW fruit were preferred to continuously cold-stored fruit for flesh and ground color, overall flavor and juiciness (Artes et al., 1998a,b).

Kluge et al. (2003) observed that the incidence of cold injury on ‘Tahiti’ lime (*Citrus spp.*) kept continually at 5 °C was 60%, but fruit warmed to 38 °C for 24 h every 7 days showed an incidence of 10-12.5%. On the other hand, fruit warmed to 38 °C for 24 h every 14 days showed no cold injury symptoms at all. However, after 60 days of cold storage, IW fruit warmed to 38 °C for 24 h every 14 days showed the greatest weight loss and respiration rate and the lowest titratability acidity and juice percentage. These effects are comparable with those observed in fruit stored under controlled atmospheres (Fernandez-Trujillo and Artes, 1997; Kluge et al., 2001).

As for physiological aspects of IW versus continuously cold-stored fruit, IW fruit have shown higher ethylene emission, higher respiration, minimal modification of the lipid profile, lower superoxide dismutase activity, and higher polygalacturonase, endo- $\beta$ -1,4-glucanase, ACO and ACS mRNA levels (Fernandez-Trujillo and Artes, 1997; Shirra and Cohen; 1999; Zhang, 1997; Zhou et al., 2001). Evidence suggests that IW treatments maintained the tissue capacity to ripen normally by preventing the inhibition of the ethylene pathway often observed in fruit cold stored for an extended period (Zhou et al., 2001).

Despite positive results with IW, the metabolic burst due to the temporary interruption of cold storage may cause unwanted effects such as chlorophyll degradation, fungal attack, loss of weight, exacerbation of senescence, and shriveling (Artes et al., 1998a,b; Fernandez-Trujillo and Artes, 1997; Kluge et al., 1998; Zhang et al., 1997). Moreover, when cold storage interruption was performed exposing the ‘Tahiti’ lime fruit to moderately high temperature (38–40 °C) for 24 h every 14 days, alterations in

respiration were observed, fruit developed odd aromas, and ethanol and acetaldehyde accumulated in the pulp (Kluge et al., 2003). Similarly Kluge et al. (1998) observed that, even though intermittent warming reduced chilling injury incidence in cold stored eggplant, pepper and okra fruit, severe mass loss and shriveling was observed. The application of IW techniques is not always successful. In many cases the rapid senescence observed in IW fruits significantly reduced their marketability (Kluge et al., 1998; Zhang et al., 1997).

### **Objectives**

Pawpaw [*Asimina triloba* (L.) Dunal] is becoming a high value alternative crop in the southeastern United States. However, the high perishability of the fruit is a significant obstacle to the development of a national pawpaw market. Low temperature storage is the most effective postharvest approach for prolonging fruit and vegetable shelf life. While pawpaw fruit may be stored at 4 °C for 4 weeks with minimal loss in quality (Archbold and Pomper, 2003), fruit cold stored longer than 4 weeks develop internal discoloration, dramatic loss of firmness, impaired ripening and general loss of quality once moved back to room temperature (Archbold et al., 2003; Koslanund et al., 2005; Archbold and Pomper, 2003).

Exposure to low temperatures above or below 0 °C during fruit storage can cause tissue damage in many species. Tropical species related to pawpaw in the Annonaceae family such as cherimoya are extremely sensitive to cold injury. Alique et al. (1994) observed that cherimoya stored lower than 8 °C for more than 5 days lose their ability to ripen normally and, once moved to 20 °C, develop severe skin browning and off-flavor. This response is often termed chilling injury and is common with tropical and subtropical species exposed to non-freezing temperatures below 10 – 12 °C (Arora et al., 2002). Cold storage results in some level of oxidative stress causing such injury. Though plant tissues can be protected against such stresses by their antioxidant systems, the capacity of these systems may be exceeded and injury will result.

Though prior observations suggested that cold injury may be occurring in pawpaw fruit cold-stored for more than 4 weeks, little is known about the effect that prolonged cold storage has on pawpaw post-storage ripening and fruit quality traits as well as on the

protective antioxidant pathways. In addition, there is no information available about possible genotypic differences in ripening behavior and the cold storage response.

Due to the importance of cold storage for postharvest handling and extending shelf life, many techniques have been tested with the aim of preventing, reducing or retarding cold injury, increasing crop cold tolerance and prolonging commodity storage and shelf life. Both heat treatments (Paull and Chen, 2000; Erkan et al., 2005; Lurie, 1998) and intermittent warming (Lurie, 1998; Wang et al., 2003) have been used successfully to abate cold storage injury in some fruit species. Preliminary observations indicated such treatments may alleviate injury in cold-stored pawpaw (Koslanund, 2003).

Hence, the aims of this study were:

- 1) to compare ripening behavior and selected fruit quality traits such as respiration, ethylene production, firmness, sugar and starch content, titratable acidity after harvest and after cold storage of some commercially-promising pawpaw varieties
- 2) to investigate the effect that cold storage has on fruit volatile profile and enzymes of volatile biosynthesis including alcohol acyltransferase, lipoxygenase and alcohol dehydrogenase activities
- 3) to evaluate genotypic variation in total antioxidant, phenolic, carotenoid and ascorbate content of some commercially-promising varieties at harvest
- 4) to study the effect of prolonged cold storage on fruit antioxidant protective systems including the glutamate-ascorbate system and phenolic metabolism, and
- 5) to determine if heat treatments or intermittent warming can alleviate chilling injury and extend cold storage life of pawpaw

**Table 1.1.** Pawpaw vegetative and fruit characteristics (Kral, 1960; Pomper et al., 2003; Peterson, 1991; Pomper and Layne, 2005)

<b>Characteristics</b>	
<b>Tree</b>	5 to 10 m tall Pyramidal habit in sunny locations Production of root suckers Cold hardiness and drought tolerance
<b>Leaves</b>	Oblong shape Glabrous 15 to 30 cm long, 10 to 15 cm wide
<b>Flowers</b>	Basipetal flower buds Three lobed dark – maroon petals Hypogynous Protogynous Pendant (4 cm long peduncle) Fetid aroma Flowering occurs between middle of April and middle of May Gynoecium composed of 3 to 7 carpels Natural pollinators: flies (Diptera), beetles (Nitidulidae), and nocturnal insects
<b>Fruits</b>	Oblong thin skinned berry Green color at ripening 3 to 15 cm long and 3 to 10 cm wide Single fruit or cluster (3 to 7 fruits) From 100 g to 1 kg of fresh weight Strong flavor (a mix of banana, pineapple and mango) 2 rows of seeds (12 to 20 seeds/fruit) Harvest period from end of August to end of September Firm, custardy, melting flesh Abscission at a firm but ripening stage Easily bruised when ripe

**Table 1.2.** Comparison of pawpaw nutritional value with other major fruit crops. The highest value among the four species is highlighted in bold (Peterson, 1982; (<http://www.pawpaw.kysu.edu/pawpaw>))

	<b>Pawpaw</b>	<b>Banana</b>	<b>Apple</b>	<b>Orange</b>
<b>Nutritional Value (g/100 g FW)</b>				
Protein	<b>1.2</b>	1.0	0.2	0.9
Total Fat	<b>1.2</b>	0.5	0.4	0.1
Carbohydrate	18.8	<b>23.4</b>	15.3	11.8
Dietary Fiber	2.6	2.4	<b>2.7</b>	2.4
<b>Vitamins</b>				
Vitamin A (retinol equivalent)	1	0.9	0.6	<b>2.3</b>
Vitamin C (international units)	30.5	15.2	9.5	<b>88.7</b>
Thiamin (mg/100gFW)	0.8	3.5	1.3	<b>6.7</b>
Riboflavin (mg/100gFW)	6.0	<b>6.7</b>	0.9	2.7
Niacin (mg/100gFW)	<b>6.5</b>	3.2	0.5	1.7
<b>Minerals (mg/100 g FW)</b>				
Potassium	9.9	<b>11.3</b>	3.3	5.2
Calcium	<b>7.9</b>	0.8	0.9	5.0
Phosphorus	<b>5.9</b>	2.5	0.9	1.8
Magnesium	<b>35.9</b>	9.2	1.6	3.2
Iron	<b>56.0</b>	2.5	1.4	0.8
Zinc	<b>6.7</b>	1.2	0.3	0.5
Copper	<b>22.2</b>	4.6	1.8	2.0
Manganese	<b>74.3</b>	4.3	1.3	0.7
<b>Essential Amino Acids (mg/100 g FW)</b>				
Histidine	21	<b>81</b>	3	18
Isoleucine	<b>70</b>	33	8	25
Leucine	<b>81</b>	71	12	23
Lysine	<b>60</b>	48	12	47
Methionine	15	11	2	<b>20</b>
Cysteine	4	<b>17</b>	3	10
Phenylalanine	<b>51</b>	38	5	31
Tyrosine	<b>25</b>	24	4	16
Threonine	<b>46</b>	34	7	15
Tryptophan	9	<b>12</b>	2	9
Valine	<b>58</b>	47	9	40

**Table 1.3.:** Some examples of heat treatment responses.

<b>Species - Cultivar</b>	<b>Treatments</b>	<b>Results</b>	<b>Reference</b>
'Valencia' oranges	<u>HWD</u> : 48 °C/12 min, 53 °C/3 min, 53 °C/6 min. <u>C</u> : 48 °C/12 min; 53 °C/3 and 6 min.	Every temperature / time combination applied decreased fruit decay after 2, 4 and 6 weeks of cold storage and increased consumer acceptance	Erkan et al., 2005
'Colt' tomatoes, mature green	<u>HWD</u> : 42 °C/30 or 60 min; <u>C</u> : 38 °C/72 h	Both HWD and C treatments applied before cold storage reduced total acidity, color development and increased acetaldehyde accumulation and fruit firmness	Polenta et al., 2006
'Dasher' cucumber	<u>HWD</u> : 25, 38 and 42 °C/ 30 min	Reduced cold injury symptoms	McCollum et al., 1995
'Latundan' banana, 'Carabao' mango, 'Indian' mango	<u>HWD</u> : 47-49 °C/10 min	Treatment delayed fruit ripening, disease development and improved shelf life	Acedo et al., 2002
Grapefruits, lemon, oroblanco, kumquat	<u>HWD</u> : 53 °C/ 2- 3 min <u>C</u> : 36 °C/72 h	Hot water dip more effective than curing on cold injury prevention	Rodov et al., 1995
'McIntosh', 'Cortland', 'Jonagold' and 'Northern Spy' apples	<u>C</u> : 46 °C 4, 8 or 12 h	Severe flesh browning and increased ethanol concentration of fruit treated for 12 h	Fan et al., 2005
'Kensington' mango	<u>C</u> : 47 °C/15 min; 40 °C/ 4 or 16 h + 47 °C/15 min	Heat-treated fruit showed starchy pulp patches due to $\alpha$ -amylase inactivation	Jacobi et al., 2002
'Golden Delicious' apple	<u>C</u> : 38 °C/4 days	Heating treatment slowed apple softening during storage and after storage ripening	Lurie et al., 1995
'Golden Delicious' apple	<u>C</u> : 38 °C/1, 2 and 4 days	Heat treatment shorter than 4 days at 38 °C caused extensive lipid catabolism, increased membrane leakage and accelerated fruit yellowing	Whitaker et al., 1997
'Hass' avocado	<u>C</u> : 38 °C/ 6, 12, 24, 36 and 48 h	6–12 h gave maximum protection against cold injury symptoms in preclimacteric fruit, but not in ripe fruit	Florissen et al. 1996

HWD = Hot Water Dip; C = Curing (hot air treatment)

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## CHAPTER 2

### LOSS OF RIPENING CAPACITY OF PAWPAP FRUIT WITH EXTENDED COLD STORAGE. I. RESPIRATION, ETHYLENE PRODUCTION AND NON-VOLATILE QUALITY TRAITS

#### INTRODUCTION

Pawpaw (*Asimina triloba* (L.) Dunal) is the only temperate member of the otherwise tropical Annonaceae family that includes cherimoya (*A. cherimola* Mill.), sugar apple (*A. squamosa* L.), soursop (*A. muricata* L.), custard apple (*A. reticulata* L) and atemoya (*A. squamosa* x *A. cherimola*) (Pomper and Layne, 2005). Pawpaw grows widely in the mesic hardwood forests of 26 states in the eastern United States ranging from northern Florida to southern Ohio and as far west as eastern Nebraska (Pomper and Layne, 2005). The trees grow up to 5–10 m in height, and they are the only member of this tropical family able to grow in a temperate climate (USDA growing zone 5). They are the largest tree fruit native to North America.

Pawpaw fruit are large, oval to oblong, thin-skinned berries containing two rows of almond-sized seeds (Pomper and Layne, 2005). They have a custard-like pulp and a pleasant aroma typical of tropical fruit. The pawpaw's unique taste, a mix of banana, mango and pineapple flavors, make it an interesting and potentially high-value alternative crop in the southeastern United States. As observed by McGrath and Karahadian (1994a), pawpaw ripening is characterized by rapid changes in firmness, peel color, soluble solids content and aroma volatile production. The decline in green color intensity is not a consistent trait among the different genotypes (Archbold, unpublished), and thus it is difficult to visually determine when pawpaw fruit ripening has commenced.

Pawpaw fruit are climacteric with both respiratory and ethylene peaks detected within 3 days after harvest (Archbold and Pomper, 2003; Archbold et al., 2003; Koslanund et al., 2005). During this same period, fruit soften rapidly. The most firm fruit at harvest are too soft for handling within 5 days. The decline in fruit firmness was correlated to the activity of cell wall-degrading enzymes such as polygalacturonase,



endo-(1→4)-glucanase, endo-β-1,4-mannase and pectin methylesterase (Koslanund et al., 2005). Due to the rapid decline in fruit firmness, pawpaw fruit are extremely perishable, a characteristic that they share with other members of the Annonaceae family, and this presents an obstacle in the development of a broader commercial market (Archbold et al., 2003; Koslanund et al., 2005a,b).

Archbold et al. (2003) reported that pawpaw fruit can be stored for one month at 4 °C with little loss in quality. Cold storage delayed the ripening process of both ripe and unripe fruit and significantly delayed the loss of firmness. However, upon removal from cold storage, firmness declined rapidly accompanied by climacteric increases in ethylene production and respiration. For cold-stored fruit the ethylene peak was higher than of fruit immediately after harvest, and it was detected within 4 days upon removal from storage. Preliminary observations indicated that longer storage periods resulted in external and internal black discoloration of the fruit tissues (Koslanund, 2003), possibly symptoms of cold injury similar to that found with other Annonaceae like cherimoya (Martinez-Tellez and Lafuente, 1997).

Cold injury symptoms are a response of many species to temperatures at or near freezing and of many tropical and subtropical species to non-freezing temperatures below 10–12 °C, often termed chilling injury (Arora et al., 2002). Fruit in cold storage may be susceptible to this problem. External symptoms of cold injury include scald or skin discoloration, skin desiccation, pitting, internal breakdown, uneven ripening, development of large sunken areas, poor flavor and poor color development (Al-Haq and Sugiyama, 2004; Perez-Tello et al., 2001). Physiological responses may include low ethylene production, impaired photosynthesis, accumulation of acetaldehyde, ethanol and other toxic compounds, activation of proteolytic enzymes, and programmed cell death after prolonged cold storage (Kratsch and Wise, 2000; Sabehat et al., 1996; Salvador et al., 2005; Valdenegro et al., 2005).

Cherimoya, in the same family as pawpaw, stored lower than 8 °C for more than 5 days lost their ability to ripen normally, and, once moved to 20 °C, developed severe skin browning and off-flavor (Alique et al., 1994). Munoz et al. (2001) reported that, compared to fruit stored at room temperature, cold-injured cherimoya fruit cells had a lower pH, higher phosphorous concentration, lower phosphorylation potential

(ATP/ADP, Pi), and higher total soluble concentration. These results, associated with the low respiration rate and impaired mitochondrial function often observed in cold-stored fruit, suggested that a switch from aerobic to anaerobic metabolism took place in the tissues (Munoz et al., 2001).

Other than the observation that cold injury may be occurring in pawpaw fruit cold stored for more than 4 weeks, little is known about the effect that prolonged cold storage has on post-storage ripening. In addition, there is no information available about possible genotypic differences in ripening behavior and the cold storage response. Hence the aims of this study were (a) to compare ripening behavior and the cold storage response of some commercially-promising pawpaw varieties, and (b) to investigate the effect that cold storage length has on selected ripening parameters including respiration, ethylene production, sugar content, and titratable acidity.

## **MATERIAL AND METHODS**

### ***Fruit harvest and storage***

Pawpaw fruit exhibiting some tissue softening as determined by touch were harvested from the Kentucky State University Research Farm, Frankfort, KY, on several dates during the August to October harvest season in 2004 and 2005. Based on fruit availability and tree yields in 2004, fruit were harvested from the following cultivars and advanced selections from the PawPaw Foundation breeding effort: 8-20, 9-58, 'Middletown', 'PA Golden', 'Taylor' and 'Taytwo'. In 2005 fruit were harvested from 1-7-2, 8-20, 'Middletown', 'PA Golden', 'Shenandoah', 'Taytwo', 'Taylor', 'Wells' and 'Wilson'. In addition 10 unripe fruit were collected from 'Taytwo' one week before normal harvest in 2005. Immediately following harvest, fruit were transported to the laboratory at the University of Kentucky, Lexington, KY.

On each harvest date ripening fruit were separated by variety and allocated to sub-groups for pre-determined storage lengths. In 2004 every storage group (after harvest, or 2, 4, 6, or 8 weeks at 4 °C) contained 15 fruit/variety. In 2005 every storage group (after harvest, or 2, 4, 6, or 8 weeks at 4 °C) contained 10 fruit/variety. During cold storage individual fruit were sealed in poly bags (permeable to CO<sub>2</sub>, O<sub>2</sub>, and C<sub>2</sub>H<sub>4</sub>), and ethylene

traps were added to the cooler. Upon harvest or removal from cold storage fruit were moved to ambient temperature ( $21 \pm 2$  °C) and ripened. Daily for 3 days in 2004, and on days 1 and 3 in 2005, fruit weight, firmness, respiration rate and ethylene production were assessed on 5 randomly-selected fruit, and after these measurements the fruit were peeled, sectioned and individually frozen in -80 °C storage.

### ***Ethylene production and respiration***

Rates of C<sub>2</sub>H<sub>4</sub> and respiratory CO<sub>2</sub> production by individual fruit were obtained by analyzing headspace composition of fruit held for periods in enclosed bottles as described by Archbold and Pomper (2003).

### ***Firmness***

The external firmness of each fruit was measured by compression with a Chatillon Force Gauge as described by Archbold and Pomper (2003).

### ***HPLC sugar analysis***

High performance liquid chromatography using pulsed electrochemical detection (HPLC-PED) analysis were performed on 3 replicate extractions from 8-20 fruit harvested in 2004 at the ripe stage. Soluble sugar samples were obtained through ethanol extraction as described below and the reconstituted aqueous solution was filtered through 0.45 µm Nylon filters (Costar Spin-X, Corning Inc., Corning, NY, USA) and diluted 1000X to be within the linear range of HPLC sensitivity. Sugars were quantified using a Dionex BioLC (Dionex Corp., CA, USA), with degassed NaOH (200 mM) and degassed water as mobile phase (9-91%, v/v), a sample loop volume of 25 µL, and on a CarboPac PA1 Analytical (P/N 46110) column at a flow rate of 1.0 mL/min. Quantitative determinations were made by comparing the sample results with an external standard mixture of sugars. Pawpaw sugars were identified by their retention times. 2-Deoxyglucose was used as an internal standard from which calculations of percent recovery were estimated. The amount of the major sugars identified, sucrose, glucose and fructose, were determined as amount per g fresh weight based on peak areas and percent recovery.

### ***Soluble sugar and starch quantification***

Soluble sugar and starch content were quantified from 'Taytwo' fruit harvested in 2005. Samples for analysis represented fruit that were unripe (one week before the start of harvest), ripening at harvest, and after 2, 4, 6 or 8 weeks of cold storage followed by 4 and 72 h of bench ripening. As described by Holland et al. (2002), 1 mL of 80% ethanol was added to 200 mg of frozen fruit pulp. The mixture was vortexed, incubated at 37 °C for 30 min and centrifuged at 14000 X g for 6 minutes. The supernatant was collected and the pellet was re-extracted 2 more times in the same manner. The combined supernatants were used for the determination of soluble sugars.

For sucrose and glucose quantification, the supernatant was dried and re-dissolved in 1 mL of Millipore water. For glucose analysis, 20 µL of the reconstituted soluble sugar fraction were added to 80 µL of water. For sucrose analysis, 20 µL of the soluble sugar fraction were added to 480 µL of Tris buffer (10 mM, pH 4.5), and 50 µL of invertase (10 mg invertase/mL Tris buffer), previously desalted through a Sephadex G-50 column, was then added. The mixture was vortexed, and incubated in a water bath at 55 °C overnight.

The pellet remaining after soluble sugar extraction was used for starch determination. To 5 mg of washed pellet, 1 mL of sodium acetate buffer (100 mM, pH 4.5) was added. The mixture was vortexed, boiled for 1 hour and cooled to room temperature. Then, 50 µL of desalted amyloglucosidase( 0.1 g in 1 ml sodium acetate buffer) (Sigma, St. Louis, MO) were added to each sample, and the mixture was vortexed, incubated in a water bath at 55 °C overnight, and centrifuged for 5 min at 14000 X g. The supernatant was diluted 100-fold with distilled water for analysis of the glucose released from starch.

Glucose in each resulting fraction above was quantified using the PGO enzyme color reagent kit (Sigma, St. Louis, MO). To 0.1 mL of diluted supernatant, invertase-treated supernatant, or amyloglucosidase-treated pellet solution, 1 mL of PGO reagent was added. The resulting solution was incubated at 37 °C for 30 min. Absorbance was read at 450 nm, and a glucose standard curve was used for quantification. Free glucose in pawpaw tissue extracts, and glucose released from sucrose and from starch were

determined. For determining glucose from sucrose, the free glucose was subtracted from that after invertase treatment with the resulting difference as that from sucrose only. Amounts for each are reported as mg glucose per g FW.

Pawpaw flesh starch content was also visually assessed by cutting fruit in half perpendicular to the stem-blossom axis, and soaking the halves in iodine solution (0.1% iodine, 1% potassium iodide in water).

#### ***Titrateable acidity***

Titrateable acidity (TA) was determined by mixing 10 g of pawpaw pulp with 20 ml of distilled water. The homogenate was filtered and the solution was titrated with 0.1 M NaOH to pH 8.1 as described by Polenta et al. (2006). Results were expressed as mmol malic acid equivalents/100 g FW since, as reported by Maldonado et al. (2004), this is the predominant organic acid in cherimoya, a species related to pawpaw fruit.

#### ***Statistical analysis***

All data were subjected to analysis of variance using SAS version 9.1 software (SAS Institute Inc., Cary, NC). Means were compared by Fisher's protected least significance difference (LSD,  $P=0.05$ ) when the number of replications was constant among treatments and by Tukey's test ( $P=0.05$ ) when the number of replications varied among treatments. Sugars, titrateable acidity and pH data were subjected to analysis of variance. Single degree of freedom contrasts were used to compare 4 vs 72 h values and determine if there were linear or quadratic trends across storage periods.

## **RESULTS AND DISCUSSION**

#### ***Ethylene production and respiration***

Ethylene production and respiration were measured after harvest and periods of cold storage in 2004 with 6 pawpaw cultivars: 8-20, 9-58, 'Middletown', 'PA Golden', 'Taylor' and 'Taytwo'. Both  $C_2H_4$  production (Table 2.1) and respiratory peaks (Table 2.2) were generally detected within 48 hours after harvest, and no major differences were observed among the cultivars. The highest ethylene and respiratory peak values for a

single fruit were 7.7  $\mu\text{g C}_2\text{H}_4/\text{kg/h}$  ('Middletown' one day after harvest) and 264  $\text{mg CO}_2/\text{kg/h}$  ('Taytwo' 4 h after harvest), respectively. The highest mean ethylene production was 2.3  $\mu\text{g C}_2\text{H}_4/\text{kg/h}$  for 'Middletown' 1 day after harvest and the highest mean respiration rate was 151  $\text{mg CO}_2/\text{kg/h}$  for 'Taytwo' 4 h after harvest.

Since the decline of fruit firmness is the only obvious indicator of fruit ripening, usually fruit are harvested after softening, and thus ripening, has begun (Archbold et al., 2003). Moreover, as shown by Archbold and Pomper (2003), firmer fruit at harvest exhibit a delayed ethylene peak when compared to softer fruit. Thus, the variability of the timing of the peaks among cultivars could be linked to the fact that fruit were at varying ripening stages at harvest.

No obvious or major differences were observed among cultivars in response to cold storage. Once returned to room temperature, fruit exhibited ethylene and respiration patterns consistent with those immediately after harvest with peaks within 3 days (Tables 2.1, 2.2). Only 'PA Golden' and 'Taytwo' fruit stayed mold-free until the end of the 8 weeks of cold storage. Thus, no data have been shown for 4 cultivars at 8 weeks due to the loss of samples. In addition to the loss due to mold, both the pulp and the skin of remaining fruit exhibited a black discoloration, a probable symptom of cold injury. Cherimoya, another Annonaceous species, stored at 8 °C for at least 5 days developed severe skin browning once moved to 20 °C (Alique et al., 1994), indicative of chilling or cold injury.

Due to fruit availability in 2005, 9-58 was not used, but the cultivars 1-7-2, 'Shenandoah', 'Wells' and 'Wilson' were added. In addition, since 2004 data were in agreement with the data already reported (Archbold and Pomper, 2003; Koslanund et al., 2005b), respiration and ethylene production data were only collected at 4 and 72 h after harvest or removal from cold storage.

At harvest, mean ethylene production ranged from 0.2 to 1.7  $\mu\text{g C}_2\text{H}_4/\text{kg/h}$  and respiration ranged from 73 to 129  $\text{mg CO}_2/\text{kg/h}$  (Table 2.3). For individual fruit, ethylene production ranged from none detected ('Taytwo') to 5.4  $\mu\text{g C}_2\text{H}_4/\text{kg/h}$  ('PA Golden'), and respiration from 39  $\text{mg CO}_2/\text{kg/h}$  ('Shenandoah') to 163  $\text{mg CO}_2/\text{kg/h}$  ('Wilson'). After 3 days of bench ripening, mean ethylene production ranged from 0.3 ('Taylor') to 2.7 (1-7-2)  $\mu\text{g C}_2\text{H}_4/\text{kg/h}$  and mean respiration ranged from 96 ('Shenandoah') to 146

(‘Taytwo’) mg CO<sub>2</sub>/kg/h. For individual fruit at 3 days of bench ripening, ethylene production and respiration values ranged from 0.25 µg C<sub>2</sub>H<sub>4</sub>/kg/h (‘Wells’) to 32 µg C<sub>2</sub>H<sub>4</sub>/kg/h (‘PA Golden’) and 66 mg CO<sub>2</sub>/kg/h (‘Taylor’) to 205 mg CO<sub>2</sub>/kg/h (8-20), respectively. The 2005 harvest data was consistent with both the 2004 data, though ethylene values were generally lower than those recorded in 2004, and those reported by Archbold and Pomper (2003) and Koslanund et al. (2005b).

During the 2005 cold storage period, many fruit were lost due to mold development as in 2004. Post-storage ethylene production means ranged from none detected (fruit of several varieties and cold storage periods) to 28 µg C<sub>2</sub>H<sub>4</sub>/kg/h (‘PA Golden’ at 2 weeks and 72 h), and respiration ranged from none detected (fruit of several cultivars and cold storage periods) to 214 mg CO<sub>2</sub>/kg/h (‘PA Golden’ at 2 weeks and 72 h). The data re-confirmed the 2004 results that there were no significant differences among the cultivars. However, in 2005, mean ethylene production after 2 weeks of cold storage was generally higher than that measured after harvest or after 4, 6 and 8 weeks of cold storage (Table 2.4).

#### ***Overall ethylene production and respiration***

Since the values and relative times of the ethylene and respiration peaks were comparable to those reported by Archbold and Pomper (2003) and Koslanund (2005b), we can conclude that after harvest pawpaw fruit respiration and ethylene peaks are reached within 3 days and range from 1–15 µg C<sub>2</sub>H<sub>4</sub>/kg/h and 90-242 mg CO<sub>2</sub>/kg/h, respectively (Tables 2.1, 2.2, 2.3). An overall assessment of ethylene production maxima as well as mean levels of production from 2004 and 2005 indicate that ethylene production is higher after 2 weeks of cold storage than after harvest (Tables 2.4, 2.5). Archbold and Pomper (2003) also reported that, once the fruit were moved back to room temperature after cold storage, the ethylene peak was significantly higher than just after harvest. The rapid ethylene production observed in fruit after removal from cold storage suggests that no irreparable damage occurred to the ethylene biosynthetic machine (Field et al., 1990).

A burst in CO<sub>2</sub> production following cold storage removal has been observed in many species. However, pawpaw failed to show such an increase in contrast to chilling

sensitive species such as squash, tomato and banana respiration where this burst is extremely amplified (Lyons and Breidenbach, 1990). Alferez et al. (2005) reported that a transient increase in ethylene production in 'Navelate' orange was an indicator of the initial cell damage occurring in citrus cultivars under low temperature storage. Specifically, the temporary increase in ethylene production was registered before the appearance of peel pitting resulting from cold injury. Field et al. (1990) observed a rapid increase in 1-aminocyclopropane-1-carboxylic acid (ACC) synthesis, ACC concentration, and ethylene production but not chilling injury symptoms were observed in cold stored cucumber upon transfer to room temperature. Even though a respiration increase could be an indication of cold sensitivity, the respiratory spike is not always related to cold injury development and cannot be used as a chilling injury index (Lyons and Breidenbach, 1990).

In pawpaw fruit the highest respiratory peak and mean values were most often registered after harvest and 2 weeks of cold storage (Tables 2.4, 2.5). In fact, fruit stored longer than 4 weeks had significantly lower post-storage respiration rates than fruit at harvest. As reported by Lyons and Breidenbach (1990) and Uritani (1978), mitochondrial metabolism in chilling sensitive plant tissue just removed from cold storage is significantly depressed by exposure to temperatures below 10 °C; hence the immediate response of sensitive tissues to chilling is the depression of respiratory activity. However, when tissue exposure to low temperature is prolonged, both vacuolar and mitochondrial membranes are permanently damaged causing a permanent imbalance in the cell respiratory activity. Moreover, the accumulation of fermentation products such as ethanol (Chapter 4) and acetaldehyde synthesized from glycolytic intermediates reflects the formation of an anaerobic environment inside the cell and the presence of a permanent imbalance between respiration and glycolysis in cold sensitive species (Lyons and Breidenbach, 1990). Monuz et al. (2001) observed a permanent switch from aerobic to anaerobic metabolism in cold-injured cherimoya fruit stored too long below 10 °C. Even though Archbold and Pomper (2003) didn't observe any differences between respiration values measured after harvest versus after cold storage up to 4 weeks, cold-stored pawpaw showed an obvious decline in respiration rate from 4 to 72 h after 4 weeks when compared to fruit at harvest (Table 2.5).



### ***Fruit firmness***

There was a large variation in fruit firmness at harvest among the varieties both seasons (Table 2.6). We have arbitrarily defined 15 N as a cutoff point for firm and shippable if  $> 15$  N and for immediate fresh market if  $< 15$  N. During the 2004 harvest, 9-58 and 'Middletown' fruit were most firm (32 N and 27 N, respectively) and 'Taytwo' fruit was least firm at 13 N. On the other hand, in 2005, 'Taytwo' and 'Middletown' fruit were the most firm (28 N and 26 N, respectively) and 'Taylor', 'Wells' and 'Wilson' were below the defined 15 N cutoff value.

After harvest, most varieties lost firmness very fast at ambient temperature. Across varieties fruit firmness decreased by 38% during the first 72 h. For all varieties firmness declined during cold storage as well. This decline in firmness during cold storage indicated that 4 °C storage did not stop fruit softening. The results of Archbold and Pomper (2003) showed little firmness loss by fully-ripe fruit (firmness  $\sim 6$  N) during cold storage. A separate category of unripe fruit with firmness  $> 50$  N at the start of cold storage showed some loss of firmness during cold storage, similar to the present work. A positive correlation between cold storage length and loss of firmness was observed in peach (Forlani et al., 2004) and cherimoya (Alique et al., 1994) as well.

After 2 weeks of cold storage the overall number of fruit with firmness greater than 15 N decreased from 71% (at harvest) to 19% (Table 2.7). By 6 weeks of cold storage the percentage of fruit with firmness greater than 15 N decreased to 9%. Even though no obvious differences in cold storage response was observed among the genotypes, as a general guideline we can say that firmer fruit are the best candidates for longer term cold storage as suggested by Archbold and Pomper (2003), as more will exhibit higher firmness values upon removal though they will be less firm than at the start of cold storage.

### ***Starch content***

Starch content of unripe fruit was 45 mg glucose/g FW (Table 2.8). Like McGrath and Karahadian's observations (1994a), starch content gradually decreased during ripening and during cold storage to levels  $\leq 10$  mg glucose/g FW at 4 weeks and after. This decline was also seen in iodine-stained fruit cross-sections (Figure 2.1). Starch

content of pawpaw at harvest is comparable to that of other starch-rich fruit like banana (Bierhals et al., 2004), and 2-fold higher than in apple (Wegrzyn et al., 2000). Loss of starch is a common phenomenon during ripening of many species, including banana (Bierhals et al., 2004), custard apple (Prasanna et al., 2000), cherimoya (Merodio and De La Plaza, 1997), and apple (Dunque et al., 1999a)

### ***Soluble sugar content***

Sucrose, fructose and glucose were the most abundant soluble sugars found in pawpaw fruit. HPLC analysis showed that ripe fruit contained sucrose at  $66 \pm 6$  mg/g FW, fructose at  $9 \pm 2$  mg/g FW, and glucose at  $5 \pm 2$  mg/g FW. Small quantities of other sugars such as myo-inositol at  $1.5 \pm 0.3$  mg/g FW, galactinol at  $0.02 \pm 0.01$  mg/g FW, sorbitol at  $0.2 \pm 0.1$  mg/g FW, mannitol at  $0.06 \pm 0.03$  mg/g FW, and galactose at  $0.23 \pm 0.02$  mg/g FW were detected as well. Peterson et al. (1982) reported that pawpaw fruit at harvest contained 60 mg/g FW sucrose, 13 mg/g FW fructose and 18 mg/g FW glucose, values comparable to the present work, while McGrath and Karahadian (1994a) reported 219 mg total soluble sugar/g FW in ripe pawpaw.

At harvest, 'Taytwo' fruit contained 39 mg sucrose/g FW and 3 mg glucose/g FW (Table 2.8). Compared to other species, pawpaw fruit contained 2-fold more sucrose and 2-fold less glucose than apple (Sun et al., 2000), 20-fold more sucrose and a comparable amount of glucose than carambola fruit (Perez-Tello et al., 2001), 3-fold less soluble sugars than *Annona muricata* (Peters et al., 2001), and 2-fold less sucrose and 13-fold less glucose than banana (Ni and Eads, 1993).

No significant differences in sucrose or glucose content were observed between unripe and fruit at harvest. However, after 72 h of bench ripening, both significantly increased, accompanied by the loss of starch previously described. Specifically, after 72 h of bench ripening, starch decreased by 32% and glucose and sucrose increased by 4- and 3-fold, respectively. Starch degradation accounted for only 13% of the soluble sugars synthesized by the fruit during ripening. In many species, starch breakdown and the consequent rise in glucose, fructose and sucrose concentration are characteristic ripening events (Tucker, 1993; Kader, 2002). Starch breakdown often explains only part of the increase in soluble sugars, especially sucrose (Hubbard et al., 1991; Bierhals et al., 2004).

For example, in mango (Hubbard et al., 1991) and apple (Dunque et al., 1999a), starch loss only accounted for approximately 50% of the increase in sucrose. Thus, alternative sources of C are probably used for sucrose synthesis. These C sources are unknown, though Hubbard et al. (1999) hypothesized that fructan reserves could play a role in soluble sugar synthesis in ripening mango.

During cold storage, sucrose content increased linearly for both 4 and 72 h measurements reaching its highest concentration by 4 weeks. However, no differences were observed between the beginning and end of bench ripening. Accumulation of soluble sugars during cold storage have been reported in many species such as the Annonaceous cherimoya (Alique et al., 1994) and custard apple (Prasanna et al., 2000) as well as banana (Narayana et al., 2002), mango (Narayana and Singh, 2000), peach (Oliveira et al., 2001), and apple (Dunque et al., 1999a)

The lowest glucose content was in unripe and harvested pawpaw fruit and the highest content was detected in fruit cold stored for 8 weeks (Table 2.8). During cold storage, glucose concentration increased linearly when measured at 4 or 72 h, though no differences were observed between 4 and 72 h at each storage period. Since glucose is involved in both ascorbic acid synthesis (Smirnoff, 1996) and pentose phosphate pathways (Dunque et al, 1999a), the decrease in ascorbic acid biosynthesis (Chapter 5) and respiration (Table 2.5) could partly explain the accumulation of this sugar in overstored fruit. In fact the decrease in ascorbic acid pools discussed in Chapter 5 is significantly correlated with the accumulation of glucose ( $r = 0.61$ ,  $P < 0.05$ ), but not with the accumulation of sucrose ( $r = 0.38$ ) in fruit pulp.

Starch content decreased linearly during cold storage when measured at 4 or 72 h. When plant tissues are exposed to low temperatures, starch may be hydrolyzed to soluble carbohydrates by  $\beta$ -amylase, sucrose may increase due to the activation of a cold-induced form of sucrose phosphate synthase, and hexose phosphates may accumulate in the cytosol as a result of the cold-lability of phosphofructokinase and acid invertase increasing sucrose hydrolysis (Deiting et al., 1998; Purvis, 1990). Even though accumulation of sugars increases tissue resistance to low temperature (Wismer, 2003), low temperature sweetening is an oxidative stress response of some plant tissues during prolonged storage. It has been shown that in both cold-stored potato tubers and apple

decreased respiration caused ATP accumulation and the consequent activation of the alternative oxidase pathway (cyanide-resistant pathway). This alternative pathway allows carbon flow through glycolysis and the citric acid cycle to remove excess carbohydrate and providing biosynthetic precursors. Overall, the alternative pathway reduces tissue carbohydrate imbalance and ATP excess, but it increases active oxygen species and consequent oxidative stress levels (Wismer, 2003; Dunque et al., 1999b)

### ***Titrateable acidity and pH***

Pawpaw fruit at harvest exhibited an average TA of 0.94 mmol malic acid equivalents /100 g FW (Table 2.9) and an average pH of 6.4. These values were comparable to those reported for cherimoya (Maldonado et al., 2004; Berger and Galletti, 2005) and 5-times lower than for peach (Fernandez-Trujillo and Artes, 1997) and apple (Defilippi et al., 2004a).

In contrast to cold-stored cherimoya (Maldonado et al., 2004), pawpaw TA did not significantly change during ripening or cold storage. However, pH significantly increased with ripening after harvest and 4 or 6 weeks of cold storage. After 8 weeks of cold storage the fruit pH had significantly decreased. Single degree-of-freedom analysis indicated that values at 4 h were significantly greater than those at 72 h across storage dates, and values at both times significantly decreased in linear (4 and 72 h) and quadratic (4 h only) trends. In cherimoya, malate accumulation in the cell was responsible for cytoplasmic acidification (Munoz et al., 2001). In fact, a high concentration of malate coupled with low respiration could be related to a switch from aerobic to anaerobic metabolism and a consequent cell accumulation of pyruvate and ethanol.

## **CONCLUSIONS**

The analysis of the ripening behavior of 10 different pawpaw cultivars (1-7-2, 8-20, 9-58, 'Middletown', 'PA Golden', 'Shenandoah', 'Taytwo', 'Taylor', 'Wells', and 'Wilson') over 2 seasons showed that all genotypes ripened similarly after harvest. For fruit held for up to 3 days at room temperature, CO<sub>2</sub> production ranged from 48 to 174 mg CO<sub>2</sub>/kg FW/h, ethylene production ranged from 0.2 to 2.7 µg C<sub>2</sub>H<sub>4</sub>/kg FW/h, and

firmness decreased by 30–50%. In both harvest years ethylene and CO<sub>2</sub> peaks were generally detected within 48 hours from harvest.

No differences in the cold storage response were found among the different genotypes. After cold storage, ethylene production ranged from none detected to 28 µg C<sub>2</sub>H<sub>4</sub>/kg/h, respiration ranged from 0 to 214 mg CO<sub>2</sub>/kg FW/h, and firmness declined as the storage period increased.

Interestingly, during bench ripening, pawpaw cold-stored for 2 weeks showed greater ethylene production and but not respiration rate across years. Upon removal from cold storage a dramatic increase in ethylene production may be observed in chilling-sensitive species, but it is not directly correlated with development of chilling injury symptoms and overstorage (Field et al., 1990; Lyons and Breidenbach, 1990; Purvis, 1990). However in pawpaw stored for periods longer than 2 weeks there were no increases in respiration or ethylene production. In fact pawpaw respiration declined as storage time lengthened. Prolonged exposure to low temperature may cause permanent damage to mitochondrial membranes and consequently lower tissue respiration rate (Uritani, 1978; Lester, 2003).

Once moved back to room temperature, fruit stored longer than 4 weeks developed a brown discoloration in both pulp and peel and had lower pH and higher soluble sugar content than fruit cold-stored for shorter periods. Low temperature sweetening, tissue acidification, reductions in respiration rate, and development of black discoloration of overstored fruit not only caused a loss of pawpaw fruit quality making them unmarketable, but they may be cold injury symptoms.

Overall, no obvious differences in ripening behavior or cold storage response were observed among the genotypes in this study, and low temperature storage did not stop fruit softening. After only 2 weeks of cold storage, fruit firmness had decreased by 38% with an average firmness lower than 15 N. Since firmer fruit of all genotypes at harvest still had a greater proportion of fruit with firmness > 15 N at 2 and 4 weeks of cold storage, as a general guideline, only firmer fruit (> 15 N) should be cold stored.

**Table 2.1.** Pawpaw fruit ethylene production by 6 cultivars upon harvest or removal from cold storage in 2004. Fruit were cold stored at 4 °C for 0 (Harvest) 2, 4, 6 or 8 weeks. Measurements were collected daily for 3 days after beginning bench ripening, and are expressed as  $\mu\text{g C}_2\text{H}_4/\text{kg FW/h}$ . The cultivars used include: 8-20, 9-58, ‘Middletown’ (MT), ‘PA Golden’ (PAG), ‘Taylor’ (TY), and ‘Taytwo’ (TT). Data are the average value of  $n = 5$  measurements. The maximum value for each cultivar is indicated by bold font. Overall SE = 0.09; Tukey’s critical value at  $P = 0.05$  is 5.04

Cultivar	Ethylene Production ( $\mu\text{g C}_2\text{H}_4/\text{kg FW/h}$ )					
	8-20	9-58	MT	PAG	TY	TT
<b>Day</b>	<b>Harvest</b>					
<b>0</b>	0.18	0.36	0.89	0.21	0.35	<b>0.61</b>
<b>1</b>	0.86	0.51	<b>2.31</b>	<b>0.78</b>	0.30	0.39
<b>2</b>	<b>1.22</b>	<b>1.13</b>	0.78	0.38	0.71	0.37
<b>3</b>	0.15	0.97	0.78	0.48	<b>1.05</b>	0.46
2 Weeks						
<b>0</b>	0.21	0.30	<b>7.00</b>	1.12	0.54	0.15
<b>1</b>	0.57	<b>0.49</b>	3.63	<b>1.26</b>	<b>1.29</b>	<b>1.88</b>
<b>2</b>	<b>1.82</b>	0.20	3.18	0.45	0.24	1.02
<b>3</b>	0.87	0.38	0.79	0.49	0.27	0.47
4 Weeks						
<b>0</b>	0.37	0.27	<b>3.77</b>	0.83	1.56	0.36
<b>1</b>	0.83	0.44	2.93	2.58	<b>2.61</b>	0.41
<b>2</b>	<b>2.27</b>	<b>1.18</b>	0.38	0.51	1.37	<b>1.30</b>
<b>3</b>	0.91	0.39	0.49	<b>4.87</b>	0.40	0.26
6 Weeks						
<b>0</b>	0.55	<b>0.62</b>	2.19	0.40	1.15	0.47
<b>1</b>	1.01	0.57	<b>2.77</b>	<b>0.80</b>	<b>2.14</b>	<b>1.91</b>
<b>2</b>	<b>3.18</b>	0.50	1.11	0.36	0.66	0.10
<b>3</b>	1.37	0.27	1.41	0.34	0.49	0.31
8 Weeks						
<b>0</b>	NA	NA	NA	0.47	1.80	NA
<b>1</b>	NA	NA	NA	0.25	<b>3.95</b>	NA
<b>2</b>	NA	NA	NA	<b>1.46</b>	1.87	NA
<b>3</b>	NA	NA	NA	0.25	0.49	NA

NA= data not available due to loss of fruit to mold.

**Table 2.2.** Pawpaw fruit respiration by 6 cultivars upon harvest or removal from cold storage in 2004. Fruit were cold stored at 4 °C for 0 (Harvest), 2, 4, 6 or 8 weeks. Measurements were collected daily for 3 days after beginning bench ripening, and are expressed as mg CO<sub>2</sub> / kg FW / h.. The cultivars used include: 8-20, 9-58, ‘Middletown’,(MT), ‘PA Golden’ (PAG), ‘Taylor’ (TY), and ‘Taytwo’ (TT). Data are the average value of n = 5 measurements. The maximum value for each cultivar is indicated by bold font. Overall SE = 2; Tukey’s critical value at P = 0.05 is 5.

Cultivar	Respiration (mg CO <sub>2</sub> /kg FW/h)					
	8-20	9-58	MT	PAG	TY	TT
<b>Day</b>	<b>Harvest</b>					
<b>0</b>	87	127	48	135	110	<b>152</b>
<b>1</b>	111	<b>156</b>	160	<b>156</b>	92	122
<b>2</b>	<b>143</b>	145	<b>174</b>	103	<b>131</b>	145
<b>3</b>	97	111	145	86	96	108
	<b>2 Weeks</b>					
<b>0</b>	66	74	109	<b>156</b>	105	48
<b>1</b>	92	109	109	148	<b>187</b>	<b>140</b>
<b>2</b>	<b>139</b>	<b>165</b>	<b>127</b>	98	104	131
<b>3</b>	122	108	93	110	99	113
	<b>4 Weeks</b>					
<b>0</b>	78	79	79	<b>90</b>	112	99
<b>1</b>	103	<b>101</b>	150	<b>90</b>	121	106
<b>2</b>	<b>128</b>	95	<b>129</b>	68	<b>122</b>	114
<b>3</b>	106	83	90	79	115	<b>115</b>
	<b>6 Weeks</b>					
<b>0</b>	67	68	61	94	57	38
<b>1</b>	<b>94</b>	98	112	<b>123</b>	63	30
<b>2</b>	90	85	<b>114</b>	61	<b>84</b>	45
<b>3</b>	64	<b>104</b>	60	8	62	<b>55</b>
	<b>8 Weeks</b>					
<b>0</b>	NA	NA	NA	<b>100</b>	<b>108</b>	NA
<b>1</b>	NA	NA	NA	94	106	NA
<b>2</b>	NA	NA	NA	73	55	NA
<b>3</b>	NA	NA	NA	68	41	NA

NA= data not available due to loss of fruit to mold.

**Table 2.3.** Ethylene production and respiration of pawpaw fruit in 2005 upon harvest or removal from cold storage. Fruit were cold stored at 4 °C for 2, 4, 6, or 8 weeks, and measurements were collected at the start and after 3 days of bench ripening. The cultivars used include: 1-7-2, ‘Middletown’ (MT), ‘PA Golden’ (PAG), ‘Taylor’ (TY), ‘Taytwo’ (TT), ‘Shenandoah’ (SH), ‘Wells’ (WE) and ‘Wilson’(WI). Data are the mean of n = 5 fruit. Ethylene: overall SE = 0.40, Tukey’s critical value = 4.50. Respiration: overall SE = 6; Tukey’s critical value at P = 0.05 is 0.5

<b>Cold Storage (weeks) and Bench Ripening (days)</b>										
<b>Week</b>	<b>Harvest</b>	<b>Harvest</b>	<b>2</b>	<b>2</b>	<b>4</b>	<b>4</b>	<b>6</b>	<b>6</b>	<b>8</b>	<b>8</b>
<b>Day</b>	<b>0</b>	<b>3</b>	<b>0</b>	<b>3</b>	<b>0</b>	<b>3</b>	<b>0</b>	<b>3</b>	<b>0</b>	<b>3</b>
<b>Cultivar</b>	<b>Ethylene production (<math>\mu\text{g C}_2\text{H}_4/\text{kg / h}</math>)</b>									
<b>MT</b>	0.33	0.90	0.95	0.46	0.97	0.30	0.54	7.59	0.27	2.27
<b>PAG</b>	1.65	13.0	0.71	28.12	0.48	0.76	NA	NA	NA	NA
<b>TY</b>	0.22	0.31	0.44	3.45	NA	NA	NA	NA	NA	NA
<b>TT</b>	0.22	1.41	0.22	7.34	0.67	5.78	1.07	0.10	0.86	0.01
<b>1-7-2</b>	1.68	2.71	NA	NA	0.14	4.55	NA	NA	NA	NA
<b>SH</b>	0.23	0.79	NA	NA	NA	NA	NA	NA	NA	NA
<b>WE</b>	0.83	0.48	0.01	10.70	0.57	4.50	0.00	0.05	0.05	2.17
<b>WI</b>	0.83	2.01	0.01	0.00	0.10	0.07	NA	NA	NA	NA
<b>Respiration (<math>\text{mg CO}_2/\text{kg / h}</math>)</b>										
<b>MT</b>	122	109	24	48	16	34	55	135	0	0
<b>PAG</b>	95	130	92	214	61	119	NA	NA	NA	NA
<b>TY</b>	129	100	83	155	NA	NA	NA	NA	NA	NA
<b>TT</b>	111	146	80	213	54	166	25	10	27	15
<b>1-7-2</b>	123	143	NA	NA	46	142	NA	NA	NA	NA
<b>SH</b>	73	96	NA	NA	NA	NA	NA	NA	NA	NA
<b>WE</b>	120	111	56	139	4	72	0	0	0	0
<b>WI</b>	120	102	0	0	0	0	NA	NA	NA	NA

NA= data not available due to fruit loss to mold.



**Table 2.4:** Pawpaw fruit ethylene production and respiratory peak and mean values following harvest or removal from cold storage in 2004. Fruit were cold stored at 4 °C for 0 (Harvest), 2, 4, 6 or 8 weeks. Measurements were collected daily for 3 days after beginning bench ripening, and are expressed as  $\mu\text{g C}_2\text{H}_2/\text{kg FW/h}$  and  $\text{mg CO}_2/\text{kg FW/h}$  for ethylene production and respiration rate, respectively. The cultivars used include: 8-20, 9-58, ‘Middletown’, ‘PA Golden’, ‘Taylor’, and ‘Taytwo’. Data are the average value of at least  $n = 2$  measurements. Values within each year followed by the same letter are not statistically different by Fisher’s LSD at  $P=0.05$ .

<b>Cold Storage (weeks)</b>	<b>Ethylene peak <math>\mu\text{g C}_2\text{H}_2/\text{kg FW/h}</math></b>	<b>Ethylene mean <math>\mu\text{g C}_2\text{H}_2/\text{kg FW/h}</math></b>	<b>Respiration peak <math>\text{mg CO}_2/\text{kg FW/h}</math></b>	<b>Respiration mean <math>\text{mg CO}_2/\text{kg FW/h}</math></b>
<b>2004</b>				
<b>Harvest</b>	1.18 NS	0.64 NS	152 a	123 a
<b>2</b>	2.29	1.29	152 a	118 a
<b>4</b>	2.67	1.31	118 bc	99 b
<b>6</b>	1.90	1.07	96 c	72 c
<b>8</b>	2.71	1.09	105 bc	82 bc
<b>2005</b>				
<b>Harvest</b>	NA	1.87 b	NA	115 ab
<b>2</b>	NA	4.62 a	NA	107 b
<b>4</b>	NA	1.76 b	NA	59 c
<b>6</b>	NA	0.93 b	NA	133 a
<b>8</b>	NA	0.93 b	NA	7 d

NA= due to data collection strategy these data could not be determined.

**Table 2.5.** Mean ethylene production and respiration of all pawpaw fruit harvested in 2004 and 2005. Fruit were cold stored at 4 °C for 2, 4, 6 or 8 weeks, and measurements were collected at the beginning and after 3 days of bench ripening. The cultivars used include: 1-7-2, 8-20, 9-58, ‘Middletown’, ‘PA Golden’, ‘Taylor’, ‘Taytwo’, ‘Shenandoah’, ‘Wells’ and ‘Wilson’. Data are the mean of at least 25 fruit. Ethylene production was expressed as  $\mu\text{g C}_2\text{H}_4 / \text{kg} / \text{h}$ , respiration as  $\text{mg CO}_2 / \text{kg} / \text{h}$  and firmness as N. Data within columns followed by different letters are significantly different by Fisher’s LSD at  $P = 0.05$ .

<b>Cold Storage (weeks)</b>	<b>Day</b>	<b>Ethylene (<math>\mu\text{g C}_2\text{H}_4 / \text{kg} / \text{h}</math>)</b>	<b>Respiration (<math>\text{mg CO}_2 / \text{kg} / \text{h}</math>)</b>
<b>Harvest</b>	0	0.58 c	111 b
<b>Harvest</b>	3	2.05 bc	113 ab
<b>2</b>	0	3.92 ab	81 cd
<b>2</b>	3	5.17 a	125 a
<b>4</b>	0	0.82 c	59 de
<b>4</b>	3	1.96 bc	94 bc
<b>6</b>	0	0.77 c	113 ab
<b>6</b>	3	1.55 bc	51 e
<b>8</b>	0	0.65 c	57 de
<b>8</b>	3	1.01 c	28 e

**Table 2.6.** Firmness of pawpaw fruit in 2004 and 2005 upon harvest or removal from cold storage. Measurements were collected at the start and after 4 and 72 hours of bench ripening. Fruit were cold stored at 4 °C for 2, 4, 6 or 8 weeks. The cultivars used include: 1-7-2, 8-20 ‘Middletown’ (MT), ‘PA Golden’ (PAG), ‘Taylor’ (TY), ‘Taytwo’ (TT), ‘Shenandoah’ (SH), ‘Wells’ (WE) and ‘Wilson’ (WI). Data are the mean of n = 4 fruit.

2004: Overall SE = 1; Tukey’s critical value at P = 0.05 is 5.

2005: Overall SE = 1; Tukey’s critical value at P = 0.05 is 5

Across years: Overall SE = 0.4; Tukey’s critical value at P = 0.05 is 5.

<b>Cold Storage (weeks) and Bench Ripening (days)</b>										
<b>Week</b>	<b>Harvest</b>	<b>Harvest</b>	<b>2</b>	<b>2</b>	<b>4</b>	<b>4</b>	<b>6</b>	<b>6</b>	<b>8</b>	<b>8</b>
<b>Day</b>	<b>0</b>	<b>3</b>	<b>0</b>	<b>3</b>	<b>0</b>	<b>3</b>	<b>0</b>	<b>3</b>	<b>0</b>	<b>3</b>
<b>Firmness (N)</b>										
<b>Cultivar</b>	<b>2004</b>									
<b>MT</b>	27	10	23	9	17	8	12	10	NA	NA
<b>PAG</b>	19	10	8	5	9	9	4	4	9	5
<b>TY</b>	16	13	8	4	8	4	5	3	5	5
<b>TT</b>	13	7	16	5	9	3	5	3	NA	NA
<b>9-58</b>	32	13	16	7	17	5	7	10	NA	NA
<b>8-20</b>	25	14	17	7	15	8	13	7	NA	NA
<b>2005</b>										
<b>MT</b>	26	11	10	8	6	6	7	5	6	3
<b>PAG</b>	20	7	11	8	4	4	NA	NA	NA	NA
<b>TY</b>	13	10	7	7	NA	NA	NA	NA	NA	NA
<b>TT</b>	28	13	25	10	12	2	7	4	4	3
<b>1-7-2</b>	23	23	NA	NA	6	5	NA	NA	NA	NA
<b>SH</b>	18	10	NA	NA	NA	NA	NA	NA	NA	NA
<b>WE</b>	14	14	13	10	4	7	5	4	5	4
<b>WI</b>	14	7	6	1	5	5	NA	NA	NA	NA
<b>Across years</b>										
<b>All</b>	21	13	14	9	10	6	7	5	6	4

NA= data not available due to fruit loss to mold.

**Table 2.7.** Proportion (%) of fruit with a firmness > 15 N in 2004 and 2005 recorded shortly after harvest or removal from cold storage. Fruit were cold stored at 4 °C for 2, 4, 6 or 8 weeks. The cultivars used include: 1-7-2, 8-20, 9-58, ‘Middletown’ (MT), ‘PA Golden’ (PAG), ‘Taylor’ (TY), ‘Taytwo’ (TT), ‘Shenandoah’ (SH), ‘Wells’ (WE) and ‘Wilson’ (WI).

		<b>Fruit with firmness &gt; 15 N (%)</b>			
<b>Week</b>	<b>Harvest</b>	<b>2</b>	<b>4</b>	<b>6</b>	<b>8</b>
<b>2004</b>					
<b>MT</b>	100	100	40	20	NA
<b>PAG</b>	60	20	20	0	0
<b>TY</b>	20	0	0	0	NA
<b>TT</b>	20	40	0	0	0
<b>8-20</b>	100	60	40	60	NA
<b>9-58</b>	100	80	40	0	NA
<b>2005</b>					
<b>MT</b>	100	0	0	0	0
<b>PAG</b>	67	20	0	NA	NA
<b>TY</b>	20	0	NA	NA	NA
<b>TT</b>	83	29	20	0	0
<b>1-7-2</b>	100	NA	0	NA	NA
<b>SH</b>	40	NA	NA	NA	NA
<b>WE</b>	40	20	0	0	0
<b>WI</b>	40	0	0	NA	NA
<b>Combined Years</b>					
<b>Overall</b>	71	38	17	9	0

NA= data not available due to fruit loss to mold.

**Table 2.8.** Glucose, sucrose and starch content of unripe (collected 1 week before harvest), ripe (on the harvest day) and cold-stored ‘Taytwo’ pawpaw fruit. Fruit were cold stored at 4 °C for 2, 4, 6 or 8 weeks and bench ripened for 4 or 72 h after harvest or removal from cold storage. Sucrose and starch content were expressed as mg of glucose released/g FW. To calculate mg sucrose, multiply the value by 0.52, and to calculate the total starch multiply by 0.9. Data are the average value of n = 3 measurements. Values followed by different letters are significantly different by Fisher’s LSD at P=0.05.

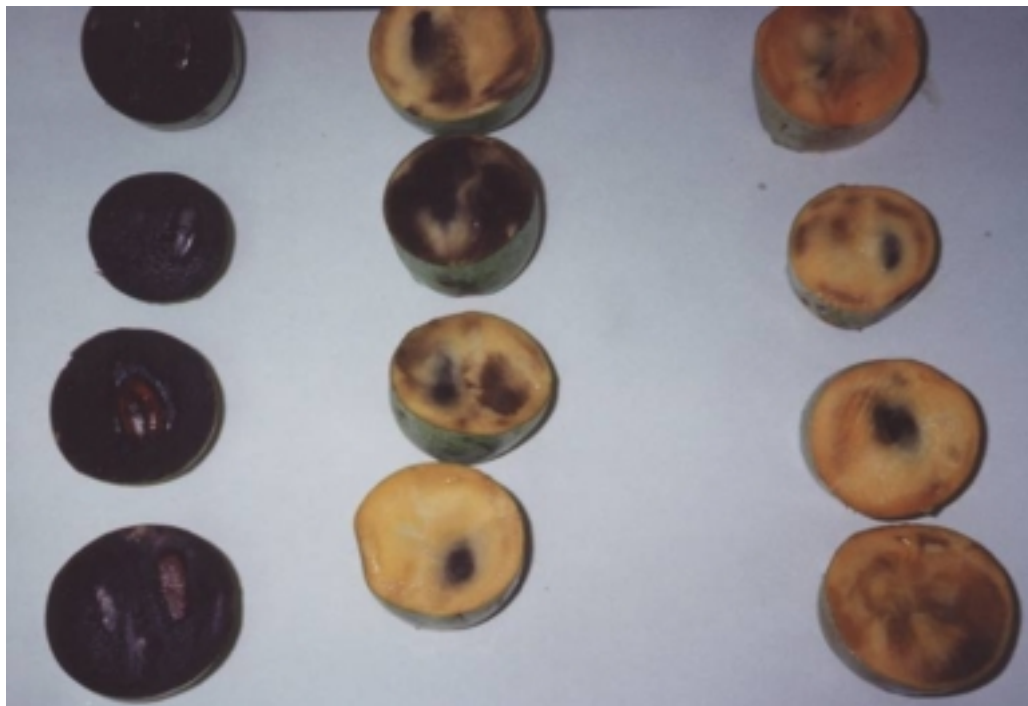
<b>Sugar content (mg glucose/ g FW)</b>				
<b>Cold storage (weeks)</b>	<b>Day</b>	<b>Glucose</b>	<b>Sucrose</b>	<b>Starch</b>
<b>Unripe</b>	0	3 d	22 c	45 a
<b>Harvest</b>	0	3 d	20 c	31 ab
<b>Harvest</b>	3	16 c	86 ab	21 bcd
<b>2</b>	0	8 cd	42 bc	31 ab
<b>2</b>	3	19 bc	89 a	8 cd
<b>4</b>	0	11 cd	80 ab	8 cd
<b>4</b>	3	14 cd	94 a	24 bc
<b>6</b>	0	19 bc	55 abc	9 cd
<b>6</b>	3	19 bc	94 a	6 d
<b>8</b>	0	38 a	100 a	10 cd
<b>8</b>	3	29 ab	59 abc	8 cd
<b>Single degree of freedom contrasts (P)</b>				
<b>4 vs 72 h</b>		0.8810	0.5330	0.9108
<b>4 h linear</b>		0.1220	0.0012	0.0314
<b>4 h quadratic</b>		0.0019	0.0005	0.5091
<b>72 h linear</b>		0.4313	0.0014	0.0403
<b>72 h quadratic</b>		0.0675	0.9616	0.8275

**Table 2.9.** ‘Taytwo’ pawpaw fruit titratable acidity and pH measured 1 week before commercial harvest (unripe), at harvest, and after 2, 4, 6, or 8 weeks of cold storage at 4 °C. Titratable acidity has been expressed as mmol of malic acid (MA) equivalents \* 100/100 g FW. Data are the mean of n = 4 samples ± SE. Data within columns followed by different letters are significantly different by Fisher’s LSD at P = 0.05. NS indicate no significant differences. Single degree of freedom contrasts have been indicated.

<b>Cold storage (weeks)</b>	<b>Day</b>	<b>Titratable acidity (mmol MA eq * 100 / 100 g FW)</b>	<b>pH</b>
<b>Unripe</b>	0	96 NS	7.48 a
<b>Harvest</b>	0	96	6.54 c
<b>Harvest</b>	3	95	7.38 ab
<b>2</b>	0	98	7.23 ab
<b>2</b>	3	97	7.40 ab
<b>4</b>	0	99	6.35 c
<b>4</b>	3	98	7.24 ab
<b>6</b>	0	100	6.48 c
<b>6</b>	3	96	7.16 b
<b>8</b>	0	100	5.34 d
<b>8</b>	3	101	5.21 d
<b>Single degree of freedom contrasts</b>			
<b>4 vs 72 h</b>		0.5595	0.0053
<b>4 h linear</b>		0.1918	< 0.0001
<b>4 h quadratic</b>		0.6497	< 0.0001
<b>72 h linear</b>		0.0645	< 0.0001
<b>72 h quadratic</b>		0.3535	0.0044

**Figure 2.1:** Iodine–starch assay performed on pawpaw fruit sections. Unripe fruit are on the left, ripe fruit with firmness between 5 and 25 N are in the center, and very soft, overripe fruit collected from the ground after natural drop are on the right. A. View of several fruit in each category. B. View of one fruit representative of each category.

A.



B.



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## CHAPTER 3

### LOSS OF RIPENING CAPACITY OF PAWPAW FRUIT WITH EXTENDED COLD STORAGE. II. AROMA VOLATILE PRODUCTION

#### INTRODUCTION

One of the most important parameters that determines fruit quality and consumer acceptability is aroma or flavor. The aroma characteristics of pawpaw fruit are due to the high concentration of methyl and ethyl esters in fruit headspace (McGrath and Karahadian, 1994b). The concentration of ethyl esters in pawpaw fruit at harvest ranged from 46% to 90%, and ethyl hexanoate was the dominant compound (Shiota, 1991; McGrath and Karahadian, 1994b). Aldehydes and alcohols were almost undetectable. Other major esters included ethyl butanoate and methyl octanoate which are also important contributors to the aroma of banana, pineapple, guava and mango (Engel et al., 1990; Nogueira et al., 2003; Sakho et al., 1985; Torres et al., 2007) and are the likely reason for their similarity to pawpaw.

Analysis of other Annonaceae family members showed that the main fruit volatile components were methyl 2-hexanoate (23.9%), ethyl 2-hexanoate (8.6%), methyl 2-octanoate (5.4%), and methyl 2-butanoate (2.4%) in soursoup (*Annona muricata*) (Jirovez et al., 1998); methyl 2-hexanoate, methyl butanoate, methyl butanoate and methyl hexanoate in sugar apple (*Annona squamosa*) (El Monem et al., 2003); and, 3-methyl-2-butenyl acetate (16.7%), methyl octanoate (15.6%), 3-methyl-3-buten-1-ol (9.6%) and methyl hexanoate (7.5%) in wild soursop (*Annona montana*) (Pino et al., 2002).

Fruit aroma is determined by a large number of volatile compounds, the distribution and biosynthesis of which are dependent on many factors, such as species, cultivar, maturity, and postharvest conditions (Dixon and Hewett, 2000; Olias et al., 2002; Shalit et al., 2001). Olias et al. (2002) showed that even though most commercial strawberry cultivars have similar aroma profiles, with few differences between European and American cultivars, both groups develop a significantly different aroma than the



related species *Fragaria vesca*, or wild strawberry. Shalit et al. (2001) found differences in aroma amount and composition among melon cultivars, as well as among melons harvested from the same genotypes but at different ripening stages. In pawpaw, some variation in total volatile production, ethyl hexanoate content, concentration of other fruit-like esters and fatty acid precursors have been observed among three cultivars after harvest (McGrath and Karahadian 1994b). Postharvest storage temperature can also affect volatile production. For example, volatile concentration in apples increased as storage temperature increased from -1 to 10 °C with a maximum intensity when storage temperature was 22 °C during 12 weeks of storage (Dixon and Hewett, 2000).

Aldehydes and alcohols are important components of fruit aroma in many species such as strawberry and banana. These compounds may not only directly define fruit aroma, but also have important roles as precursors in the biosynthesis of volatile esters (Olias et al., 2002; Wyllie and Fellman, 2000). The importance of esters in fruit aroma makes alcohol acyltransferase (AAT) and esterase, the enzymes catalyzing the formation and the hydrolysis of the ester bonds between an acyl moiety and an alcohol, the main enzymes in aroma formation. However, most agree that AAT and not esterase should be considered the key enzyme in fruit aroma biosynthesis (Olias et al., 2002; Wyllie and Fellman, 2000; Perez et al., 1996).

Since aroma is determined by alcohols, aldehydes, and other minor compounds, the enzymes involved in the synthesis of these compounds such as hydrolase, lipoxygenase (LOX), hydroperoxide-lyase, and alcohol dehydrogenase (ADH) may also play important roles in the synthesis of aroma components (Defilippi et al., 2005a,b; Perez et al., 2003). Ke et al. (1994) showed that the biosynthesis of ethyl acetate and ethyl butanoate by strawberry were related not only to AAT activity but to ADH activity as well. This is not always the case, though, as ADH activity in banana and apple apparently did not influence the characteristics of the final aroma profile (Wyllie and Fellman, 2000).

Prolonged cold storage at temperatures above freezing but below 10 °C causes volatile profile modification in tropical and subtropical fruit symptomatic of cold injury. Nair et al. (2003) showed that volatile production in mango was significantly correlated to cold storage temperature and length. Fruit stored below 15 °C up to 25 days had lower

total volatile production than fruit stored at a higher temperature and also developed cold injury symptoms such as black discoloration once moved back to room temperature. Storage of avocado, mango and grapefruit at cold injury-inducing temperatures caused the accumulation of anaerobic metabolites such as acetaldehyde and ethanol (Feygenberg et al., 2005; Vazquez et al., 2005). Cold storage might alter aroma by partial or complete inhibition of the activities of key aroma biosynthetic enzymes, causing changes in the fruit aroma profile. Luaces et al. (2005) reported a decrease in LOX activity in cold-stored olive, and Perez et al. (1996) observed that cold-stored strawberry had significantly lower AAT activity than fruit stored at room temperature.

Previous work (Chapter 2; Koslanund, 2003) indicated that pawpaw fruit stored at 4 °C longer than 4 weeks developed cold injury symptoms such as black discoloration, and low respiration rate. These fruit experienced a significant loss of ripening capacity and a general loss of quality. Since little is known about the effect of cold storage on pawpaw aroma biosynthesis and the volatile profile, the aims of this work were to (a) identify the major compounds of pawpaw aroma in ripening fruit, (b) determine if pawpaw cultivars vary in aroma profile, and (c) analyze the effect of cold storage on the pawpaw volatile profile and LOX and ADH activities.

## **MATERIALS AND METHODS**

### ***Fruit harvest and storage***

Pawpaw fruit were harvested from the Kentucky State University Research Farm, Frankfort, KY, on several dates during the August to October harvest season in 2005. Based on fruit availability and tree yields, fruit were harvested from the following cultivars and advanced selections from the PawPaw Foundation breeding effort: 1-7-2, 8-20, 'Middletown', 'PA Golden', 'Shenandoah', 'Taytwo', 'Taylor', 'Wells' and 'Wilson'. Immediately following harvest, fruit were transported to the laboratory at the University of Kentucky, Lexington, KY.

On each harvest date, fruit of each cultivar were allocated to different storage duration groups. Every group (after harvest or 2, 4, 6, or 8 weeks at 4 °C) contained 10 fruit/variety. Due to low yields and/or fruit loss to mold, not all varieties were used for

analysis after periods of cold storage. For cold storage, fruit harvested from ‘Taytwo’, ‘Wells’ and ‘Wilson’ were placed in poly bags (permeable to CO<sub>2</sub>, O<sub>2</sub>, and C<sub>2</sub>H<sub>4</sub>), and ethylene traps were added to the cooler. Weekly, cold-stored fruit were inspected and the ones that showed mold were eliminated. Upon harvest or removal from cold storage, fruit were moved to ambient temperature for 3 days of ripening. Five fruit/variety were peeled, sectioned and frozen in -80 °C storage at 4 and 72 h after harvest or cold storage removal.

### ***Volatile measurement***

Volatile profile at harvest was assayed for every cultivar. The volatile profiles of cold-stored fruit were measured after 4 and 72 h of bench ripening. Frozen tissue collected as described above was used for volatile measurement. Approximately 10 g-tissue samples were analyzed by methods described by Hamilton-Kemp et al. (2003). Briefly, samples were thawed in 30 mL glass jars sealed with Teflon-lined plastic screw caps containing a 3-layer septum. Samples were equilibrated in a water bath to 26 °C for 2 h and then placed at ambient temperature. The headspace in the bottle was sampled for 15 min using solid phase microextraction (SPME) employing a 100 µm poly(dimethylsiloxane) (PDMS) fiber. The SPME fiber was removed and inserted into a gas chromatograph (GC) (Model Hewlett Packard 5890 Series II, Agilent Technology, Wilmington, DE) equipped with a DB-5 column (60 m x 0.32 mm i.d., 1 µm film thickness) and a flame ionization detection (FID) detector. Volatiles were desorbed in the GC injection port for 5 min. Conditions for analysis were as follows: injection port temperature, 220 °C; FID detector, 240 °C; initial oven temperature, 35 °C held for 5 min and then increased to 184 °C at 2 °C min<sup>-1</sup>; injector splitless for 5 min. A modified splitless injection port was used so that both the septum and inlet purges were interrupted during SPME injections. The volatile compounds were ethyl alcohol, ethyl acetate, ethyl butanoate, methyl hexanoate, ethyl hexanoate, methyl octanoate, ethyl octanoate, ethyl decanoate, ethyl propionate, and methyl butanoate. With the exception of ethyl propionate, methyl butanoate, hexanoic acid and octanoic acid which were identified from retention times matching those of authentic standards, all other volatile compounds were identified using mass spectrometric analysis. Total volatile production (or that

adsorbed to the SPME fiber) of 3 fruit per sampling time was calculated summing the area units (AU) of both identified and unknown volatiles with AU > 20 (an arbitrary cut-off point). Data are presented as AU of volatile compound adsorbed to the SPME fiber during sampling, and are expressed as AU/g FW for each compound.

#### ***Alcohol acyltransferase assay***

Extraction and assay methods of AAT were based on those described by Ke et al. (1994) and Defilippi et al. (2005b). For each replicate, 6 g of pawpaw pulp tissue were homogenized in a Polytron homogenizer in 18 mL of 100 mM potassium phosphate buffer (pH 7.5) containing 2 g of polyvinylpolypyrrolidone (PVPP). The homogenate was filtered through four layers of cheesecloth and centrifuged at 27000 X g for 15 min at 4 °C. The supernatant was recovered, passed through a Sephadex 50 column pre-equilibrated with assay buffer, and used for the enzyme assay. The AAT activity assay was performed by adding 750 µL of potassium phosphate buffer (100 mM at pH 7.5), 10 µL of 1 M MgCl<sub>2</sub>, 100 µL of 10 mM 5,5'-dithiobisnitrobenzoic acid (DTNB), 20 µL of 20 mM isoamyl alcohol, and 20 µL of 50 mM acetyl CoA. The assay solution was gently mixed in a cuvette and 100 µl of fruit extract was added to a total volume of 1 mL. The increase in absorbance at 421 nm over time was measured spectrophotometrically (Model Cary 50 Bio, Varian Analytical Instruments, Walnut Creek, CA) to follow the production of the yellow thiophenol product (2-nitro-5-thiobenzoic acid) formed by the reaction of DTNB with free CoA released from the AAT esterification reaction. One activity unit (U) was defined as the increase in one unit of absorbance per minute, and results were expressed as specific activity (mU mg<sup>-1</sup> of protein). Protein was determined according to Bradford (1976) using BSA as a standard.

#### ***Alcohol dehydrogenase assay***

Based on methods for ADH assay in Defilippi et al. (2005a), 3 g of pawpaw pulp tissue of each replicate were homogenized in a Polytron homogenizer in 10 mL of 100 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 6.5) containing 2 mM dithiothreitol and 1% (w/v) PVPP. The homogenate was filtered through four layers of cheesecloth and centrifuged at 27000 X g for 15 min at 4 °C. The supernatant was

recovered, purified through a Sephadex 50 column and used for the enzyme assay. The reduction of acetaldehyde was followed spectrophotometrically at 25 °C by measuring the change in absorbance at 340 nm for 2 min of a reaction mixture containing 800 µL of MES buffer (100 mM at pH 6.5), 50 µL of 1.6 mM NADH, 100 µL of enzyme extract, and 50 µL of 80 mM acetaldehyde. The reaction was initiated by the addition of acetaldehyde. One activity unit (U) was defined as the increase in one unit of absorbance per minute, and results were expressed as specific activity (U mg<sup>-1</sup> of protein). Protein was determined according to Bradford (1976) using BSA as a standard.

#### ***Lipoxygenase assay***

LOX extraction and activity were measured modifying the methodology used by Myung et al. (2006). For each replicate, 3 g of pawpaw pulp tissue were homogenized in a Polytron homogenizer in 10 mL of 1 M Tris HCl buffer (pH 8.5), containing 15% sucrose, 10% (w/v) Triton X-100, 0.5% (w/v) PVPP, 1 M KCl, 10 mM MgCl<sub>2</sub>, and 1:50 protease inhibitor cocktail (Plant Fractionated Protein Extraction Kit (Sigma)). The homogenate was filtered through four layers of cheesecloth and centrifuged at 25000 X *g* for 15 min at 4 °C. The supernatant was recovered and used for the enzyme assay. LOX activity was assayed spectrophotometrically by mixing 980 µL of assay solution (40 mM Na acetate buffer, pH 4.5 containing 1 mM linolenic acid (10% ethanol solution), 0.14% (v/v) Tween-20, and 1 M KOH) with 20 µL of enzyme extract. The activity was followed by measuring the increase in absorbance at 235 nm due to the formation of hydroperoxides from linolenic acid for 1 minute. One activity unit (U) was defined as the increase in one unit of absorbance per minute, and results were expressed as specific activity (U mg<sup>-1</sup> of protein). Protein was determined according to Bradford (1976) using BSA as a standard.

#### ***Lipid peroxidation product measurement***

As described by Du and Bramlage (1992), the content of thiobarbituric acid–reactive substances (TBARS), indicative of the degree of lipid peroxidation, was analyzed by homogenizing 1 g of pawpaw pulp in 16 mL of 80% ethanol containing 0.01 % butylated hydroxytoluene (BHT). One mL of the supernatant collected after centrifugation

at 3000 x g for 10 min was added to 1 mL of 20% aqueous TCA solution containing 0.65% thiobarbituric acid. The mixture was placed in a hot water bath (95 °C) for 25 min and then cooled on ice for 10 min. After centrifugation at 3000 x g for 10 min, the absorbance of the supernatant was read at 440 nm for sucrose and fructose absorbance and 532 nm for lipid peroxidation products absorbance using a Cary Bio 50 UV spectrophotometer. TBARS was quantified using the equation of Du and Bramlage (1992).. Lipid peroxidation products were expressed as nmol malondialdehyde (MDA) equivalents/g FW.

### ***Statistical analysis***

All data were subjected to analysis of variance. Single degree of freedom contrasts were used to compare 4 vs 72 h values and to determine if there were linear or quadratic trends across storage periods. Means were compared by Fisher's protected least significance difference (LSD, P=0.05) using SAS version 9.1 software (S.A.S. Institute Inc., Cary, NC).

## **RESULTS AND DISCUSSION**

### ***Pawpaw volatile profile during ripening after harvest***

At harvest, when fruit were considered in the early stage of ripening, 'Wilson' produced a significantly higher amount of total volatile compounds, 10-fold or more, than the other cultivars which were similar to one another (Table 3.1). Total volatile production ranged from  $119 \pm 39$  ('Taytwo') to  $11550 \pm 1170$  ('Wilson' fruit)  $\text{AU} * 10^{-3} / \text{g FW}$ . However, as reported in Chapter 2, no unique characteristics indicative of advanced ripening such as unusually soft texture or high respiration and ethylene values were observed for 'Wilson'. Varieties with firmness comparable to 'Wilson', such as 'Shenandoah' and 'Taylor' produced significantly less total volatile.

Differences in fruit volatile profile composition and magnitude have previously been observed among pawpaw cultivars. McGrath and Karahadian (1994b) reported up to a 4-fold variation in total volatile production by 3 cultivars. Cultivars within other species have shown similar variation. Shalit et al. (2001) reported a 10-fold greater total ester production between melon cultivars. McLeod and Snyder (1985) reported significant

differences among total headspace volatiles of mango cultivars grown in Egypt, Florida and India; even though car-3-diene and  $\alpha$ -pinene accounted for 60% and 22% of total volatile production, respectively, in the Florida variety ‘Tommy Atkins’, these compounds were almost undetectable in the Indian variety ‘Jaffna’.

Classifying the known esters by their acyl (acetate, propionate, butanoate, hexanoate, octanoate, and decanoate) or alcohol (methyl and ethyl) groups, the dominant alcohol group at harvest for all but ‘Wilson’ was methyl accounting for 37% (‘Taytwo’, ‘Shenandoah’, ‘Middletown’) to 69% (‘PA Golden’) of total volatile emission (Table 3.1). The dominant acyl group was octanoate accounting for 38% (‘Shenandoah’) to 69% (‘PA Golden’) of total volatile production. Consequently, the most abundant volatile at harvest was methyl octanoate, accounting for 34% (‘Shenandoah’) to 65% (‘PA Golden’) of the total volatile profile. Ethyl esters accounted for 16% (‘Taytwo’) to 40% (1-7-2) of total volatile production, and ethyl hexanoate accounted for 2% (‘Taytwo’) to 18% (1-7-2) of total volatile compounds at harvest. Overall, in declining order after octanoates and hexanoates, decanoate or butanoate esters were the most abundant groups.

‘Wilson’ was unique in that the prevalent acyls and alcohols were the hexanoate and the ethyl and groups (Table 3.1). Hence the most abundant volatile was ethyl hexanoate at 49% of total volatile production. However, ‘Wilson’ fruit showed not only the highest total volatile production, but they also produced considerable free hexanoic and octanoic acids, considered off-flavor compounds (Defilippi et al., 2005a,b). This may indicate that ‘Wilson’ was nearly overripe at harvest, as overripe pawpaw fruit produced a higher amount of volatile acids including hexanoic and octanoic acids (Shiota, 1991). Since neither ‘Wells’ nor ‘Taytwo’ ever reached the same level of volatile production as ‘Wilson’, we may infer that ‘Wilson’ has a greater inherent capacity for volatile production than other cultivars.

During bench ripening for 72 h after harvest, total volatile production of ‘Taytwo’ and ‘Wells’ fruit were similar and increased significantly, 26-fold and 10-fold, respectively, compared to values at harvest (Tables 3.2, 3.3). In contrast, total volatile production of ‘Wilson’ significantly decreased from  $11150 \pm 1139 \text{ AU} \cdot 10^{-3}/\text{g FW}$  to  $2684 \pm 369 \text{ AU} \cdot 10^{-3}/\text{g FW}$  between the start and end of ripening. The rise of volatile production during ripening of ‘Taytwo’ and ‘Wells’ is consistent with the observation of

McGrath and Karahadian (1994a) that fruit volatile production dramatically increased during pawpaw ripening on the tree and on the bench for a few days after harvest. During bench ripening the rise in volatile production was principally linked to an increased emission of hexanoate and octanoate esters for both ‘Taytwo’ (Table 3.2) and ‘Wells’ (Table 3.3). The concentration of hexanoates increased by more than 700-fold during ripening.

The volatile analysis results obtained with ‘Wells’ fruit (Table 3.3), 56% ethyl esters and 37% methyl esters with ethyl hexanoate the dominant ester, were in agreement with those reported by Shiota (1991) and McGrath and Karahadian (1994b). Shiota (1991) observed that ethyl and methyl esters represented the principal components of ripe pawpaw aroma. Ethyl esters accounted for more than 80% and methyl esters accounted for about 2% of the total volatile profile. The most common compounds were ethyl hexanoate at 50% of the total aroma production and methyl octanoate at 2% of total aroma profile. Similarly, McGrath and Karahadian (1994b) showed that ethyl compounds by themselves accounted for 85% of the total volatile profile of pawpaw fruit harvest. Ethyl hexanoate was highest (31 to 65%) followed by ethyl butanoate (3 to 11%), methyl hexanoate (1 to 4%), methyl octanoate (2 to 5%) and ethyl octanoate (4 to 31%). In contrast, ripe ‘Taytwo’ fruit produced 18% ethyl esters and 65% methyl esters with methyl octanoate the dominant ester (Table 3.2).

Both McGrath and Karahadian (1994a,b) and Shiota (1991) found that ethyl butanoate accounted for 3% to 11% of total volatile production, but its presence was very low in the present work for both cultivars after harvest (Tables 3.3, 3.4, 3.5). This difference and others among these studies may be explained not only by the difference in ripening stage and varieties used, but also by the differences in volatile extraction methodology. While Shiota (1991) and McGrath and Karahadian (1994b) used organic solvents such as pentane/dichloromethane and diethyl ether to extract volatiles, these volatile compounds were collected from the headspace around thawed pulp in the current work. Buttery et al. (1969) reported that the amount of ester volatiles released by an aqueous solution such as the fruit pulp in the present work will increase with increasing volatile molecular weight. Thus, lower headspace values were associated with alcohols, intermediate values were associated with ketones and higher values were associated with



esters. Applied to the current study, the octanoates may be released somewhat more than hexanoates, and those more than butanoates. In addition, the adsorption characteristics of the SPME fiber for octanoates versus hexanoates versus butanoates may also have played some role.

Generally, across cultivars (Tables 3.1-3.4), four compounds, methyl octanoate, ethyl octanoate, ethyl hexanoate and methyl hexanoate, were the dominant esters produced at the time of harvest and subsequent ripening. Interestingly, some individual fruit produced only small amounts of methyl octanoate with no other compounds evident, suggesting it is the first volatile product indicative of ripening (data not shown). Since octanoic acid represented more than 20% of the total pawpaw pulp fatty acid (Wood et al., 1999; McGrath and Karahadian, 1994b), and octanoic acid was present at higher levels than hexanoic acid in the headspace (Tables 3.1-3.4), the presence of volatile esters of this fatty acid may be explained by its abundance.

Some of the esters produced by pawpaw fruit such as ethyl butanoate, ethyl hexanoate and methyl butanoate were observed in other Annonaceae like soursop, cherimoya, and custard apple (Merodio and De La Plaza, 1997; Taylor, 1993), and in tropical species such as banana, and mango (Engel et al., 1990; McLeod et al., 1988; Torres et al., 2007). Thus, the similar types and combination of volatiles produced by pawpaw may account for its tropical-like aroma and similarity to pineapple, mango and banana.

‘Taytwo’ and ‘Wells’ fruit produced similar total volatiles at each storage period and ripening time (Tables 3.2, 3.3). Both varieties produced the highest total volatiles after 2 and 4 weeks of cold storage and 72 h of bench ripening, which was shown in the combined data as well (Table 3.4). Across storage periods, there was a significant increase in total volatile production from 4 to 72 h of bench ripening (Table 3.5), an increase evident for each of the major volatiles produced. No linear or quadratic trends for total volatile production at 4 h after removal from cold storage across storage periods was found, nor was there a linear trend for values at 72 h after removal. However, there was a significant quadratic trend for total volatile production as well as all individual volatiles at 72 h after removal across storage periods. This reflects the fact that peak values occurred at 2 and 4 weeks of storage with lower values both before and after that.

Though total volatile production did not exhibit trends at 4 h or a linear trend at 72, some of the individual compounds did show trends over storage time. Of note, there were linear increases in those considered off-flavors including ethyl acetate, ethyl propionate, and hexanoic and decanoic acids.

Similar to that observed with mango (Nair et al., 2003), apple (Lopez et al., 1998) and strawberry (Perez et al., 1996), total volatile production of pawpaw fruit just removed from cold storage (at 4 h) were generally comparable to levels at harvest. On the other hand, the increasing volatile production observed in pawpaw during post-storage ripening (at 72 h) up through 4 weeks of cold storage was similar to the trend observed in apple cold stored for 3 and 5 months, where volatile production increased during bench ripening at room temperature (Lopez et al., 1998).

At harvest and through 4 weeks of cold storage, ester compounds were the major components of the total volatile profile. Depending on variety, they accounted for 38% to 96% of the total volatile pool (Tables 3.1-3.4). However their contribution to the volatile profile declined beyond 4 weeks of cold storage. For ‘Taytwo’ and ‘Wells’, esters represented 51% of total volatile profile at harvest and 74%, 72%, 48%, 44% of the total volatile profile of fruit just removed from 2, 4, 6, and 8 weeks of cold storage, respectively. For bench ripened fruit after removal from storage, esters also decreased after 4 weeks. Esters accounted for 90% of total volatile profile after harvest and for 95%, 92%, 68% and 49% of fruit bench ripened for 72 h after 2, 4, 6 and 8 weeks of cold storage, respectively.

The primary volatile compounds produced by ‘Taytwo’ and ‘Wells’ fruit from harvest through 4 weeks of cold storage were ethyl octanoate, methyl octanoate, ethyl hexanoate and methyl hexanoate (Tables 3.2, 3.3, 3.4), comprising 49% (at 4 h) to 83% (at 72 h) of the total volatile profile. The production of ethyl butanoate also increased over this period. However, once storage duration exceeded 4 weeks, the compounds indicative of cold injury and/or altered volatile metabolism, ethanol, ethyl acetate, ethyl propionate, octanoic acid and hexanoic acid, significantly increased from 13% (fruit at harvest) harvest to 45% by 6 weeks and 50% by 8 weeks of cold storage, while methyl and ethyl hexanoates and methyl and ethyl octanoates declined from 49% at harvest to 35% after 8 weeks of cold storage (Table 3.4). Similarly in bench ripened fruit, ethanol,

ethyl acetate, ethyl propionate, octanoic acid and hexanoic acid significantly increased from 2% (after harvest) to 18% by 6 weeks and 41% by 8 weeks. On the other hand, methyl and ethyl hexanoates and methyl and ethyl octanoates declined from 83% to 28% after 8 weeks (Table 3.4).

Ethyl esters were the dominant group at 4 weeks of cold storage and after (Table 3.4). The similarities among volatile profiles observed at harvest and after 2 and 4 weeks of cold storage demonstrated that pawpaw fruit can be cold-stored as long as 4 weeks without any major loss in quality as indicated by Archbold and Pomper (2003). After 4 weeks of cold storage, fruit headspace composition changed and would likely affect consumer perception.

Though the increased production of one ester may be related to the increase of all the other esters that share the same alcohol or acyl moiety, substrate/product relationships aren't necessarily so clear. Many carbon chain donors such as amino acids and lipids can also be used as substrates (Baldwin, 2002). As an example, in pawpaw an increase of the octanoic acid concentration was not related to an increase of methyl octanoate or ethyl octanoate production. Similarly, the increase in ethyl acetate concentration in fruit headspace coincided not only with an increase in its direct precursor ethanol, but also with a decrease of ethyl butanoate and hexanoate. Analogous volatile trends have been observed in strawberry by Ke et al. (1994).

Anaerobic respiration can cause the accumulation of acetaldehyde, ethanol and ethyl acetate (Almenar et al., 2006; Perez et al., 1996; Salvador et al., 2005). In strawberry stored under modified atmospheres with O<sub>2</sub> lower than 2%, a rise in ethyl acetate and ethanol concentrations in the headspace was related to a switch between aerobic and anaerobic metabolism (Ke et al., 1994; Perez et al., 1996). Perez et al. (1996) indicated that methyl and ethyl acetates, and ethanol, were important contributors to off-flavor development. Accumulation of acetaldehyde and ethanol were also observed in cold-sensitive fruit cold stored under regular atmosphere of the pawpaw-related cherimoya (Merodio et al., 1998) as well as by grapefruit (Vazquez et al., 2005), mango, and avocado (Feygenberg et al., 2005).

Independent of atmospheric gas composition, long-term exposure to cold temperature may cause mitochondrial impairment and a switch between aerobic and

anaerobic metabolism (Merodio et al., 1998; Feygenberg et al., 2005). Sharom et al. (1994) found that membrane electrolyte leakage observed in cold-stored tomatoes increased significantly over 4 days of bench ripening that followed the end of cold storage. Evidence for a pronounced lateral phase separation of lipids within mitochondrial membranes was observed. Since membrane damage is a characteristic symptom of cold injury, it is not surprising that a switch to anaerobic metabolism and the accumulation of acetaldehyde and ethanol in sensitive species were accompanied by the development of other cold injury symptoms such as peel pitting and pulp discoloration (Eaks and Morris, 1956; Salvador et al., 2005; Sapitnitskaya et al., 2006; Sharom et al., 1994; Vazquez et al., 2005). These events would also cause impaired respiration in the affected fruit and the activation of the anaerobic pathway for energy production (Eaks and Morris, 1956). In the current study with pawpaw, the evidence for this switch includes both the accumulation of ethanol in fruit pulp (Tables 3.2, 3.3) and a decrease in respiration rate (Chapter 2) by fruit cold stored longer than 4 weeks.

#### ***AAT activity during ripening and cold storage***

In ‘Taytwo’ pawpaw, AAT activity was detected at an early stage of ripening. At harvest, AAT was 44 mU/mg protein (Table 3.6). These values are comparable to those reported for apple (Defilippi et al., 2005a), strawberry (Perez et al., 1996), and are double the activity of banana (Harada et al., 1985). No differences have been observed among AAT activity data measured after 4 and 72 upon removal from cold storage or harvest; however, both data groups linearly decreased with cold storage length.

Despite the correlation between AAT activity and total volatile production observed in melon by Shalit et al. (2001), no significant correlation ( $P > 0.05$ ) was observed between AAT and total volatile production ( $r = 0.51$ ), or between AAT activity and total esters ( $r = 0.51$ ) in pawpaw. Though the highest total volatile production was observed after 2 or 4 weeks of cold storage followed by 72 h of bench ripening (Table 3.6), AAT activity at these times did not differ from other storage times.

Factors other than AAT activity may be responsible for limiting volatile production in many species. In ‘Greensleeves’ apple, Defilippi et al. (2005a) observed that high levels of AAT activity were accompanied by low levels of ester accumulation.

‘Fuji’ apple cold stored under air or under three different controlled atmosphere conditions showed no difference in AAT activity even though aroma profile was modified among different cold storage groups (Lara et al., 2006). These results suggest that substrate availability was a more decisive factor than enzyme activity for volatile production after cold storage. The availability of primary precursor substrates, such as fatty acids and amino acids, may play an important role in the final volatile profile (Defilippi et al., 2005a; Perez et al., 1996; Wyllie and Fellman, 2000). In general, fatty acids and amino acids are considered the major precursors of aroma volatiles in many species. These compounds are produced during fruit ripening and transformed to aldehydes, ketones, acids, alcohols, and esters by many biosynthetic pathways such as  $\beta$ -oxidation, hydroperoxy acid cleavage, and LOX- and ADH-mediated modification (Defilippi et al., 2005a; Griffiths et al., 1999; Wyllie and Fellman, 2000).

#### ***ADH activity during ripening and cold storage***

ADH activity in ‘Taytwo’ fruit (Table 3.6) was almost 50- and 3-times lower than ADH activity measured in apple (Defilippi et al., 2005a) and tomato at harvest (Speirs et al., 2002), respectively. Even though the variability of the data was high due to the limited replication and the variability in ripening stage at harvest, enzyme activity did not change during ripening after harvest, though differences in ADH activity at different stages of ripening have been observed in tomato (Chen and Chase, 1993) and grape (Sarni-Manchado et al., 1997). Speirs et al. (2002) reported that a genetically-manipulated tomato fruit line with enhanced ADH activity showed an increase in C6 alcohols, hexanol and Z-3-hexenol in the aroma profile.

Similar to peach (Bellincontro et al., 2005), no clear changes in ADH activity were observed during cold storage of pawpaw, with the exception of a high value in fruit cold-stored for 2 weeks after 72 h of bench ripening. No significant linear or quadratic trends were observed in the present data, and no significant differences were observed between data collected after 4 versus 72 h upon harvest or removal from cold storage (data not shown). Thus, ADH activity did not limit alcohol availability for ester production. The low concentration of aldehydes in the pawpaw aroma profile observed by McGrath and Karahadian (1994b) could be partially explained by the low or

negligible ADH activity observed in the present work. However, aldehydes were not quantified in this study.

### ***LOX activity during ripening and cold storage***

LOX activity at harvest (Table 3.6) was comparable to that reported for apple (Defilippi et al., 2005a) and strawberry (Perez et al., 1999). As with the ADH activity above, the variability of the LOX data was high.

Though no significant differences were observed in LOX activity at harvest versus just after removal from 2, 4, 6, or 8 weeks of cold storage, significantly higher activities were registered in fruit ripened just after harvest or after 2 weeks of cold storage. In fact single degree-of-freedom contrast analysis of 4 h data across cold storage periods showed a quadratic trend ( $P < 0.01$ ). This ripening-related increase was lost by 4 weeks. No other trends could describe LOX activity.

A TBARS assay showed that some lipid peroxidation had occurred in both harvested and cold stored fruit. However no significant difference were found among TBARS values measured in fruit bench ripened for 4 or 72 h upon harvest or removal from cold storage (Table 3.7). Lipid peroxidation product concentration was significantly correlated ( $P > 0.05$ ) with LOX activity ( $r = 0.64$ ). Autocatalytic lipid degradation occurs not only during senescence or oxidative stress but also during normal fruit development (Lester, 2003). During ripening, some of the products of lipid catabolism enter into the volatile biosynthetic pathway and some are recycled to regenerate cell membranes. On the other hand, in senescent tissues the membrane regeneration capacity is lost. Since fruit can ripen normally after harvest or 2 weeks of cold storage (Chapter 2), the high level of LOX activity and lipid peroxidation products in these fruit at 72 h may be explained by lipid degradation typical of ripening tissues. However, in fruit stored for 4 weeks or longer, no differences in LOX activity at 4 or 72 h were observed (Table 3.6). Similarly, in kiwifruit (Xu et al., 2003) and guava (Gonzalez-Aguilar et al., 2004), LOX activity slowly decreased during the first weeks of cold storage and, once it reached its minimum value, remained essentially constant thereafter

There were no significant correlations ( $P > 0.05$ ) of ADH ( $r = 0.24$ ) or LOX ( $r = 0.07$ ) activity with AAT activity, nor between ADH activity ( $r = 0.52$ ) or LOX ( $r = 0.40$ ) and total volatile production.

Lara et al. (2003) studied long-term cold storage of pears, and ADH and LOX activity *in vivo* were reduced by substrate limitation and by the accumulation of ethanol. Ethanol accumulation, an event typical of an hypoxic environment such as with overstored fruit (Chapter 2), has been shown to inhibit ADH activity (Lara et al., 2003). However, the data collected for pawpaw fruit did not suggest any direct inhibition. With the exception of fruit cold stored for 2 weeks and bench ripened for 72 h, activity of both LOX and ADH stayed fairly constant during cold storage, and no significant trends were observed in overstored fruit. As reported by Gonzalez-Aguilar et al. (2004) for guava, no differences were observed in LOX activity measured in fruit just after harvest or removal from cold storage. However, cold-stored guava transferred to 25 °C showed a large increase in LOX activity consistent with ripening initiation promoted by the temperature change. The lack of a LOX activity rise in pawpaw fruit cold stored longer than 2 weeks could indicate that these fruit already suffered some metabolic impairment, even though they still ripened normally once move back to room temperature.

## CONCLUSIONS

No significant differences were observed in total volatile production among all but one variety. ‘Wilson’ showed significantly greater total volatile production than the other varieties. Despite the high values of volatile production at harvest, total volatile production by ‘Wilson’ rapidly decreased during both bench ripening and cold storage, whereas all other varieties exhibited significant increases in volatile production. Since ‘Wilson’ did not show characteristics related to an advanced ripening stage of the fruit at the time of harvest (Chapter 2), this cultivar may exhibit an altered pattern of volatile production within the framework of overall ripening and is unfit for cold storage, but rather should be consumed fresh shortly after harvest.

With the exception of ‘Wilson’, after harvest total volatile production ranged from 100 AU \* 10<sup>-3</sup> / g FW (‘Taytwo’) to 1178 AU \* 10<sup>-3</sup> / g FW (‘PA Golden’). The most

common compounds were methyl and ethyl octanoates and hexanoates. Ethyl and methyl esters represented 4% and 47% of total volatile production at harvest and 39% and 52% of total volatile production after 72 h of bench ripening, respectively. Cold storage for up to 4 weeks resulted in no significant differences in the volatile profile compared to freshly-harvested fruit, when considered immediately following removal from storage. However, fruit cold stored for 2 and 4 weeks and bench ripened for 72 h had significantly higher total volatile production than fruit bench ripened directly after harvest.

The enhanced aroma production and similarity to at-harvest profiles confirmed the marketability of these fruit as a fresh product through 4 weeks. Prior studies had already indicated that pawpaw fruit can be stored up to 4 weeks without any major loss in other fruit quality traits like firmness and appearance (Archbold et al., 2003; Archbold and Pomper, 2003; Koslanund, 2003).

In fruit stored longer than 4 weeks, methyl octanoate and ethyl hexanoate levels decreased and ethanol, ethyl acetate, octanoic and hexanoic acid levels increased. Many studies have considered these off-flavour compounds that could be used as indicators of anaerobic metabolism and cytoplasmic acidification, typical events that occur in cold-injured fruits like grapefruit, apple, cherimoya, avocado and mango (Feygenberg et al., 2005; Lopez et al., 1998; Merodio et al., 1998; Vazquez et al., 2005). In pawpaw, increased ethanol in overstored fruit suggest that long term exposure to cold caused mitochondrial impairment and a switch from aerobic to anaerobic metabolism as shown for cherimoya (Merodio et al., 1998), avocado and mango (Feygenberg et al., 2005).

AAT is considered the key enzyme in the volatile production of fruit of many species (Olias et al., 2002; Perez et al., 1996). However, there was no clear relationship between AAT activity and volatile production of pawpaw fruit. The lack of significant relationships suggest that other factors such as substrate availability could play an important role defining pawpaw aroma. The rise in enzyme activity at 8 weeks could be related to a detoxification response to ethanol accumulation in the cells as in strawberry (Perez et al., 1996).

Neither cold storage nor bench ripening had any effect on ADH activity. However, LOX activity rose during ripening after harvest and 2 weeks of cold storage. Lipid peroxidation and a rise in LOX activity occurs in ripening fruit (Gonzalez-Aguilar



et al., 2004; Lester, 2003); thus the high level of LOX activity observed in ripening pawpaw was likely evidence of normal fruit ripening. In fruit stored longer than 2 weeks, LOX did not increase during ripening. This could indicate that fruit stored longer than 2 weeks already exhibit some metabolic impairment, perhaps an early indicator of cold storage injury.

**Table 3.1.** Headspace volatile profile composition of pawpaw cultivars at harvest. The cultivars were: 1-7-2, 8-20, ‘Middletown’ (MT), ‘PA Golden’ (PAG), ‘Shenandoah’ (SH), ‘Taytwo’ (TT), ‘Taylor’ (TY), ‘Wells’ (WE), and ‘Wilson’ (WI). The major volatiles detected were: ethyl alcohol (EA), ethyl acetate (EAC), ethyl butanoate (EB), methyl hexanoate (MH), ethyl hexanoate (EH), methyl octanoate (MO), octanoic acid (OA), ethyl octanoate (EO), ethyl decanoate (ED), ethyl propionate (EP), hexanoic acid (HA), methyl butanoate (MB). Data are average of n=3 measurements. Data are expressed as area units (AU) \* 10<sup>-3</sup> / g FW. Data within rows followed by the same letter are not statistically different (Fisher’s LSD, P=0.05).

<b>Headspace volatile composition (AU * 10<sup>-3</sup> / g FW)</b>									
<b>Cultivar</b>									
<b>Volatile compound</b>	<b>1-7-2</b>	<b>8-20</b>	<b>MT</b>	<b>PAG</b>	<b>SH</b>	<b>TT</b>	<b>TY</b>	<b>WE</b>	<b>WI</b>
<b>Total</b>	1146 b	200 b	251 b	1178 b	354 b	100 b	119 b	246 b	11550 a
<b>Groups</b>									
<b>Ethyl</b>	455 b	36 b	90 b	112 b	62 b	16 b	45 b	41 b	9961 a
<b>Methyl</b>	548 b	86 c	94 c	809 ab	130 c	37 c	73 c	126 c	1133 a
<b>Acetate</b>	0 b	0 b	0 b	0 b	0 b	0 b	0 b	0 b	10 a
<b>Propionate</b>	2 b	0 b	1 b	2 b	0 b	0 b	0 b	1 b	7 a
<b>Butanoate</b>	38 b	0 b	5 b	1 b	0 b	0 b	0 b	0 b	1188 a
<b>Hexanoate</b>	227 b	14 b	19 b	102 b	39 b	0 b	3 b	2 b	6164 a
<b>Octanoate</b>	697 b	94 c	123 c	816 b	136 c	39 c	81 c	135 c	3668 a
<b>Decanoate</b>	6 b	3 b	4 b	3 b	1 b	2 b	2 b	3 b	82 a
<b>Individual Compounds</b>									
<b>EA</b>	24 abc	13 cd	31 ab	6 d	17 a-d	15 bcd	33 a	30 abc	30 abc
<b>EAC</b>	14 a	0 b	3 ab	0 b	0 b	0 b	0 b	0 b	1 b
<b>EP</b>	0 b	0 b	1 b	2 b	0 b	0 b	0 b	1 b	7 a
<b>MB</b>	0 b	0 b	0 b	0 b	0 b	0 b	0 b	0 b	39 a
<b>EB</b>	38 b	0 b	4 b	1 b	0 b	0 b	0 b	0 b	1149 a
<b>MH</b>	15 b	3 b	2 b	49 b	9 b	0 b	0 b	0 b	532 a
<b>HA</b>	0 b	0 b	0 b	0 b	0 b	0 b	0 b	0 b	10 a
<b>EH</b>	211 b	11 b	17 b	53 b	31 b	0 b	3 b	2 b	5633 a
<b>MO</b>	532 a	83 b	91 b	760 a	121 b	37 b	73 b	126 b	562 a
<b>OA</b>	3 b	1 b	1 b	9 b	1 b	2 b	1 b	2 b	46 a
<b>EO</b>	162 b	10 b	30 b	47 b	13 b	0 b	7 b	7 b	3060 a
<b>ED</b>	6 b	3 b	4 b	3 b	1 b	2 b	2 b	3 b	82 a

**Table 3.2.** Headspace volatile profile composition of ‘Taytwo’ pawpaw. The major volatiles detected were (following retention time): ethyl alcohol (EA), ethyl acetate (EAC), ethyl butanoate (EB), methyl hexanoate (MH), ethyl hexanoate (EH), methyl octanoate (MO), octanoic acid (OA), ethyl octanoate (EO), ethyl decanoate (ED), ethyl propionate (EP), hexanoic acid (HA), methyl butanoate (MB). Data are expressed as an average of n=3 measurements. Data are expressed as area units (AU) \* 10<sup>-3</sup> / g FW. Data within columns followed by the same letter are not statistically different (Fisher’s LSD, P=0.05).

**Headspace volatile composition of ‘Taytwo’ fruit (AU \* 10<sup>-3</sup> / g FW)**

Storage Duration (weeks)	Ripening period (h)	EA	EAC	EP	MB	EB	MH	HA	EH	MO	OA	EO	ED	Total
0	4	15 c	0 c	0 c	0 b	0 b	0 d	0 c	0 c	37 c	2 c	0 c	2 de	100 d
0	72	29 c	0 c	2 c	0 b	2 b	342 b	5 bc	446 c	1395 a	24 c	125 c	4 cde	2654 b
2	4	7 c	0 c	0 c	0 b	0 b	1d	0 bc	2 c	127 bc	3 c	3 c	cde	251d
2	72	24 c	0 c	2 c	0 b	29 b	779 a	9 bc	2221 b	1847 a	35 c	559 b	9 bc	6105 a
4	4	8 c	0 c	0 c	0 b	0 b	2 d	0 c	3 c	186 bc	4 c	5 c	0 e	482 cd
4	72	157 ab	3 bc	58 bc	0b	381 a	264 b	6 bc	3694 a	360 bc	18 c	1321 a	21 a	6987 a
6	4	139 ab	64 c	93 bc	0 b	5 b	36 cd	96 bc	94 c	145 bc	119 bc	198 c	5 cde	1305 bed
6	72	31 c	0 c	2 bc	0 b	25 b	179 bc	3 bc	683 c	669 b	10 c	254 bc	5 cde	2397 bc
8	4	119 c	48 ab	134 ab	0 b	4 b	12 cd	115 ab	41 c	95 c	348 a	263 bc	14 b	1353 bed
8	72	222 a	70 a	222 a	39 a	12 b	47 cd	217 a	154 c	152 bc	242 ab	312 bc	8 bed	2087 bed

**Table 3.3.** Headspace volatile profile composition of ‘Wells’ pawpaw fruit. Measurements were collected 4 and 72 h after harvest or after removal from cold storage at 4 °C for 2, 4, 6 or 8 weeks. The volatiles detected were (following retention time): ethyl alcohol (EA), ethyl acetate (EAC), ethyl butanoate (EB), methyl hexanoate (MH), ethyl hexanoate (EH), methyl octanoate (MO), octanoic acid (OA), ethyl octanoate (EO), ethyl decanoate (ED), ethyl propionate (EP), hexanoic acid (HA), methyl butanoate (MB). Data are an average of n=3 measurements. Data are expressed as area units (AU) \* 10<sup>-3</sup> / g FW Data within column followed by the same letter are not statistically different (Fisher’s LSD, P=0.05)

**Headspace volatile composition of ‘Wells’ fruit (AU \* 10<sup>-3</sup> / g FW)**

<b>Storage Duration (weeks)</b>	<b>Ripening period (h)</b>	<b>EA</b>	<b>EAC</b>	<b>EP</b>	<b>MB</b>	<b>EB</b>	<b>MH</b>	<b>HA</b>	<b>EH</b>	<b>MO</b>	<b>OA</b>	<b>EO</b>	<b>ED</b>	<b>Total</b>
<b>0</b>	4	28 d	0 c	0 c	0 a	0 a	0 c	0 c	2 b	126 b	2 d	7 b	3 b	246 b
<b>0</b>	72	14 d	1 c	0 c	0 a	30 a	378 a	4 c	1133 ab	576 a	20 cd	260 b	8 b	2569 ab
<b>2</b>	4	1 d	0 c	0 c	0 a	1a	19 c	0 c	10 b	214 b	4 d	21 b	0 b	293 b
<b>2</b>	72	19 d	1 c	21 c	14 a	1478 a	285 ab	11 c	4909 a	230 b	28 bcd	1702 ab	91 a	8891 a
<b>4</b>	4	178 ab	46 b	151 b	10 a	866 a	17 c	43 bc	536 b	69 b	185 ab	481 ab	25 ab	2847 ab
<b>4</b>	72	76 cd	13 bc	61 c	18 a	1485 a	110 bc	12 c	4274 ab	238 b	36 bcd	2263 a	93 a	8884 a
<b>6</b>	4	185 ab	50 b	147 b	0 a	6 a	5 c	45 bc	36 b	38 b	141 abc	202 b	9 b	1040 ab
<b>6</b>	72	177 ab	50 b	195 ab	0 a	7 a	8 c	69 b	44 b	49 b	179 abc	226 b	20 ab	1171 ab
<b>8</b>	4	110 bc	29 bc	141 b	0 a	3 a	6 c	80 c	24 b	50 b	211 a	138 b	7 b	918 ab
<b>8</b>	72	223 a	154 a	227 a	3 a	16 a	20 c	185 a	89 b	97 b	288 a	341 ab	21 ab	1857 ab

**Table 3.4.** Individual headspace volatile profile composition of ‘pawpaw fruit harvest from the cultivars ‘Taytwo’ and ‘Wells’. The major volatiles detected were (following retention time): ethyl alcohol (EA), ethyl acetate (EAC), ethyl butanoate (EB), methyl hexanoate (MH), ethyl hexanoate (EH), methyl octanoate (MO), octanoic acid (OA), ethyl octanoate (EO), ethyl decanoate (ED), ethyl propionate (EP), hexanoic acid (HA), methyl butanoate (MB). Data are expressed as an average of n=3 measurements. Data are expressed as area units (AU) \* 10<sup>-3</sup> / g FW. Data within columns followed by the same letter are not statistically different (Fisher’s LSD, P=0.05).

**Headspace volatile composition of ‘Taytwo’ and ‘Wells’ fruit (AU \* 10<sup>-3</sup> / g FW)**

<b>Storage Duration (weeks)</b>	<b>Ripening period (h)</b>	<b>EA</b>	<b>EAC</b>	<b>EP</b>	<b>MB</b>	<b>EB</b>	<b>MH</b>	<b>HA</b>	<b>EH</b>	<b>MO</b>	<b>OA</b>	<b>EO</b>	<b>ED</b>	<b>Total</b>
<b>0</b>	4	21 c	0 d	0 e	0 b	0 b	0 d	0 d	1 b	82 b	2 c	4 c	2 c	173 b
<b>0</b>	72	22 c	1 d	1 e	0 b	16 b	360 b	5 d	789 b	986 a	22 c	193 c	6 c	2611 b
<b>2</b>	4	4 c	0 d	0 e	0 b	1 b	10 d	0 d	6 b	171 b	4 c	12 c	1 c	272 b
<b>2</b>	72	22 c	1 d	12 de	8 b	754 ab	532 a	10 d	3565 a	1038 a	32 bc	1131ab	50 ab	7498 a
<b>4</b>	4	93 b	23 bcd	75 bcd	5 b	433 ab	9 d	21 cd	270 b	128 b	94 bc	243 bc	13 bc	1664 b
<b>4</b>	72	116 b	8 cd	59 cde	19 a	933 a	187 c	9 d	3984 a	299 b	27 bc	1793 a	57 a	7936 a
<b>6</b>	4	162 ab	57 b	120 bc	0 b	6 b	20 d	70 bc	65 b	91 b	130 b	200 c	7 c	1173 b
<b>6</b>	72	104 b	25 bcd	99 bc	2 b	16 b	94 cd	36 cd	364 b	359 b	95 bc	240 bc	12 bc	1784 b
<b>8</b>	4	115 b	39 bc	137 b	0 b	4 b	9 d	97 b	33 b	73 b	279 a	200 c	10 bc	1136 b
<b>8</b>	72	223 a	112 a	225 a	1 b	14 b	33 cd	201 a	121 b	124 b	265 a	327 bc	14 bc	1972 b

**Table 3.5.** Statistical analysis of the differences in volatile production between 4 and 72 h across storage times and for linear (L) and quadratic (Q) trends in volatile production at 4 or 72 h across storage times of fruit harvested from ‘Taytwo’ and ‘Wells’. The major volatiles detected were: ethyl alcohol (EA), ethyl acetate (EAC), ethyl butanoate (EB), methyl hexanoate (MH), ethyl hexanoate (EH), methyl octanoate (MO), octanoic acid (OA), ethyl octanoate (EO), ethyl decanoate (ED), ethyl propionate (EP), hexanoic acid (HA), methyl butanoate (MB).

<b>Single degree of freedom contrasts (P)</b>					
<b>Volatile</b>	<b>4 vs 72</b>	<b>4 L</b>	<b>4 Q</b>	<b>72 L</b>	<b>72 Q</b>
<b>EA</b>	0.2449	< 0.0001	0.3878	< 0.0001	0.1637
<b>EAC</b>	0.4928	0.0012	0.5993	< 0.0001	0.0002
<b>EP</b>	0.4550	< 0.0001	0.9472	< 0.0001	0.0233
<b>MB</b>	0.0184	1	0.4283	0.7261	0.0005
<b>EB</b>	0.1943	0.9839	0.4655	0.4615	0.0254
<b>MH</b>	0.0211	0.0004	0.0026	0.0484	< 0.0001
<b>HA</b>	0.3343	0.0002	0.2350	< 0.0001	< 0.0001
<b>EH</b>	0.0032	0.5149	0.6953	0.1877	< 0.0001
<b>MO</b>	0.3828	0.0005	0.0115	0.2542	0.0124
<b>OA</b>	0.3666	< 0.0001	0.0536	< 0.0001	0.0161
<b>EO</b>	0.0126	0.8428	0.9418	0.8125	0.0009
<b>ED</b>	0.0372	0.7581	0.9959	0.7631	0.0107
<b>Total</b>	0.0004	0.6226	0.5814	0.6119	< 0.0001

**Table 3.6.** Alcohol acyltransferase (AAT), alcohol dehydrogenase (ADH) and lipoxygenase (LOX) activity of ‘Taytwo’ fruit at 4 and 72 h after harvest or after 2, 4, 6, or 8 weeks of cold storage. Enzyme activities are expressed as mUnits (mU) /mg protein. One AAT, ADH or LOX activity unit is defined as the increase in one unit of absorbance per minute. Data are expressed as the mean of n=3 measurements. Data in columns followed by the same letter are not significantly different (Fisher LSD, P=0.05). Single degree of freedom contrasts have been indicated: NS = not significant, \*\* Significant at P = 0.01, \* Significant at P = 0.05.

		<b>Enzyme activity (mU / mg protein)</b>		
<b>Storage Time (Weeks)</b>	<b>Ripening period (h)</b>	<b>AAT</b>	<b>ADH</b>	<b>LOX</b>
<b>0</b>	4	44 bc	1326 b	3314 c
<b>0</b>	72	85 ab	0 b	18997 ab
<b>2</b>	4	31 cd	556 b	5062 bc
<b>2</b>	72	36 cd	6873 a	25498 a
<b>4</b>	4	25 cd	365 b	0 c
<b>4</b>	72	10 cd	261 b	1435 c
<b>6</b>	4	34 cd	307 b	11145 bc
<b>6</b>	72	17 cd	33 b	2880 c
<b>8</b>	4	1018 a	460 b	3492 c
<b>8</b>	72	0 d	0 b	2284 c
<b>Single degree of freedom contrasts (P)</b>				
<b>4 vs 72 h</b>		0.0741	0.4511	0.0550
<b>4 h linear</b>		0.0163	0.6716	0.6546
<b>4 h quadratic</b>		0.0032	0.7202	0.8843
<b>72 h linear</b>		0.0006	0.1865	0.0006
<b>72 h quadratic</b>		0.0713	0.3576	0.4942

**Table 3.7.** Lipid peroxidation product content (TBARS assay) of ‘Taytwo’ fruit at 4 and 72 h after harvest or after 2, 4, 6, or 8 weeks of cold storage. TBARS values are expressed as nmol MDA eq/g FW. Data are expressed as the mean of n=3 measurements. NS= no significant differences.

<b>TBARS content (nmol MDA eq/g FW)</b>		
<b>Storage Time (Weeks)</b>	<b>Ripening period (h)</b>	<b>TBARS values</b>
<b>0</b>	4	30 NS
<b>0</b>	72	41
<b>2</b>	4	0
<b>2</b>	72	33
<b>4</b>	4	0
<b>4</b>	72	0
<b>6</b>	4	14
<b>6</b>	72	33
<b>8</b>	4	9
<b>8</b>	72	7

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## CHAPTER 4

### LOSS OF RIPENING CAPACITY DURING COLD STORAGE. III. COLD STORAGE INJURY AND ANTIOXIDANT SYSTEMS

#### INTRODUCTION

Though pawpaw [*Asimina triloba* (L.) Dunal] is becoming a high value alternative crop in the southeastern United States, the rapid perishability of the fruit is a big obstacle to the development of a national fresh pawpaw market. Low temperature storage is the most common and effective postharvest approach for prolonging fruit and vegetable shelf life. Pawpaw fruit may be stored at 4 °C for 4 weeks with minimal loss in quality (Archbold and Pomper, 2003). However, pawpaw fruit cold stored longer than 4 weeks develop internal discoloration and off-flavor aroma (Archbold et al., 2003; Koslanund, 2003; Chapter 2). The brown-to-black discoloration could be related to the activation of degenerative pathways such as those for phenolic oxidation and/or to a general loss of antioxidant protection in the tissue (Dogan et al., 2005).

The production of reactive oxygen species (ROS) and/or free radicals is an unavoidable consequence of the electron transport involved in respiratory and photosynthetic pathways that normally occurs in plant tissue (Arora et al., 2002). However, many kinds of stress events, such as exposure to temperature extremes, pollutants or herbicides, phytotoxic metals, drought, ripening, and senescence, increase the production of free radicals and reactive oxygen compounds in plant tissue. The generation of reactive oxygen species in tissue is a non-specific stress response. However, lipid content and composition, membrane fluidity, low adenylate energy charge, and cytoplasmic acidification favor ROS production and the successive peroxidation of lipids (Blokhina, 2000).

Detoxification of ROS products, such as H<sub>2</sub>O<sub>2</sub>, results in phenoxyl radical production, compounds with powerful prooxidant activity. These compounds can attack fatty acids and initiate lipid peroxidation (Blokhina, 2000; Sakihama et al., 2002). Reductions in antioxidant defenses, generation of ROS, and associated lipid peroxidation

can result in membrane damage and eventually cell death (Greggains et al., 2001; Taulavuori et al., 2001). Thus, lipid peroxidation is a widely-used plant membrane stress indicator. Since lipid peroxidation occurs when oxygen radicals or free radicals produced from oxidative reactions attack membrane lipids, both membrane phospholipid composition and the degree of fatty acid unsaturation play important roles in tissue stress resistance (Zhang and Kirkham, 1996). Two basic traits are associated with the rate of aging and with the maximum cell life span, the presence of low rates of ROS production and low degrees of membrane fatty acid unsaturation. A lower degree of fatty acid unsaturation may confer this advantage by less sensitivity to lipid peroxidation and, consequently, less damage to other macromolecules such as DNA and proteins (Pamplona et al. 2002).

Under normal stress conditions adequate protection against destructive ROS reactions is provided by cellular antioxidant defense systems. Major components of these systems include peroxidase (POD), glutathione reductase (GR), ascorbate peroxidase (APX), and antioxidant compounds such as phenolics, carotenoids, ascorbate, and glutathione (Arora et al., 2002; Lata et al., 2005). While these handle ROS species during mild, transient stresses, the antioxidant defense system may only be moderately upregulated as stress levels increase, causing an imbalance between oxidative and antioxidative reactions with permanent tissue damage resulting (Arora et al., 2002).

Species and/or cultivars with higher antioxidant levels should exhibit better stress resistance (Arora et al., 2002; Lata et al., 2005). Davey and Keulemans (2004) showed that apple cultivars known to have poor storage qualities such as ‘Sunrise’ and ‘Gravenstein’ experienced significant losses of ascorbic acid and glutathione content during the first month of cold storage. On the other hand, varieties able to withstand long-term storage maintained and even increased their antioxidant content during 6 months of cold storage.

The increases in respiration and ethylene production observed in ripening climacteric fruit are important factors causing tissue peroxidation and tissue senescence. The conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene in plant tissue is a peroxidation reaction. Thus, inhibition of ethylene slows peroxidation and prolongs commodity shelf life (Arora et al., 2002). Moreover, the enhanced production

of hydrogen peroxide and superoxides linked to alternate respiration pathway activation and observed in fruit tissue during the respiratory climacteric peak not only increases membrane breakdown but also increases ROS concentration in cells (Arora et al., 2002). Since ethylene production in pawpaw fruit is lower than for many climacteric species such as soursop and apple, but respiration is comparable to that observed in other Annonaceae and significantly higher than for other climacteric species such as apple and peach (Archbold and Pomper, 2003; Koslanund et al., 2005), respiration may be a major cause of ROS production and tissue peroxidation in pawpaw fruit.

Along with ascorbate peroxidase (APX) and GR, ascorbate and glutathione are essential in the hydrogen peroxide scavenging system also known as the Asada–Halliwell pathway or ascorbate–glutathione cycle (Figure 4.1) (Arora et al., 2002, Blokhina, 2000; Lata et al., 2005). In this pathway, reduced glutathione (GSH) is used as a reducing agent for the enzymatic regeneration of dehydroascorbic acid (DHA). The DHA is used in the reduction of  $H_2O_2$  to  $H_2O$ , protecting tissue from damage. The capacity of this cycle for tissue protection is dependent on antioxidant concentration and the activity of the aforementioned enzymes (Lata et al., 2005). The enzyme activities of the ascorbate–glutathione cycle may be increased by stresses such as drought and low temperature. In general, progressive oxidation and degradation of the ascorbate and glutathione pools are inseparably linked to senescence and cell death (Lata et al., 2005; Smirnoff, 1996).

Under conditions of severe stress, phenolic compounds may become a backup defense system and work cooperatively with the ascorbate–glutathione–dependent pathway. Phenolics can scavenge ROS through direct and enzymatic reactions. Phenolic compounds such as flavonoids and anthocyanins are considered among the most active antioxidant compounds in plant tissues (Llorach et al., 2002; Orak, 2006). The antioxidant properties of phenolic compounds is mainly due to their redox properties which allow them to act as reducing agents, hydrogen donors, oxygen scavengers and metal chelators (Kahkonen et al., 1999). Maldonado et al. (2002) reported that lignins and tannin polyphenols accumulated in the pawpaw-related cherimoya fruit and played an important role in the cell wall hardening observed in cold-injured fruits.

During these processes phenoxyl radicals are produced from the phenolic compounds. In healthy tissue phenoxyl radicals can be reduced to their parent compounds

by monodehydroascorbate reductase (MDHAR) or by non-enzymatic reactions with ascorbate (Sakihama et al., 2002). However, as noted above, in stress-damaged tissues, phenoxyl radicals can initiate free radical chain reactions in membranes and cross-link with DNA and cell proteins. Phenolics aren't the only compounds in cells that can act both as antioxidant and prooxidant. Fukomoto and Mazza (2000) showed that under certain conditions such as low concentrations and presence of metallic ions, many antioxidant compounds such as ascorbic acid (AA) have shown prooxidant activity.

Despite their protective role in fruit tissue, phenolic metabolism may be related to tissue browning and cold injury development (Concellon et al., 2004). Enzymatic browning is caused by oxidation of natural phenolic compounds and, when uncontrolled, it can cause significant economic loss in both fresh and processed fruit (Concellon et al., 2004; Gooding et al., 2001). The degree of tissue browning depends on the nature and amount of endogenous phenolic compounds produced by phenolic oxidation, and on the presence of oxygen, reducing substances, and metallic ions (Dogan et al., 2005). Oxidation of the *o*-diphenols to *o*-quinones by polyphenol oxidase (PPO) produces brown-colored polymers that change the composition of the phenolic pool. This may be the most important cause of the browning in plant tissues.

Enzymatic browning also causes a loss in the nutritional value and in the antioxidant pools through loss of free phenolic compounds and phenolic-mediated oxidation of ascorbic acid (Dogan et al., 2005). Enzymes involved in phenolic oxidation include PPO, POD and phenylalanine ammonia lyase (PAL). It has been shown that an increase in the activity of this last enzyme indirectly boosted the phenolic oxidation pathway since it increased the concentration of PPO substrates such as mono- and diphenols in the cell (Nguyen et al., 2003). PAL catalyzes the elimination of ammonia from L-phenylalanine and the consequent production of *trans*-cinnamate and *trans*-4-hydroxycinnamate. These compounds can be transformed via the coumarate pathway into phenolic compounds such as chlorogenic acid and caffeic acid derivatives. Once produced, these phenolics become substrates for POD and PPO (Gooding et al., 2001; Maldonado et al., 2002b, Nguyen et al., 2003). Polyphenols are transformed by these enzymes into complex polyphenols such as *o*-quinones. The complex polyphenols can polymerize and covalently bind nucleophilic amino acids producing the dark pigments

responsible for the discoloration observed in fruit after cold damage or improper storage (Nguyen et al., 2003; Gooding et al., 2001).

Despite the role played by PAL and POD in phenolic evolution, PPO is recognized as the key enzyme in tissue browning of cold-damaged fruits and vegetables (Concellon et al., 2004; Tang and Newton, 2004). A decline in total phenolic concentration and rise in PPO activity were correlated with the development of cold injury symptoms including discoloration in cold-stored banana (Nguyen et al., 2003) and apple (Leja et al., 2003). Even though an increase in PPO activity in carambola fruit in response to low temperature was only temporary, there was a significant correlation between PPO activity and cold injury development (Perez-Tello et al., 2001). In contrast, an increase in PPO activity during the first two weeks of cold storage of 'Fortune' mandarin was not related to peel pitting (Martinez-Tellez and Lafuente, 1997).

POD activity is often correlated with ethylene production; hence, the activity of this enzyme increases during the onset of ripening but decreases during cold storage, exposing the tissue to a higher risk of free oxygen radical attack (Leja et al., 2003; Masia et al., 1998; Perez-Tello et al., 2001). This is because, in healthy tissues, POD may protect plant cells against the attack of ROS by acting as a peroxide scavenger (Xu and Huang, 2005; Leja et al., 2003).

The orange-yellow pulp color of pawpaw suggests that it contains carotenoids, pigments contributing to such color in many species. Carotenoids protect membranes from ROS that cause lipid peroxidation and absorb excess chlorophyll excitation energy (Aboul-Einen et al., 2003; Zhang et al., 1999). The oxygen scavenging activity of carotenoids is linked to their conjugated double bonds, and they reach maximum scavenging activity in compounds that have 9 or more of these (Rodriguez-Amaya and Kimura, 2004). The qualitative and quantitative content of carotenoids in fruit vary according to factors such as variety, stage of maturity, site of production, and pre-harvest production and postharvest handling and storage conditions (Rodriguez-Amaya and Kimura, 2004). Being highly unsaturated compounds, carotenoids are prone to isomerization and oxidation and may decrease during cold storage (Mayer-Miebach and Spiesz, 2003; Rodriguez-Amaya, 1997).

Though tissue discoloration and loss of ripening of pawpaw have been observed for periods of cold storage exceeding 4 weeks, nothing is known about the effect such cold storage has on pawpaw antioxidant compound content and/or metabolism, nor is there information about possible genotype differences in phenolic, carotenoid and ascorbate content of the fruit at harvest. Thus, the aims of this study were (a) to compare total antioxidant, phenolic, carotenoid and ascorbate content of some commercially-promising varieties at harvest, and (b) to investigate the effect that cold storage length has on antioxidant compound concentration, and key components of the glutamate-ascorbate pathway and of phenolic oxidative metabolism.

## **MATERIALS AND METHODS**

### ***Fruit harvest and storage***

Pawpaw fruit were harvested from the Kentucky State University Research Farm, Frankfort, KY, on several dates during the August to October harvest season in 2005. Based on fruit availability and tree yields fruit were harvested from the following cultivars and selections from the PawPaw Foundation breeding effort: 1-7-2, 8-20, 'Middletown', 'PA Golden', 'Shenandoah', 'Taytwo', 'Taylor', 'Wells' and 'Wilson'. Immediately following harvest, fruit were transported to the laboratory at the University of Kentucky, Lexington, KY.

On each harvest date fruit were divided by variety and allocated to sub-groups for pre-determined storage lengths. In 2005, every storage group (at harvest, or 2, 4, 6, or 8 weeks at 4 °C) contained 10 fruit/variety. Upon harvest or removal from cold storage fruit were moved to ambient temperature ( $21 \pm 2$  °C). After 4 and 72 h in 2005, fruit were examined for signs of cold injury such as discoloration, and 5 fruit/cultivar were peeled, sectioned and frozen in -80 °C storage.

### ***Glutathione and ascorbic acid extraction and assay***

Frozen pawpaw ('Taytwo') tissue (0.5 g) was homogenized in 2.5 mL of 5% (w/v) metaphosphoric acid, filtered through 4 layers of Miracloth and centrifuged at 20,000 X g

for 15 min at 4 °C. The supernatant was collected and used for glutathione and ascorbic acid determination.

Components of the glutathione pool were assayed according to Griffiths (1980). Total glutathione was measured in a 1.2 mL mixture containing 0.4 mL reagent I (110 mM Na<sub>2</sub>HPO<sub>4</sub>\*7H<sub>2</sub>O; 40 mM NaH<sub>2</sub>PO<sub>4</sub>\*H<sub>2</sub>O, 15 mM EDTA, 0.3 mM 5-5–dithio-bis(2-nitrobenzoic acid) (DTNB) and 0.04% BSA), 0.32 mL reagent II (1 mM EDTA, 50 mM imidazole, 0.02% BSA, 1.5 unit GR (baker's yeast, Type III, Sigma)/mL) and 0.4 mL of a 1:10 dilution of extract in 5% Na<sub>2</sub>HPO<sub>4</sub> (pH 7.5). The reaction was initiated with 80 µL of 3 mM NADPH. The change in absorbance at 412 nm was recorded. Oxidized glutathione (GSSG) was determined by first incubating the mixture with 1 mL of the 1:10 dilution extract and 40 µL of 2-vinylpyridine for 60 min at 25 °C. Standard curves were prepared by using GSH and GSSG. Reduced glutathione (GSH) was obtained as the difference between total glutathione and GSSG.

Components of the ascorbate pool were measured according to Foyer et al. (1983) with some modification. To determine total ascorbate, 0.2 mL of the pawpaw extract was initially reacted with 0.1 mL of 10 mM dithiothreitol (DTT) which converted dehydroascorbate (DHA) into its reduced form, AA. The mixture was incubated for 10 min at room temperature, then 0.5 mL of 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA and 0.1 mL of 0.5 M N-ethylmaleimide (NEM) was added. After adding 0.4 mL of 10% (w/v) TCA, 0.4 mL of 44% (v/v) orthophosphoric acid, 0.4 mL (w/v) 2,2'-dipyridyl in 70% ethanol and 0.2 mL of 3% (w/v) FeCl<sub>3</sub>, the mixture was incubated at 37 °C for 60 min and the absorbance at 525 nm was read. AA concentration was measured using the same methodology but DTT and NEM were replaced with 0.2 mL of H<sub>2</sub>O. A standard curve was produced with AA. DHA level was calculated as the difference between total ascorbate and AA.

#### ***GR and APX extraction and assay***

Frozen pawpaw tissue ('Taytwo') (0.5 g) was homogenized in 5 mL of 50 mM Tris-HCl (pH 7.0) containing 20% (w/v) glycerol, 1 mM GSH and 5 mM MgCl<sub>2</sub>. The mixture was filtered through 4 layers of Miracloth and centrifuged at 25,000 X g for 15 min at 4 °C. The supernatant was used for enzyme activity determination.

GR activity was assayed by following the change in absorbance at 340 nm according to Foyer and Halliwell (1976) in 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM MgCl<sub>2</sub>, 0.5 mM GSSH, 0.2 mM NADPH and extract in a final volume of 1 mL. One unit of GR was defined as the amount of enzyme that utilized 1 μmol NADPH/min/mg protein under the assay conditions. Protein was determined by the method of Bradford (1976) with bovine serum albumin as a standard

APX activity was assayed by monitoring the change at 290 nm according to the method of Nakano and Asada (1987). The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 1 mM AA, 2.5 mM H<sub>2</sub>O<sub>2</sub> and extract in a final volume of 1 mL. One unit of APX was defined as the amount of enzyme that oxidized 1 μmol AA/min/mg protein under the assay conditions. Protein was determined as above.

#### ***Total antioxidant activity and phenolic content***

Antioxidant and phenolic analysis were performed according to Fukomoto and Mazza (2000) and Arnous et al. (2002) using pawpaw fruit frozen immediately after harvest (cvs. 1-7-2, 8-20, 'Middletown', 'PA Golden', 'Shenandoah', 'Taytwo', 'Taylor', 'Wells' and 'Wilson') and after 2, 4, 6 or 8 weeks of cold storage follow by 4 or 72 h at room temperature ('Taytwo'). Three g of frozen fruit pulp were combined with 4 mL of 80% methanol and homogenized in a Polytron homogenizer. The homogenate was filtered through two layers of cheesecloth and centrifuged at 1500 X g for 5 – 6 min. A portion of supernatant was diluted with deionized water to achieve a 5% methanol concentration for analysis.

Total antioxidant activity was measured using the ferric reducing/antioxidant power (FRAP) assay. For this assay, 0.2 mL of the 5% methanol pawpaw extract was combined with 0.042 mL of 3 mM ferric chloride in 5 mM citric acid. This solution was vortexed and incubated for 30 minutes in a 37 °C water bath. After incubation 0.758 mL of 1 mM 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ) was added and absorption was read at 620 nm. Results were derived using an AA standard curve in 5% methanol and are expressed as g AA equivalents/100 g FW.

Total phenolic content was measured using a modified version of the Glories' method as described by Fukomoto and Mazza (2000). For this assay, 0.5 mL of 5%



methanol pawpaw extract was combined with 0.24 mL 0.1% HCl in 95% ethanol and 4.31 mL of 2% ethanol. The solution was vortexed and the absorbance was read at 280 nm against a blank containing 5% methanol. Results were derived from a chlorogenic acid standard curve and are expressed as mg chlorogenic acid/100 g FW.

#### ***PPO, PAL, and POD enzyme extraction and activity***

As described by Ruoyi et al. (2005) and Lee et al. (2004), 3 g of frozen pawpaw tissue ('Taytwo') was homogenized in 9 mL of 100 mM phosphate buffer (pH 6.2) containing 2 g of PVPP. The supernatant from the homogenate was filtered through four layers of cheesecloth and Miracloth and centrifuged at 14,000 X g for 15 min at 4 °C. The supernatant was collected as crude enzyme and used for enzyme activity assay. According to Ruoyi et al. (2005), PPO activity was determined in a 1 mL reaction mixture containing 0.150 mL 10 mM catechol, 0.550 mL phosphate extraction buffer and 0.3 mL crude extract. Catechol oxidation was followed over time at 420 nm, and PPO activity was expressed as  $\Delta A_{420}$  /min/mg protein. According to Maldonado et al. (2002a), PAL activity was determined in a 1 mL reaction mixture containing 0.10 mL 10 mM L-phenylalanine, 0.570 mL 30 mM Tris buffer (pH 8.4), and 0.330 mL crude extract. Production of cinnamate was followed over time at 290 nm, and PAL activity was expressed as  $\Delta A_{290}$  /min/mg protein. According to Flurkey and Jen (1978), POD activity was determined in a 1 mL reaction mixture containing 0.170 mL 0.03% (v/v) H<sub>2</sub>O<sub>2</sub> , 0.333 mL 40 mM guaiacol, 0.333 mL Tris buffer (pH 5.6), and 0.164 mL crude extract. Guaiacol oxidation was followed over time at 470 nm, and POD activity was expressed as  $\Delta A_{470}$  /min/mg protein. Protein was determined as above.

#### ***Total carotenoid content***

Total carotenoid content was assayed as described by Schaub and Islam (2004) in pawpaw fruit frozen right after harvest (cvs. 1-7-2, 8-20, 'Middletown', 'PA Golden', 'Shenandoah', 'Taytwo', 'Taylor', 'Wells' and 'Wilson') and after 2 or 4 weeks of cold storage follow by 4 or 72 h at room temperature ('PA Golden'). Frozen pawpaw tissue (0.5 g) was ground in liquid N<sub>2</sub>, and 6 mL of ethanol containing 1 mg butylated hydroxytoluene (BHT)/mL. Samples were incubated at 85 °C in a water bath for 3

minutes, vortexed and moved back to the water bath. After 6 min, 120  $\mu$ L of KOH (1g/mL) were added, samples were vortexed and incubated at 85 °C in a water bath for a total of 10 min, with incubation interrupted by vortexing at 5 min.

Once incubated, the samples were cooled on ice, and 4 mL of distilled water were added followed by 3 mL of 3:2 (v/v) petroleum ether:diethyl ether solution. Tubes were vortexed and centrifuged for 10 min at 1400 X g. Once centrifuged, the upper phase was collected and transferred to a new tube. These last steps were repeated twice and the final volume was brought to 10 mL by adding petroleum ether:diethyl ether solution (3:2, v/v). Samples were read spectrophotometrically at 450 nm, and total carotenoid concentration was calculated using  $E = 130056 \text{ l/mol/cm}$ .

### ***Statistical analysis***

All data were subjected to analysis of variance. Single degree of freedom contrasts were used to compare 4 vs 72 h values and determine if there were linear or quadratic trends across storage periods. Means were compared by Fisher's protected least significance difference (LSD,  $P=0.05$ ) using SAS version 9.1 software (SAS Institute Inc., Cary, NC).

## **RESULTS AND DISCUSSION**

### ***Glutamate-ascorbate pathway at harvest and during cold storage***

In both unripe and ripe fruit, GSH represented almost 97% of the total glutathione pool in pawpaw (Table 4.1). Total glutathione content was similar to that in peach (Wang et al., 2006) and 2-fold higher than in apple (Lata et al., 2005). The highest total glutathione and GSH concentrations were measured in fruit bench ripened for 72 h after harvest and after 2 and 4 weeks of cold storage. In contrast, few differences were observed in GSSG concentration before or after cold storage and bench ripening. The highest GSSG concentration was in fruit cold stored for 2 weeks and the lowest concentrations were measured in fruit cold stored for 2 weeks and bench ripened for 72 h. Total glutathione, GSH and the GSH/GSSG ratio were generally higher in fruit ripened for 72 h after harvest or cold storage (or 4 vs 72 h). Moreover, total glutathione content

decreased linearly with storage time when measured at 4 or 72 h, and GSH and GSH/GSSG values declined linearly at 72 h (Table 4.1).

Even though GSH and the GSH/GSSG ratio generally increased during bench ripening, this was not observed among fruit cold stored for 8 weeks. This indicated that overstored pawpaw fruit may have low oxidative stress tolerance due to low total glutathione and GSH concentrations. In stressed tissues, reductions in GSH concentration and low GSH/GSSG values have been observed (Blokina, 2000; Sofu et al., 2005; Zhang and Kirkham, 1996). A decrease in the GSH/GSSG ratio is related to the failure of the antioxidant protection system that can occur under prolonged stress conditions.

In pawpaw GR activity reached its maximum value in fruit cold stored for 6 weeks and bench ripened for 72 h (Table 4.1). However, no significant 4 versus 72 h differences or trends over storage time were generally observed. GR activity in pawpaw at harvest was comparable to that in mature green tomato (Mondal et al., 2004) and peach (Wang et al., 2006). Mahan and Wanjura (2005) reported that GR activity in cotton grown under water stress conditions was upregulated in tissue exposed to short-term stress, but it was down-regulated in tissue exposed to long-term stress. An increase in GR activity in tissues exposed to drought or low temperature stress has been related to an increase in GSH concentration and a general protection against oxidative stress (Mondal et al., 2004; Sofu et al., 2005). The lack of GR upregulation in cold-stored pawpaw fruit may explain both the decline of GSH and the GSH/GSSG ratio observed during cold storage.

The ascorbate content of 'Taytwo' fruit at harvest (Table 4.2) was similar to that reported by Peterson (1982). The highest total ascorbate concentration was in fruit at harvest. By 2 weeks of cold storage values had significantly dropped, and there were significant declining trends over storage time when measured at 4 or 72 h. However, bench ripening had no effect on total ascorbate.

At harvest, 41% of the total ascorbate pool was AA and 59% was DHA. Interestingly after 2, 6, and 8 weeks of cold storage followed by 72 hours of bench ripening the dominant ascorbate form present was AA and only small amount of DHA were present.

No significant trends were observed in AA concentration across cold storage periods. However, across storage periods, fruit bench ripened for 72 h had significantly higher AA than fruit at 4 h. Moreover, there was a linear decline in DHA values measured after 4 or 72 h across cold storage periods. No significant differences between 4 vs 72 h values or trends across storage time were observed among AA/DHA values.

Since an increase in AA/DHA ratio with cold storage may indicate an increase in tissue protection against oxidative stress (Wang et al., 2006), the lack of any significant change in the AA/DHA ratio in pawpaw could indicate the lack of an oxidative stress protection response in the tissue. Many stress events that cause oxidative damage such as drought and exposure to low temperatures enhance reduced forms of both ascorbic acid and glutathione and upregulate the ascorbate–glutathione machinery (Smirnoff, 1996; Sundar et al., 2004). Hence the decrease in total ascorbate and total glutathione in cold stored fruit at both 4 and 72 h upon harvest or removal from cold storage indicates that this system is not upregulated in pawpaw when cold-stored and could partially explain pawpaw sensitivity to cold temperature.

APX activity did not change during fruit ripening or cold storage, though it reached a maximum value after 2 weeks of cold storage and very low values at 4 weeks and beyond (Table 4.2). However no linear or quadratic trends of APX activity were found. APX activity in pawpaw was 2-fold lower than in melon (Barreiro et al., 2001) and 2.5-fold higher than in peach (Wang et al., 2006). A decrease in APX activity in stressed tissues may be related to either reduced synthesis or enhanced degradation of this enzyme. Both tomato (Mondal et al., 2006) and melon (Barreiro et al., 2001) exhibited a decline in APX activity during cold storage that was related to oxidative stress and postharvest senescence development. A decrease in APX activity impairs the H<sub>2</sub>O<sub>2</sub> scavenging system and causes accumulation of both H<sub>2</sub>O<sub>2</sub> and AA (Zhang and Kirkham, 1996). In contrast to tomato (Mondal et al., 2004), neither APX nor GR activities decreased during the 72 h of bench ripening that followed fruit harvest or removal from cold storage.

Overall, as shown by the reduction in the total glutathione and ascorbate pools and in APX activity, fruit stored over 4 weeks were becoming increasingly exposed to ROS damage since antioxidant protection was being lost. As discussed in Chapter 2, the

decrease in ascorbic acid was significantly correlated with the increase in glucose, the substrate for ascorbic acid biosynthesis.

#### ***Total antioxidant activity at harvest and during cold storage***

Pawpaw fruit at harvest had an average total antioxidant activity of  $0.20 \pm 0.03$  g AAE /100 g FW in the FRAP assay. This amount is comparable to FRAP assay values measured by Pellegrini et al. (2003) for some of the most popular fruit. Pawpaw contained 16% more total antioxidant than strawberry, 28% more than orange, 25% more than kiwifruit, 158% more than grapefruit, 600% more than apple, and 700% more than banana. Among the pawpaw cultivars tested, total antioxidant values ranged from 0.104 to 0.340 g AAE/100 g FW, with 'Middletown' highest and 'PA Golden' lowest (Table 4.3). Yang (2004) and Lata et al. (2005) have reported that antioxidant activity of fruits and vegetables may vary by variety, time of harvest, and post-storage handling factors.

As for total antioxidant activity during storage and ripening, no differences were found among values at 1 week before commercial harvest (unripe), at harvest, or after 2, 4, 6, 8 weeks of cold storage, irrespective of 4 or 72 h of bench ripening (data not shown). Across storage periods, pawpaw antioxidant content was  $0.26 \pm 0.02$  g AAE/100 g FW. Thus, pawpaw ascorbic acid content accounted for about 6% of the total antioxidant pool. However, as suggested by Maldonado et al. (2002b), even though total antioxidant concentration in the fruit did not change, the composition of the pool could have varied among the different storage periods. The change in the ascorbate pool described above would seem to confirm this.

#### ***Phenolic content at harvest and during cold storage***

At harvest, the average phenolic content of pawpaw fruit was  $52 \pm 1$  mg CA/100 g FW. This value was more than 1.5-fold lower than the phenolic content reported for apple (Lata and al., 2005), 4-fold lower than for black grapes and 5-fold lower than for pomegranate (*Punica granatum*) and plum (Kaur and Kapoor, 2005). Phenolic content varied among pawpaw cultivars, similar to that observed in apple (Lata and al., 2005) and grape (Orak, 2006). Specifically, 'Shenandoah' was lowest and 'PA Golden', 'Wilson' and 1-7-2 were highest (Table 4.3). There was no clear relationship ( $P > 0.05$ ) between

total phenolic and total antioxidant activity ( $r = 0.33$ ). In contrast, in cranberry, apple and grape (Orak, 2006; Proteggente et al., 2002), a linear correlation was observed between phenolic and total antioxidant content.

Even though cold storage did not affect total antioxidant content, phenolic concentration varied with storage length (Table 4.4). There was a quadratic trend for 4 and 72 h with maximum values from 2 to 6 weeks of cold storage with 4 h values generally declining over time. Ripening (4 vs 72 h values) generally resulted in an increase in phenolic content. As observed for other species such as apple (Lattanzio et al., 2001) and banana (Nguyen et al., 2003), accumulation of phenolic compounds in pawpaw fruit may be an acclimatizing response to cold storage. Lattanzio et al. (2001) found that phenolic biosynthesis in 'Golden Delicious' apple temporarily increased during the first 2 months of cold storage and subsequently decreased when stored for longer periods. Nguyen et al. (2003) indicated that the accumulation of phenolic compounds in cell walls may increase tolerance to cold storage. Both Lattanzio et al. (2001) and Nguyen et al. (2003) reported that loss of phenolic compounds in fruit stored for long periods was correlated with an increased susceptibility to fungal attack and cold injury symptoms.

#### ***PPO activity at harvest and during cold storage***

Three enzymes play a major role in phenolic compound oxidation and consequent tissue browning: PAL, PPO and POD (Nguyen et al., 2003). During a preliminary screening of fruit after harvest and at 4 or 8 weeks of cold storage, PAL, PPO and POD activities in pawpaw were comparable to values reported for fruit such as persimmon (Lee et al., 2005), peach (Ruoyi et al., 2005) and banana (Nguyen et al., 2003). After 4 weeks of cold storage, enzyme activities were: PAL at  $0.004 \pm 0.003 \Delta A_{290} / \text{min/mg}$  protein; POD at  $0.08 \pm 0.02 \Delta A_{420} / \text{min/mg}$  protein; and PPO at  $0.35 \pm 0.04 \Delta A_{470} / \text{min/mg}$  protein. However, only PPO activity increased between 4 and 8 weeks of cold storage (data not shown). Thus, PPO was the focus in subsequent studies.

Over storage time, PPO activity increased linearly after 4 but not 72 h of bench ripening (Table 4.4). The highest PPO activity was found at 8 weeks of cold storage and 4 h of bench ripening. No correlation ( $P > 0.05$ ) was found between PPO activity and

total phenolic concentration ( $r = 0.49$ ). The lack of correlation between these two variables may be because both PPO substrate and PPO product are part of the total polyphenol pool.

Increased PPO activity has been observed in persimmon (Lee et al., 2005), mango (Vela et al., 2003), and carambola (Perez-Tello et al., 2001) during cold storage. An inactive PPO form normally stored in cell plastids of unripe fruit is often activated during ripening, senescence and stress conditions (Ruoyi et al., 2005). When stressed tissues lack reducing substances, phenolic oxidation can occur at a higher rate and PPO-mediated browning is observed (Sakihama et al., 2002; Ruoyi et al., 2005; Dogan et al., 2005). Interestingly, at 8 weeks, PPO activity reached its maximum value and total phenolic, glutathione and ascorbate reached their minimums in pawpaw. A decline in total phenolic concentration and rise in PPO activity were correlated with the development of cold injury symptoms such as tissue discoloration in banana (Nguyen et al., 2003) and apple (Leja et al., 2003). Since an increase in cell antioxidant power may be a defense mechanism against the oxidative products formed by PPO activity (Perez-Tello et al. 2001), the decrease in ascorbate and glutathione pools in overstored pawpaw could allow an increase in PPO activity to cause subsequent browning.

Tissue browning dramatically increased once the fruit were moved back to room temperature and bench ripened for 72 h following 8 weeks of cold storage (Figure 4.2). However PPO activity did not change during this time. Since PPO activity did not change but total phenolic concentration did, (i) other metabolic events such as Maillard reactions may be involved in browning, and/or (ii) the phenolic compounds responsible for discoloration are ultimately produced by reactions downstream such as *o*-quinone polymerization (Dogan et al., 2005). Nonetheless, even though other metabolic events may result in browning, the oxidation of *o*-diphenols to *o*-quinones catalyzed by PPO may be important contributors to pawpaw tissue browning, as noted in other species during senescence or cold storage (Dogan et al., 2005; Gooding et al., 2001; Maldonado et al., 2002b; Nguyen et al., 2003).

### ***Carotenoids***

Pawpaw fruit develop flesh colors of white to yellow to orange, and contained an average  $4.1 \pm 0.3 \mu\text{g}$  total carotenoid/g FW in the present work. Based on values reported by Heinenon et al. (1989), pawpaw fruit at harvest contained 10-fold more total carotenoid than strawberry, 5-fold more than orange and apple, 2-fold more than kiwifruit, and 14-fold more than banana.

Carotenoid content varied among pawpaw cultivars. 'Wilson' had the highest carotenoid content, and 8-20 had the lowest (Table 4.3). As expected, fruit of carotenoid-rich cultivars had the most intense pulp color, and carotenoid-poor cultivars had moderate pulp color (Figure 4.3). Similar variation has been reported by Rodriguez-Amaya and Kimura. (2004) for guava fruit. There was no clear relationship ( $P > 0.05$ ) between the carotenoid value and the total antioxidant activity ( $r = 0.09$ ). Among the varieties analyzed, 'PA Golden' and 1-7-2 had high carotenoid content but among the lowest total antioxidant activity. In contrast, 'Wilson' and 'Taylor' had the highest content of both total antioxidant activity and carotenoids. This suggests that antioxidant composition may vary among pawpaw cultivars.

Due to their high carotenoid content, 'PA Golden' fruit were used to investigate possible modification of carotenoid content during cold storage. Carotenoid content at harvest was significantly higher than in unripe fruit, but no differences were observed between fruit at the beginning and at the end of bench ripening (Table 4.5). Generally, carotenoid content declined over 4 weeks of cold storage. Even though carotenoid and phenolic biosynthetic pathways aren't directly connected, carotenoid decline was significantly ( $r = 0.87$ ,  $P < 0.05$ ) correlated with phenolic content decline over 4 weeks of cold storage.

Interestingly, overripe but intact fruit collected from the ground after natural drop had significantly lower carotenoid content,  $4 \pm 2 \mu\text{g}$  carotenoid/g FW, than fruit bench ripened for 3 days. Thus, there may not be carotenogenesis after fruit ripening. Carrot (Mayer-Miebach and Spiesz, 2003) and mango (Rodriguez-Amaya and Kimura, 2004) carotenoid content also decreased when cold stored. Since carotenogenesis follows chloroplast degeneration, the increase in carotenoid concentration observed after 4 weeks of cold storage could be an indirect measurement of tissue senescence and organelle break down.



## CONCLUSIONS

Cold temperature exposure decreases the demand for ATP in plant tissue, resulting in an excess of free electrons and an increased formation of ROS (Wismer, 2003). Hence exposure to low temperature can cause oxidative damage and result in responses similar to other oxidative stress events including upregulation of the ascorbate–glutathione pathway and, under conditions of severe stress, use of polyphenol compounds as detoxifying backups for the ascorbate–glutathione pathway (Smirnoff, 1996; Sundar et al., 2004). Thus, ascorbic acid, glutathione and phenolics are the most important antioxidant compounds in plant cells.

Through 4 weeks of cold storage, phenolic content increased, while total ascorbic acid, and total glutathione concentrations were similar to the values at harvest. These data indicated that pawpaw tissue was temporarily protected from oxidative damage. However, when storage exceeded 4 weeks both the ascorbate-glutathione and phenolic scavenging systems diminished and tissue was likely exposed to ROS attack. In fact, even though no significant changes were observed in AA/DHA, APX and GR activities, total glutathione, total ascorbate, carotenoids and total phenolics steadily decreased during the 8 weeks of cold storage. These events can be considered strong evidence of increasing low temperature-mediated oxidative stress (Barreiro et al., 2001; Lattanzio et al., 2001; Nguyen et al., 2003; Smirnoff, 1996; Sundar et al., 2004; Zhang and Kirkham, 1996).

Both membrane phospholipid composition and the degree of fatty acid unsaturation can play important roles in tissue stress resistance (Blokhina, 2000). A high concentration of lipids (0.5–1% fresh weight) and unsaturated fatty acids (40% of total fatty acids) in pawpaw fruit may partly explain their sensitivity to cold storage stress (Wood and Peterson, 1999; McGrath and Karahadian, 1994b). Both the decline in the antioxidant systems and their unique fatty acid composition could be major causes of the inability of pawpaw to respond to prolonged low temperature stress.

The increase in PPO activity of overstored fruit and the consequent decline in phenolic concentration may be the primary causes of tissue browning observed in

pawpaw fruit tissue. More important than the total phenolic concentration is the balance between reduced and oxidized forms for control of oxidative stress in fruit tissue. Since ascorbic acid converts phenolic compounds from oxidized to reduced forms, and since PPO is the main enzyme in phenolic compound oxidation, the decrease in total ascorbate and the increase in PPO activity during cold storage may significantly increase the concentration of oxidized phenolics, observed as tissue discoloration. An increased concentration of these compounds would cause a decrease in phospholipid concentration in cell membranes, resulting in mitochondrial swelling and ultrastructural changes that are the ultimate causes of chilling and cold injury (Uritani, 1978).

Overall, we can conclude that the impaired ripening traits discussed in the previous chapters can all be considered oxidative stress symptoms caused by a major failure of the tissue antioxidant protection systems.

**Table 4.1.** Content of total glutathione, oxidized glutathione (GSSG), reduced glutathione (GSH) and glutathione reductase (GR) activity in ‘Taytwo’ pawpaw 1 week before commercial harvest (unripe), at harvest, and after 2, 4, 6 and 8 weeks of cold storage. Measurements were collected after 4 and 72 h of bench ripening. Glutathione data are expressed as nmol glutathione/g FW and GR activity has been expressed as  $\mu\text{M}$  NADPH/mg protein. Data are the average of n=3 replications. Values follow by the same letter are not significantly different by Fisher’s LSD Test (P=0.05).

Cold Storage (Weeks)	Bench Ripening (h)	Glutathione Pools (nmol/g FW)				GR activity
		Total	GSSG	GSH	GSH / GSSG	( $\mu\text{M}$ NADPH / mg protein)
Unripe	-	56 bcd	2.0 ab	54 bcd	27 bcd	1.37 ab
Harvest	4	46 cde	1.4 ab	45 cd	33 a-d	0.94 ab
Harvest	72	109 a	1.9 ab	108 a	57 a	3.17 ab
2	4	48 cde	2.7 a	45 cd	17 bcd	1.31 ab
2	72	71 abc	1.5 b	70 abc	46 abc	0.09 b
4	4	46 bcde	1.7 ab	44 cd	26 cd	0.13 b
4	72	95 ab	2.1 ab	93 ab	45 ab	0.54 ab
6	4	11 de	1.8 ab	9 d	5 d	1.71 ab
6	72	56 bcd	1.6 ab	54 bcd	33 a-d	6.30 a
8	4	11 de	1.7 ab	9 d	6 d	0.45 ab
8	72	9 e	1.9 ab	7 d	4 d	0.46 ab
<b>Single degree of freedom contrasts (P)</b>						
4 vs 72 h		0.0018	0.8344	0.0014	0.1398	0.3831
4 h linear		0.0429	0.8815	0.0542	0.0613	0.9338
4 h quadratic		0.5324	0.2224	0.0940	0.0299	0.9499
72 h linear		0.0003	0.9209	0.0005	0.0025	0.9088
72 h quadratic		0.1934	0.8792	0.2156	0.1280	0.9801

**Table 4.2.** Content of total ascorbic acid and ascorbate peroxidase (APX) activity in ‘Taytwo’ pawpaw 1 week before commercial harvest (unripe), at harvest, and after 2, 4, 6 and 8 weeks of cold storage. Measurements were collected after 4 and 72 h of bench ripening. Data are expressed as  $\mu\text{M}$  ascorbic acid (AA)/g FW. APX activity was expressed as  $\mu\text{M}$  AA\*100/mg protein. Data are the average value of n=3 replications. Values follow by the same letter are not significantly different by Fisher’s LSD Test (P=0.05).

Cold Storage (Weeks)	Bench Ripening (h)	Ascorbic Acid Pools ( $\mu\text{M}$ AAE/100 g FW)				APX activity ( $\mu\text{M}$ AA * 100 /mg prot )
		Total	AA	DHA	AA / DHA	
Unripe	-	57 ab	25 cdef	32 a	1 NS	23 bc
Harvest	4	61 a	25 def	36 a	1	34 bc
Harvest	72	72 a	41 a	31 ab	1	36 bc
2	4	39 bc	20 ef	19 abc	1	303 a
2	72	27 c	30 abcde	1 d	30	197 ab
4	4	35 c	27 bcdef	9 cd	3	50 bc
4	72	40 bc	38 ab	5 cd	8	10 bc
6	4	25 c	22 ef	3 cd	7	0 c
6	72	33 c	37 abc	4 cd	9	18 bc
8	4	27 c	16 ef	14 bcd	1	0 c
8	72	25 c	35 abcd	1 cd	35	0 c
<b>Single degree of freedom contrasts (P)</b>						
	<b>4 vs 72 h</b>	0.6861	<0.0001	0.9161	0.3590	0.6038
	<b>4 h linear</b>	0.0012	0.2306	<0.0001	0.6569	0.0793
	<b>4 h quadratic</b>	0.1074	0.4123	0.0745	0.5987	0.1946
	<b>72 h linear</b>	0.0006	0.7821	0.0147	0.2464	0.2293
	<b>72 h quadratic</b>	0.0478	0.6075	0.0436	0.2228	0.4899

**Table 4.3.** Pawpaw antioxidant, phenolic and carotenoid content at harvest in the cultivars 1-7-2, 8-20, ‘Middletown’ (MT), ‘PA Golden’ (PAG), ‘Shenandoah’ (SH), ‘Taylor’ (TY), ‘Taytwo’ (TT), ‘Wells’ (WE) and ‘Wilson’ (WI). Antioxidant values were expressed as g ascorbic acid equivalents (AAE)/100 g FW. Phenolic values were expressed as mg chlorogenic acid (CA)/100 g FW. Total carotenoid content was expressed as µg total carotenoid/g FW. Data are the average measurement of n=4. Data followed by the same letter are not statistically different by Fisher’s LSD Test (P=0.05).

<b>Cultivars</b>	<b>Total antioxidant ( g AAE / 100 g FW)</b>	<b>Total phenolic content (mg CA / 100 g FW)</b>	<b>Total carotenoid content (µg carotenoid/g FW)</b>
<b>1-7-2</b>	0.11 de	68 a	5.9 a
<b>8.2</b>	0.28 abcd	42 ab	1.5 d
<b>MT</b>	0.40 a	50 ab	3.2 cd
<b>PAG</b>	0.10 e	74 a	5.4 ab
<b>SH</b>	0.26 bcde	30 b	2.2 cd
<b>TY</b>	0.40 ab	49 ab	5.9 a
<b>TT</b>	0.20 cde	45 ab	3.6 bc
<b>WE</b>	0.28 abcd	47 ab	2.9 cd
<b>WI</b>	0.35 abc	68 a	6.1 a

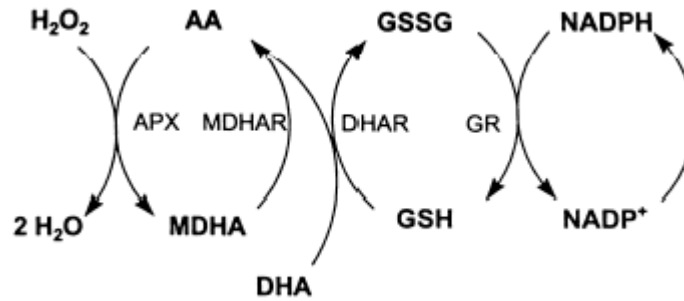
**Table 4.4.** Total phenolic content and polyphenoloxidase (PPO) activity of Taytwo pawpaw during cold storage. PPO activity was measured 1 week before commercial harvest (unripe), at harvest, and after 2, 4, 6, 8 weeks of cold storage. Measures were taken after 4 and 72 h of bench ripening. Phenolic values were expressed as g chlorogenic acid (CA)/100 g FW. PPO activity values were expressed as  $\Delta A_{420}$  /min/mg protein. Data are the average of n = 3 replicates. Data followed by the same letter are not statistically different by Fisher's LSD Test (P = 0.05). Single degree of freedom contrasts have been indicated: NS = not significant; \*\* significant at P = 0.01; \* significant at P = 0.05.

<b>Cold Storage (weeks)</b>	<b>Bench Ripening (h)</b>	<b>Phenolic content (mg CA /100 g FW)</b>	<b>PPO activity (Abs/min/mg protein)</b>
<b>Unripe</b>	-	115 ab	0.10 c
<b>Harvest</b>	4	45 defg	0.11 c
<b>Harvest</b>	72	50 def	0.17 bc
<b>2</b>	4	121 a	0.14 c
<b>2</b>	72	96 abc	0.24 abc
<b>4</b>	4	66 cde	0.14 bc
<b>4</b>	72	80 bcd	0.25 abc
<b>6</b>	4	29 efg	0.17 bc
<b>6</b>	72	113 ab	0.18 abc
<b>8</b>	4	7 g	0.33 a
<b>8</b>	72	20 fg	0.29 ab
<b>Single degree of freedom contrasts (P)</b>			
<b>4 vs 72 h</b>		0.0142	0.1408
<b>4 h linear</b>		<0.0001	0.0159
<b>4 h quadratic</b>		0.0002	0.1239
<b>72 h linear</b>		0.2355	0.2956
<b>72 h quadratic</b>		<0.0001	0.9747

**Table 4.5.** Total carotenoid content of ‘PA Golden’ pawpaw during cold storage. Values were measured 1 week before commercial harvest (unripe), at harvest, and after 2 or 4 weeks of cold storage. Measures were taken at the beginning and after 72 h of bench ripening. Total carotenoid content was expressed as  $\mu\text{g}$  total carotenoids/g FW. Data are as the average value of  $n = 4$  measurements. Values followed by the same letter aren’t statistically different by Fisher’s LSD Test ( $P = 0.05$ ). Single degree of freedom contrasts have been indicated: NS = not significant; \*\* Significant at  $P = 0.01$ ; \* Significant at  $P = 0.05$ .

<b>Cold Storage (weeks)</b>	<b>Bench Ripening (h)</b>	<b>Total carotenoid content (<math>\mu\text{g}</math> carotenoid/g FW)</b>
<b>Unripe</b>	4	1.5 c
<b>Harvest</b>	4	5.4 ab
<b>Harvest</b>	72	6.4 a
<b>2</b>	4	1.8 c
<b>2</b>	72	1.6 c
<b>4</b>	4	4.3 ab
<b>4</b>	72	1.5 c
<b>Single degree of freedom contrasts (P)</b>		
<b>4 vs 72 h</b>		0.4255
<b>4 h linear</b>		0.0007
<b>4 h quadratic</b>		0.0231
<b>72 h linear</b>		0.0261
<b>72 h quadratic</b>		0.0238

**Figure 4.1.** Asada – Halliwell pathway of hydrogen peroxide scavenging and ascorbic acid regeneration. APX, ascorbate-peroxidase; AA, ascorbate; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSH glutathione reduced form; GSSG, glutathione oxidized form (source: Blokhina, 2000)





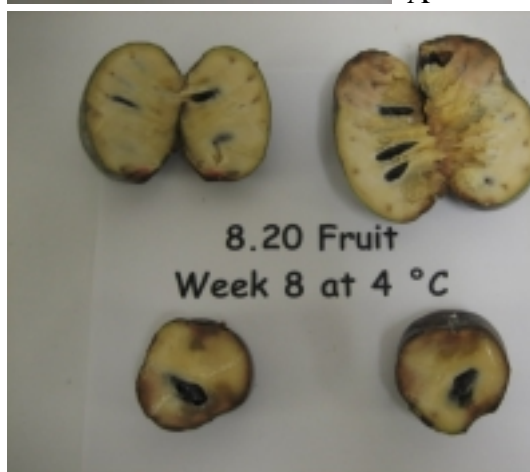
**Figure 4.2 A, B, C, D.** Pulp browning of pawpaw fruit from the cultivar 8-20. Upon removal from cold storage fruit were cut after a few hours (A and C) or after 72 h of bench ripening (B, D). Cold storage length: 4, 6 and 8 weeks.



A



B



C



D

**Figure 4.3.** Differences in pulp color of 'Wilson' (high carotenoid content) and 8-20 (low carotenoid content).



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## CHAPTER 5

### HEAT TREATMENT AND INTERMITTENT WARMING FAIL TO EXTEND SHELF LIFE OF PAWPAW FRUIT

#### INTRODUCTION

Pawpaw [*Asimina triloba* (L.) Dunal] is becoming a high value alternative crop in the southeastern United States. However, the high perishability of the fruit is a big obstacle to the development of a national pawpaw market. Low temperature storage is the most effective postharvest approach for prolonging fruit and vegetable shelf life. While pawpaw fruit may be stored at 4 °C for 4 weeks with minimal loss in quality (Archbold and Pomper, 2003), fruit cold stored longer than 4 weeks develops internal discoloration, a low sugar/acid ratio, low ethylene production, a depressed respiratory rate, and off-flavor aroma (Archbold et al., 2003; Koslanund, 2003; Chapters 2,3).

Exposure to low temperatures above or below 0 °C during fruit storage can cause tissue damage in many species. Responses of cold-sensitive fruit include scald or skin discoloration, skin desiccation, skin pitting, internal breakdown, uneven ripening, poor flavor and poor color development. Tropical species related to pawpaw in the Annonaceae family such as cherimoya are extremely sensitive to cold injury. This response is often termed chilling injury and is common with tropical and subtropical species exposed to non-freezing temperatures below 10 – 12 °C (Sabehat et al., 1996). Alique et al. (1994) observed that cherimoya stored lower than 8 °C for more than 5 days lose their ability to ripen normally and, once moved to 20 °C, develop severe skin browning and off flavor.

Due to the importance of cold storage for postharvest handling and extending shelf life, many techniques have been tested with the aim of preventing, reducing or retarding cold injury, increasing crop cold tolerance and prolonging commodity storage and shelf life. Heat treatments are commonly-applied fungal and insect control techniques (Erkan et al., 2005; Lurie, 1998). It has been shown that cold sensitive species exposed to high temperatures prior to cold storage, became more tolerant of low temperature storage

(Paull and Chen, 2000; Erkan et al., 2005; Lurie, 1998). Heat treatments can be applied using hot water, vapor heat or forced hot air. Hot water dips (50–70 °C for 10–60 s) can be safely applied directly at the storage/packaging area, and are commonly applied in the commercial postharvest warehouse (Rodov et al., 1995; Erkan et al., 2005). Vapor heat, or hot air, treatments are considered long-term treatments as they are performed by placing the commodities in heated, ventilated chambers at 38–46 °C for 12 to 96 h (Lurie, 1998). All of these treatments precede cold storage.

Both long duration/moderately high temperature and short duration/very high temperature approaches have yielded positive results in many but not all species (Paull and Chen, 2000). The discovery of an effective time/temperature combination and methodology is a key: too low of a temperature or too brief an exposure can be ineffective, but too high of a temperature or too long an exposure will cause heat damage (Paull and Chen, 2000; Erkan et al., 2005; Florissen et al., 1996). In general, heat treatments prevent the development of cold injury symptoms such as membrane damage, decay and cellular acidification during storage (Zhou et al., 2000; Merodio et al., 1998). Fruit exposure to high temperature increases heat shock protein (HSP) transcript levels in fruit, and these proteins prevent irreversible protein denaturation and confer to the fruit a temporary resistance to sub-lethal temperatures (Wang et al., 2001; Paull and Chen, 2000).

Another useful approach has been the use of intermittent warming (IW) techniques, which consist of exposing fruit to one or more periods of ambient or high temperature prior to the end of cold storage (Wang et al., 2003). Beneficial effects of IW were observed for several commodities, such as tomato (Artes et al., 1998), mango (Nyanjage et al., 1998; Zhang et al., 1997), peach (Fernandez-Trujillo and Artes, 1997; Perkins-Veazie et al., 1999; Wang et al., 2003), ‘Oroblanco’ citrus (Porat et al., 2003), oranges (Shirra and Cohen, 1999), apple (Alwan and Watkins, 1999), eggplant, okra and sweet pepper (Kluge et al., 1998). Effective IW treatments maintained fruit capacity to ripen by preventing the inhibition of the ethylene pathway often observed in fruit cold stored for an extended period (Zhou et al., 2001; Zhang et al., 1997).

Despite the benefits of heat treatment and IW observed in many species, general treatment guidelines are difficult to draw since different species, different cultivars within

a species, and fruit at different ripening stages respond differently to similar temperature/time combinations (Paull and Chen, 2000). Preliminary observations about the beneficial effect of heat treatments on slowing pawpaw ripening were reported by Koslanund (2003). Pawpaw exposed to 42, 46 or 50 °C for 15, 30 and 60 minutes prior to the beginning of bench ripening presented decreased ethylene production, a lower respiration rate and slower loss of firmness at ambient temperature when compared to untreated fruit. The activity of softening enzymes such as pectin methylesterase, endo- $\beta$ -mannanase, cellulase and polygalacturonase was, on average, 40 % lower following heat treatment than for control fruit. These preliminary results suggested that heat treatment approaches could be useful for slowing pawpaw ripening and increasing pawpaw tolerance to cold storage and, consequently, extend cold storage length for more than 4 weeks.

In this study the effects of hot air heat treatment, hot water treatment and intermittent warming on pawpaw ripening and tolerance to cold storage were investigated.

## **MATERIAL AND METHODS**

### ***Fruit harvest***

Pawpaw fruit were harvested from the Kentucky State University Research Farm, Frankfort, KY, on several dates during the August–October harvest seasons in 2003 and 2004. Because the aim of the study was the characterization of the general effect that heat treatments have on cold-stored pawpaw, and because none of the genotypes that have been studied to date exhibited different ripening patterns or cold storage responses, fruit collected from different genotypes were pooled and used to create the treatment groups. Immediately following harvest, fruit were transported to the laboratory at the University of Kentucky.

### ***Moderately high temperature/prolonged exposure time***

In 2003, fruit were placed in a heated chamber at 38 °C for 0, 24 or 72 h prior to the beginning of cold storage for 4, 8, or 12 weeks at 4 °C. During cold storage fruit were

individually sealed in poly bags (permeable to CO<sub>2</sub>, O<sub>2</sub>, and C<sub>2</sub>H<sub>4</sub>), and ethylene traps were added to the cooler. Upon removal from cold storage fruit were ripened at ambient laboratory temperature (21 ± 2 °C) for the next 3 days. Fruit firmness, respiration and ethylene production were assessed daily. Fruit that weren't exposed to a heat treatment before storage were used as controls.

#### ***Very high temperature/short exposure time***

In 2004, fruit were dipped in 60 °C water for 0, 30, 60 or 90 s prior to beginning cold storage for 4 or 8 weeks at 4 °C. Fruit were removed from cold storage, ripened, and measurements taken as noted above. Fruit that weren't exposed to a heat treatment before storage were used as controls.

#### ***Intermittent warming (IW) treatment***

After harvest in 2004, fruit were placed in a cooler at 4 °C for 4 or 8 weeks. After every 7 or 14 days of cold storage, fruit were moved to room temperature for 12 or 24 h, then returned to cold storage. After 4 or 8 weeks, fruit were removed from cold storage, ripened, and measurements taken as noted above. Cold-stored fruit that weren't exposed to IW were used as controls.

#### ***Ethylene production and respiration***

Rates of C<sub>2</sub>H<sub>4</sub> and respiratory CO<sub>2</sub> production by individual fruit were obtained as described by Archbold and Pomper (2003).

#### ***Firmness***

The external firmness of each fruit was measured by compression with a Chatillon Force Gauge as described by Archbold and Pomper (2003).

#### ***Fruit Loss***

The percentage fruit loss from mold was assessed and calculated as (((number of fruit that developed mold during cold storage and bench ripening) – (number of mold-free fruit after 72 h of bench ripening))/ (number of fruit removed from cold storage

))\*100. Mold presence was assessed weekly during cold storage and daily during bench ripening.

## RESULTS AND DISCUSSION

### *Moderately high temperature/prolonged exposure*

Using a 38 °C heat treatment for 24 or 72 h before cold storage for 4 or 8 weeks did not extend cold storage life or prevent mold attack during cold storage or during bench ripening. No differences were observed in fruit firmness upon removal from cold storage among the different treatments (Table 5.1). Moreover, after 4 weeks of cold storage, the percentage of fruit with firmness lower than 15 N was twice as high for heat-treated fruit as for control fruit. On the other hand, control fruit stored for 8 weeks had a higher mold incidence than heat-treated fruit. Apparently, even though the heat treatment did not have any effect on fruit softening, the heat treatment was effective for the control of mold during storage. Similarly, positive effects of such treatments on fruit mold control were reported in other species. Shirra et al. (1997) observed that cactus pear exposed for 24 and 72 h to 38 °C air were less sensitive to pathogen decay than non-treated fruit. Similarly, Plaza et al. (2004) and Brigati et al. (1999) observed that 30–40 °C for 60–72 h significantly decreased attack of green and blue mold on oranges and *Botrytis cinerea* attack on kiwifruit. Overall, heat treatments applied to fruit prior to cold storage reduced the incidence of stem end rot, prolonged fruit storability and didn't compromise product market value

Fruit stored for 8 weeks suffered intense mold attack that affected over 80% of the fruit. Upon request, the University of Kentucky Plant Disease Diagnostic laboratory identified *Cladosporium* spp. and *Fusarium* spp. as the major parasites of the diseased pawpaw fruit.

As observed by Archbold and Pomper (2003), ethylene and respiratory peaks of cold-stored pawpaw fruit could be detected during the first 48 h of bench ripening (Table 5.2). The ethylene peak ranged from 1 to 15 µg C<sub>2</sub>H<sub>4</sub>/kg/h and the CO<sub>2</sub> peak ranged from 90 to 242 mg CO<sub>2</sub>/kg/h (data not shown). The values for the heat-treated fruit were within these ranges; however, the ethylene peak was significantly higher for control fruit than

for heat-treated fruit after 4 weeks of cold storage (Table 5.2). On the other hand maximum respiration did not significantly vary among treatments or storage times. The reduced ethylene production of treated fruit after 4 weeks of cold storage could be interpreted as evidence of impaired ripening metabolism and loss of quality. Further, once moved back to room temperature, fruit cold stored for 4 and 8 weeks developed black discoloration in both skin and pulp. Since discoloration was not normally observed in fruit stored up to 4 weeks, their early appearance could be considered a heat injury symptom.

The results with pawpaw contrast with the successful use of similar moderately high temperature (37–38 °C) for 3 to 4 days that improved the cold storage responses of Golden Delicious apple (Lurie et al., 1995), tomato (Polenta et al., 2005), and Fortune mandarin (Holland et al., 2002). However, the results obtained with pawpaw were similar to the observation of Fan et al. (2005) with ‘McIntosh’, ‘Cortland’, ‘Jonagold’ and ‘Northern Spy’ apples, where heat treatment at 46 °C for 4, 6 or 12 h prior to the beginning of cold storage resulted in severe flesh browning once moved back to room temperature. Clearly picking the right temperature / time combination is a key factor on heat treatment success.

#### ***Very high temperature/short exposure***

A 60 °C water dip for 30, 60 or 90 s before cold storage for 4 or 8 weeks did not reduce cold storage injury symptoms or increase cold storage length. Generally, no differences in fruit firmness or mold incidence were observed between heat-treated and control fruit (Table 5.1). Overall, upon removal from cold storage, fruit were softer than fruit removed from cold storage in 2003. All fruit exhibited ethylene and respiration peaks within the first 48 h at room temperature, though heat-treatment generally reduced maximum respiration and ethylene production (Table 5.3).

#### ***Intermittent warming (IW)***

Despite the positive results obtained with other species, IW treatments decreased pawpaw storage life and increased not only loss of firmness but also fruit decay due to mold (Table 5.4). Pulp and skin developed black discoloration and mold growth was



detected after only two weeks of cold storage. After 4 weeks of cold storage, an IW cycle of 12 h every 7 days appeared to be the most successful treatment for reducing fruit decay. However, loss of firmness in these fruit was greater than that measured for control fruit. Wang et al. (2003) found that the application of IW (24 h at 20 °C every 7 or 14 days) is not always successful as peach fruit softening and senescence were extremely rapid and resulted in significantly reduced fruit marketability.

## CONCLUSIONS

Despite the positive results described for other commodities, and the promising observations with pawpaw fruit (Koslanund, 2003), 38 °C treatments for 24 or 72 h, 60 °C hot water dips for 30-90 s, and intermittent warming treatments during cold storage failed to significantly decrease cold storage sensitivity of pawpaw fruit or increase cold storage length beyond 4 weeks. Fruit softening rate was not reduced by the combination of temperatures and times tested. Mold was a persistent problem in cold storage causing the loss of many fruit. While 60 °C hot water dips for 30-90 s did reduce the incidence of mold, it may have had adverse effects on fruit ripening. The IW treatment increased the incidence of mold as well as the loss of fruit quality, so it is clearly not acceptable for pawpaw.

Since the time/temperature combination is the key element for the success of heat treatment, and only few options have been so far tested, these observations can be considered only preliminary results and shouldn't be taken as conclusive evidence that heat treatments are unsuccessful for pawpaw. In the future, other postharvest approaches that have been successful in decreasing cold injury symptoms such as methyl jasmonate infiltration, exposure to low O<sub>2</sub> or high CO<sub>2</sub> atmospheres, and Ca<sup>2+</sup> treatments could be tested for prolonging cold storage in pawpaw as they have been successful with other species (Ding et al., 2002; Maldonado et al., 2004; Lara et al., 2006; Whitaker et al., 1997).

**Table 5.1.** Fruit firmness and % fruit loss from mold of heat-treated pawpaw fruit upon removal from cold storage. Heat treatments were performed after harvest using several cultivars from the 2003 and 2004 harvest seasons. In 2003, fruit were heated at 38 °C for 0, 24 or 72 h prior to beginning cold storage. In 2004, fruit were dipped in hot water (60 °C) for 0, 30, 60 or 90 s prior to cold storage. Values are mean of at least n = 8 measurements. Data followed by the same letter are not significantly different (Fisher's LSD, P < 0.05).

<b>2003</b>				
<b>Cold storage (weeks)</b>	<b>Heat Treatment</b>	<b>Mean firmness upon removal</b>	<b>Fruit with firmness &lt; 15 N (%)</b>	<b>Fruit lost to mold (%)</b>
<b>4</b>	<b>None</b>	38 NS	17	0
	<b>38 °C / 24 h</b>	40	33	7
	<b>38 °C / 72 h</b>	31	36	0
<b>8</b>	<b>None</b>	30	50	17
	<b>38 °C / 24 h</b>	32	43	7
	<b>38 °C / 72 h</b>	33	40	0
<b>2004</b>				
<b>Cold storage (weeks)</b>	<b>60 °C Heat Treatment (s)</b>	<b>Mean firmness upon removal</b>	<b>Fruit with firmness &lt; 15 N (%)</b>	<b>Fruit lost to mold (%)</b>
<b>4</b>	<b>None</b>	12 a	80	10
	<b>30</b>	11 ab	80	0
	<b>60</b>	10 abc	87	0
	<b>90</b>	9 abcd	100	7
<b>8</b>	<b>None</b>	7 cd	100	21
	<b>30</b>	11 ab	75	20
	<b>60</b>	7 bcd	93	20
	<b>90</b>	4 d	100	25

NS = not significant differences

**Table 5.2.** Maximum ethylene production and respiration of pawpaw fruit following removal from cold storage after 4 or 8 weeks. Fruit were heated at 38 °C for 0, 24 or 72 h prior to beginning cold storage. Respiration and ethylene production were measured daily during bench ripening after removal from cold storage. Values are mean of  $n \geq 10$ . Data followed by the same letter are not significantly different (Fisher's LSD,  $P = 0.05$ ). Time of the ethylene and respiration peaks is reported as hours after removal from cold storage.

			<b>Ethylene Production</b>		<b>Respiration</b>	
<b>Cold storage (weeks)</b>	<b>Heat Treatment (h)</b>	<b>Maximum (<math>\mu\text{g C}_2\text{H}_4 / \text{kg} / \text{h}</math>)</b>	<b>Time of peak (h)</b>	<b>Maximum (<math>\text{mg CO}_2 / \text{kg} / \text{h}</math>)</b>	<b>Time of peak (h)</b>	
<b>4</b>	<b>0</b>	12.0 a	48	106 a	24	
	<b>24</b>	1.1 b	24	89 a	48	
	<b>72</b>	1.1 b	48	65 a	4	
<b>8</b>	<b>0</b>	5.8 ab	24	100 a	24	
	<b>24</b>	3.0 ab	48	72 a	4	
	<b>72</b>	2.0 b	48	80 a	48	

**Table 5.3.** Maximum ethylene production and respiration for pawpaw fruit following removal from cold storage after 4 or 8 weeks. Fruit were dipped in hot water (60 °C) for 0, 30, 60 or 90 s prior to cold storage. Respiration and ethylene production were measured daily during bench ripening after removal from cold storage. Values are mean of  $n \geq 10$ . Data followed by the same letters are not significantly different (Fisher's LSD,  $P = 0.05$ ). Time of the ethylene and respiration peaks is reported as hours after removal from cold storage.

Cold storage (weeks)	Heat Treatment (s)	Ethylene Production		Respiration	
		Maximum ( $\mu\text{g C}_2\text{H}_4 / \text{kg} / \text{h}$ )	Time of peak (h)	Maximum ( $\text{mg CO}_2 / \text{kg} / \text{h}$ )	Time of peak (h)
4	None	1.5 a	48	108 a	48
	30	1.4 b	24	73 b	24
	60	1.8 ab	24	84 ab	4
	90	0.7 bc	48	67 bc	4
8	None	1.6 a	48	103 a	4
	30	0.7 bcd	4	81 bcd	24
	60	0.4 cd	48	37 cd	24
	90	0.1 d	24	39 d	48

**Table 5.4.** Intermittent warming (IW) effect on firmness and % loss from mold of cold-stored pawpaw fruit. Fruit were stored for 4 or 8 weeks, and cold storage was interrupted every 7 or 14 days by 12 or 24 h at room temperature. The number of fruit starting storage, number of fruit remaining at the end of cold storage, % fruit loss from mold, and percentage of fruit having firmness < 15 N after removal from cold storage are reported. There were no significant effects of heat treatment on fruit firmness.

<b>Cold Storage (weeks)</b>	<b>IW Treatment</b>	<b>Fruit number at start</b>	<b>Fruit number at end</b>	<b>Fruit lost to mold (%)</b>	<b>Fruit with firmness &lt; 15 N (%)</b>
<b>4</b>	<b>None</b>	324	196	40	83
	<b>12 h every 7 d</b>	20	17	15	100
	<b>24 h every 7 d</b>	20	13	35	100
	<b>12 h every 14 d</b>	20	14	30	93
	<b>24 h every 14 d</b>	20	15	25	87
<b>8</b>	<b>None</b>	383	255	33	100
	<b>12 h every 7 d</b>	20	8	60	100
	<b>24 h every 7 d</b>	20	9	55	100
	<b>12 h every 14 d</b>	20	4	80	100
	<b>24 h every 14 d</b>	20	5	75	100

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## CHAPTER 6

### OVERALL CONCLUSIONS

The analysis of 10 different pawpaw cultivars (1-7-2, 8-20, 9-58, 'Middletown', 'PA Golden', 'Shenandoah', 'Taytwo', 'Taylor', 'Wells', and 'Wilson') over 2 seasons showed that all genotypes had similar ripening behavior after harvest. For fruit held for up to 3 days at room temperature, respiration ranged from 73 to 242 mg CO<sub>2</sub>/kg FW/h, ethylene production ranged from 0.3 to 15 µg C<sub>2</sub>H<sub>4</sub>/kg FW/h, and firmness decreased by 30–50%. In both harvest years ethylene and respiratory peaks were generally detected within 48 hours after harvest.

After storage at 4 °C for up to 8 weeks, ethylene production ranged from 0.6 µg C<sub>2</sub>H<sub>4</sub>/kg/h to 5.2 µg C<sub>2</sub>H<sub>4</sub>/kg/h, and respiration ranged from 28 mg CO<sub>2</sub>/kg/h and 125 mg CO<sub>2</sub>/kg/h. Similar to other chilling/cold sensitive species (Field, 1990; Lyons and Breidenbach, 1990; Purvis, 1990), pawpaw cold-stored for 2 weeks showed an increase in ethylene production. However respiration rate did not change and in fact declined over storage time. After only 2 weeks of cold storage, fruit firmness had decreased by 38% with an average firmness lower than 15 N. Since firmer fruit of all genotypes at harvest still had a greater proportion of fruit with firmness > 15 N at 2 and 4 weeks of cold storage, as a general guideline, only firmer fruit (> 15 N) should be cold stored. Overall, no obvious differences in ripening behavior or cold storage response were observed among the genotypes in this study. Generally, once moved back to room temperature, fruit stored longer than 4 weeks developed a brown discoloration in both pulp and peel, and had lower pH and higher soluble sugar content than fruit cold-stored for shorter periods.

No significant differences were observed in total volatile production among all varieties but one. 'Wilson' showed significantly greater total volatile production than the other varieties. However, total aroma production of 'Wilson' rapidly decreased during both bench ripening and cold storage, whereas all other varieties exhibited significant increases in volatile production. As previously reported (McGrath and Karahadian, 1994a,b; Shiota, 1991), the most common compounds in the pawpaw aroma profile were methyl and ethyl octanoates and hexanoates. Ethyl and methyl esters represented 4% and

47% of total volatile production at harvest and 39% and 52% of total volatile production after 72 h of bench ripening, respectively. Overall, cold storage for up to 4 weeks resulted in no significant differences in the volatile profile compared to freshly-harvested fruit, when considered immediately following removal from storage. However, fruit cold stored for 2 and 4 weeks and bench ripened for 72 h had significantly higher total volatile production than those ripened directly after harvest. The enhanced aroma production and similarity to at-harvest profiles confirmed the marketability of the cold-stored fruit as a fresh product through 4 weeks.

In fruit cold-stored for more than 4 weeks, methyl octanoate and ethyl hexanoate levels decreased and ethanol, ethyl acetate, and octanoic and hexanoic acid levels increased, substances often considered off-flavor compounds (Vazquez et al., 2005; Lopez et al., 1998; Merodio et al., 1998; Feygenberg et al., 2005). The increase in ethanol content, the decrease in respiration rate and the decrease in pulp pH observed in overstored pawpaw fruit suggest that long term exposure to cold during storage caused permanent mitochondrial impairment and a switch from aerobic to anaerobic metabolism

Though AAT activity was found in both harvested and cold-stored fruit, the lack of significant correlations between AAT activity, one of the most important enzymes in ester biosynthesis, and total volatile or total ester production suggested that other factors such as substrate availability could play important roles in the definition of pawpaw aroma. Moreover, neither ADH or LOX seemed to play key roles in pawpaw aroma production.

At harvest antioxidant content ranged from 0.10 to 0.40 g ascorbic acid equivalents/100 g FW (6% represented by ascorbic acid), phenolic content ranged from 30 to 74 mg chlorogenic acid/100 g FW, and carotenoid content ranged from 1.5 to 6.1 µg carotenoid/g FW among pawpaw cultivars.

Through 4 weeks of cold storage, total ascorbic acid and total glutathione concentrations were similar to at-harvest values. When storage exceeded 4 weeks components of both the ascorbate-glutathione and phenolic scavenging systems diminished, and tissue was likely exposed to reactive oxygen species attack. Though no significant changes were observed in reduced-to-oxidized ascorbate, or ascorbate peroxidase and glutathione reductase activities, total glutathione, total ascorbate, total

carotenoids and total phenolics steadily decreased during the 8 weeks of cold storage. These events are strong evidence of increasing low temperature-mediated oxidative stress and increasing levels of cold injury (Lattanzio et al., 2001; Nguyen et al., 2003; Smirnoff, 1996; Sundar et al., 2004; Barreiro et al., 2001; Zhang and Kirkham, 1996). Pawpaw fruit contain a high concentration of lipids (0.5–1% fresh weight) and unsaturated fatty acids (40% of total fatty acids) (Wood and Peterson, 1999; McGrath and Karahadian, 1994). Even though a high concentration of unsaturated fatty acids ensure low temperature membrane fluidity and temporarily protect membrane systems from cold storage damage (Lyons and Breidenbach, 1990), the high concentration of lipids and unsaturated fatty acids observed in pawpaw may predispose the fruit to cold temperature sensitivity and tissue oxidative damage. This fact combined with the decline in the protective antioxidant systems could be the major causes of the inability of pawpaw to survive prolonged low temperature stress.

The increase in polyphenoloxidase activity of overstored fruit and the decline in phenolic concentration may be the primary causes of tissue browning observed in pawpaw fruit tissue once removed from cold storage.

Despite the positive results described for other commodities, and the promising observations with pawpaw fruit (Koslanund, 2003), heat and intermittent warming treatments failed to alleviate cold injury symptoms and did not increase cold storage length beyond 4 weeks. Fruit softening rate was not slowed by the combination of temperatures and times tested. Mold was a persistent problem in cold storage causing the loss of many fruit. The 60 °C hot water dips for 30-90 s did reduce the incidence of mold at 4 weeks at cold storage, however. Intermittent warming increased the incidence of mold as well as the loss of fruit quality, so it is clearly not acceptable for pawpaw. Since the time/temperature combination is the key element for the success of heat treatments, and only a few options have been so far tested, these results can be considered as preliminary.

From the present and prior studies, some general guidelines for postharvest handling and storage of pawpaw can be derived:



1. Since cold storage doesn't slow fruit firmness loss, only firmer fruit (firmness > 15 N) should be used for cold storage at 4 °C. Thus, softer fruit should be marketed directly after harvest.
2. Pawpaw fruit can be cold-stored for up to 4 weeks at 4 °C and retain good flavor quality and appearance.
3. Due to its rapid loss of volatile production after harvest or cold storage, 'Wilson' fruit should be marketed directly after harvest.
4. From a consumer quality point of view, the high antioxidant content of 'Middletown', 8-20, 'Taylor', 'Wilson', and 'Wells' fruit suggest that these cultivars are the most health-beneficial among those used in the studies.

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## APPENDIX

### Abbreviations:

AAE – Ascorbic acid equivalent  
AAT – ALCOHOL-ACYL TRANSFERASE  
ACC – 1-Aminocyclopropane-1-carboxylic acid  
ACO – ACC OXIDASE  
ADH – ALCOHOL DEHYDROGENASE, involved in the reduction of aldehydes to alcohols  
APX - ASCORBATE-PEROXIDASE  
AA – Ascorbic acid  
AU – Area Units  
C – Curing  
DHA - Dehydroascorbate  
DHAR - DEHYDROASCORBATE REDUCTASE  
EA – Ethyl alcohol  
EAC – Ethyl acetate  
EB – Ethyl butanoate  
ED – Ethyl decanoate  
EGase – ENDO 1-4  $\beta$  D-GLUCANASE  
EH – Ethyl hexanoate  
EO – Ethyl octanoate  
EP – Ethyl propionate  
FRAP assay - Ferric reducing ability of plasma assay  
FW – Fresh Weight  
GABA - Gamma-aminobutyric acid  
GAE – Gallic acid equivalent  
GR - GLUTATHIONE REDUCTASE  
GSH – glutathione reduced form  
GSSG – glutathione oxidized form  
HA – Hexanoic acid  
HMG CoA - 3-hydroxy-3-methylglutaryl-CoA  
HPLC - High performance liquid chromatography  
HSP – High shock proteins  
HWD – Hot water dip  
IW – Intermittent warming  
LeACS – Lycopersicum esculentum ACC synthase gene family  
LOX - LIPOXYGENASE  
MA – Malic acid  
MACC- Malonyl ACC  
MAN – ENDO –  $\beta$  (1 $\rightarrow$  4) MANNASE  
MB – Methyl butanoate  
MCP – 1-Methylcyclopropene  
MDA – Malondialdehyde

MDHA - Monodehydroascorbate  
MDHAR- MONODEHYDROASCORBATE REDUCTASE  
MH – Methyl hexanoate  
MO – Methyl octanoate  
MT – ‘Middletown’  
OA – Octanoic acid  
PAG – ‘PA Golden’  
PAL – PHENYLALANINE AMMONIALYASE  
PG – ENDO-POLYGALACTURONASE  
PME – pectin methylesterase  
POD – PEROXIDASE  
PPO – POLYPHENOL OXIDASE  
ROS – Reactive oxygen species  
SDS PAGE - Sodium dodecyl sulphate polyacrylamide gel electrophoresis  
SEM - Scanning electron microscope  
SH - ‘Shenandoah’  
TA – Titratable acidity  
TBARS assay - Thiobarbituric acid reactive substances  
TSS – Total soluble sugars  
TT – ‘Taytwo’  
TY – ‘Taylor’  
VvADH – *Vitis vinifera* alcohol dehydrogenase gene family  
WE – ‘Wells’  
WI – ‘Wilson’

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