PHYSIOLOGICAL FACTORS AFFECTING ONION (ALLIUM CEPA L.) STORABILITY-CULTURAL METHODS FOR IMPROVING POSTHARVEST QUALITY

by

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(Under the Direction of Marc van Iersel)

ABSTRACT

The relationships between bulb firmness and several physiological factors were tested in onion (*Allium cepa* L). Firmness was found to be related to the amount of total cell wall material in bulbs, with the firmest bulbs having nearly twice the amount of pectin and cellulose as the softest bulbs. This was confirmed visually using transmission electron microscopy. Dry matter content was also correlated to bulb firmness. Additionally, pectin methylesterase and polygalacturonase activities were highest in those bulbs which displayed the greatest rate of softening during storage.

The effects of calcium chloride fertility as a method to improve firmness were also studied. Bulb firmness responded positively to supplemental calcium chloride. However, no link between pectin concentration and calcium regime could be established.

In a third study, a real-time pcr assay was developed for the detection and quantification of *Botrytis allii*, the causal agent of neck rot in onion. Neck rot is the most severe storage disease in onion production. The assay allowed for the detection of 1 μg of *B. allii* mycelia in onion tissue.

INDEX WORDS: onion, firmness, cell wall, middle lamella, polygalacturonase, pectin methylesterase, cellulose, calcium chloride, ammonium sulfate, disease, alkenyl cysteine sulfoxide, lachrymatory factor, real time-pcr, quantitative pcr

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Onions (*Allium cepa* L.) are highly valued as flavoring agents. They have been successfully cultivated by for thousands of years. One of the reasons for the success of onions in agriculture is their ability to be stored for long periods of time. Onions are biennial plants and the bulbs, formed after the first year of growth, are intended to be used by the plant as a nutrient source for the second year of growth. Onion bulbs are over-wintering structures that exist in a state of dormancy until environmental conditions for growth are favorable (Komochi, 1990). Humans have long since utilized the storage attributes of onion bulbs and have selected varieties based on their storability (Hanelt, 1990). Not all types of onions however, have the same storage attributes. Onions grown at low latitudes, where bulbing can be induced with 11-12 hour day-lengths, are called short-day onions (Brewster, 1990). Because the climate in these regions is mild and winters brief, short day onions usually have a limited dormancy period and are not well-suited for storage. Typically short-day onions are soft, low in dry matter, and mildly flavored with high rates of disease and softening during storage. Long day onions, in contrast are usually high in dry matter, with little decay and disease in storage (Smittle, 1988). The Granex-type bulbs grown in Georgia are short-day and soft/poor storing. Therefore it was our purpose to investigate how to improve firmness and storability

through improving plant culture and to understand what factors contribute to the softening of bulbs in storage.

Recently a number of Georgia onion growers began experimenting with supplemental calcium chloride as a way to improve bulb quality. Empirical evidence from onions grown on high calcium soils in the Western United States suggested that increased calcium fertility improved bulb quality. In addition, the sandy soils of the onion growing regions of South East Georgia are generally low or deficient in calcium, prompting several growers to apply liquid calcium chloride during the growing season. Due to its role in cell wall architecture, calcium fertility has often been considered important in determining cell wall strength and firmness of fruits and vegetables (DeEll et al., 2001; Sams and Conway, 1984). Numerous studies have reported an improvement in firmness of fruits and vegetables when supplemental calium is applied during growth (DeEll et al., 2001; Manganaris et al., 2006; Toivonen and Bowen, 1999). Therefore the first study that was conducted was a field trial investigating the effects of calcium chloride fertility on bulb firmness and storability.

In addition to determining if calcium chloride fertility affected bulb firmness and quality we wanted to investigate the differences in cell wall metabolism between good and poor storing onion bulbs. Data obtained from the analysis of onion pectin during storage of the first year of the calcium chloride field experiment indicated that significant changes were occurring in the bulb pectin over time. The roles of pectin, pectin methylesterase and polygalacturonase have been thoroughly investigated in the softening of fruits and vegetables during the ripening process (Brummell, 2006; Micheli, 2001). Recently O'Donoghue et al. (2004) examined structural and non-structural carbohydrates

in two onion cultivars. They reported greater concentrations of hemicelluloses and cellulose in the firmer, high-dry matter, long-storing cultivar, Pukekohe Longkeeper than a short-storing, soft, cultivar, Houston Grano. Their results suggested that cellulose and pectin might be linked to firmness and storability. Therefore a study was constructed that investigated the pectin composition and pectinase activity as well as a suite of other parameters in three cultivars of onion which varied in storage potential. The results of this study are presented in chapter four.

In addition to bulb degradation, storage diseases are a significant problem facing many onion growers in Georgia. The primary disease of onion storage in Georgia is neck rot, caused by the fungus *Botrytis allii*. Losses in some years can total 35% (Williams-Woodward, 2001). There are relatively few methods available to growers to combat this disease. The most commonly used practice to prevent neck rot infection in bulbs is to cure bulbs at high temperatures after harvest. Unfortunately while high temperature curing has prevented *Botrytis* neck rot losses in certain situations, the elevated temperatures can increase problems with other storage pathogens such as *Aspergillus niger* (black rot) (Sumner, 1995), *Burkholderia cepacia* (sour skin) and *Pantoea ananatis* (center rot) (Mark et al., 2002). Therefore it has become necessary to find alternative methods to reduce neck rot in storage. One approach is to test bulbs at harvest for the presence of *B. allii* inoculum. By quantifying the amount of *B. allii* inoculum present we may be able to estimate the degree of neck rot for a given storage period. A new technique that would allow us to identify and quantify *B. allii* in onion is quantitative real-time PCR. In chapter five I present the results of a real-time PCR assay developed to quantify *B. allii* mycelia in onion tissue.

CHAPTER 2

LITERATURE REVIEW

The origin and botany of onion

The common bulbing onion (*Allium cepa* L.) has been cultivated in many parts of the world for at least 5000 years. The genus *Allium* is widely distributed over temperate zones in the northern hemisphere. Present day Turkey and Afghanistan mark the primary center of diversity for the genus, being the origin for nearly 200 of the 500 species of *Allium* that have been documented. Another center of diversity is located in western North America, where high concentrations of species are confined to mountainous areas (Hanelt, 1990).

Common bulb onions belong to the class *Monocotyloneae,* superorder *Liliiflorae,* order *Asparagales*, family *Alliaceae*, tribe *Allieae*, genus *Allium,* species *cepa*, variety *cepa.* Like most *Allium*, onion has a chromosome number of eight and is diploid. Despite having relatively few chromosomes, bulb onion has an enormous genome when compared to other angiosperms. The genome of bulb onion contains about 15,290 megabase pairs of DNA per chromosome nucleus, making it roughly 107 times the size of *Arabidopsis thaliana* (Arumuganathan and Earle, 1991; Havey, 2002).

Onion leaves are hollow with longitudinal symmetry. Leaves are arranged in a distichous phyllotaxy, as new leaf blades emerge 180^{o} from the preceding leaf (DeMason, 1990). Onion leaves can be divided into two morphologically distinct parts. One, the

leaf base, which forms a sheath through which the next leaf will arise, and two, the leaf blade which is hollow, but closed at the tip and flattened on the adaxial surface (DeMason, 1990). Each succeeding leaf increases in size, until bulbing is initiated. During bulbing, newly formed leaves become smaller until no new leaf blades are formed at all (Rubatzky and Yamaguchi, 1997). The leaf bases form a stem-like structure that is called the *pseudostem*, in order to differentiate it from the true stem at the base of the plant (Nonnecke, 1989).

The vegetative axis of the onion consists of a compressed stem, from which leaves rise in a rosette pattern. At germination, a primary root emerges from the onion seed and is relatively short lived. Subsequent adventitious roots are produced continuously from the stem (DeMason, 1990). Roots typically do not branch, and have few if any root hairs. Additionally, onion roots are shallow, rarely growing more than 50 cm below the soil surface (Pierce, 1987).

Onions are biennial, and must usually undergo vernalization to flower in their second season of growth (Rabinowitch, 1990). Temperature is the primary factor affecting inflorescence development. Generally, bulbs must be exposed to temperatures of 5-10 ^{o}C for a period of one to two months in order for vernalization to occur. However, if exposed to cool temperatures for an adequate duration during their first season of growth, plants will produce a seed stalk and an inflorescence in a process called bolting (Rubatzky and Yamaguchi, 1997). Bolting can be reversed if onions are suddenly exposed to higher temperatures. If this occurs, the seed stalk will return to vegetative growth and bulbing will continue (Rabinowitch, 1990).

In onion, the last leaf formed, is actually not a leaf at all, but a leaf homologue. Called the spathe, this "pseudoleaf" encloses the floral apex (DeMason, 1990). The seed stalk, or scape, is a single elongated internodal segment that separates that last true foliage leaf and the spathe. Elongation occurs at the base of the scape through a single intercalary meristem (DeMason, 1990). When the scape reaches a height of one to two meters, the spathe splits, revealing the inflorescence. The onion's inflorescence is an umbel, and many contain from 50 to 2000 flowers (DeMason, 1990). Generally, flowers near the top of the umbel will open first, with all flowers opening over a period of two to four weeks (Rabinowitch, 1990). Individual flowers are protandrous and shed most of their pollen in two to three days. As anthers dehisce the style elongates, reaching its final length after complete dehiscence (Rabinowitch, 1990). Each flower usually consists of five whorls, consisting of three different organs each (DeMason, 1990). The two outermost whorls each consist of three perianths, while the next two whorls each contain three stamens. The innermost whorl contains three carpels arranged to form a syncarpous gynoecium, with each carpel having one locule, inside of which are two ovules (DeMason, 1990; Esau, 1977).

Onions are an outcrossing species, and are typically pollinated by bees or flies in commercial fields (Peters, 1990). Seed are collected and harvested when about 25-30% of the umbels show ripe seed. The entire umbel is harvested along with a portion of the scape, which is then dried using forced air. (Peters, 1990).

The primary organ of interest of the onion is the bulb, which is an aggregate of swollen leaf bases and the vegetative stem axis. The bulb is made up of a series of scales, which are the bases of a few outer leaves which have lost their blades, inner leaf bases

which have never formed leaf blades and a few sprout leaves in the center, which may form leaves in favorable conditions (DeMason, 1990). Outer scales are protected by a thick cuticle, which prevents dessication. When bulbing is induced, photosynthate that was produced in the leaf blades is translocated to the leaf bases. This causes swelling at the base, and the formation of a bulb (Rubatzky and Yamaguchi, 1997). Generally, the innermost (youngest) scales act as a stronger "sink" for photosynthate than the outermost (oldest) scales during active bulbing (Mann, 1983). Bulbs come in a variety of shapes including globular, ovoid, flattened disciform, bottle-like and pear-shaped (Hanelt, 1990).

Bulbing is primarily a photoperiodic response, but can also be influenced by other environmental factors including, temperature, light intensity and quality, nitrogen (N) nutrition and irrigation regime. Onions are divided into short-day, intermediate-day, or long-day cultivars, based on the photoperiod length that the plants must be exposed to in order to initiate bulbing. Short-day plants will bulb when exposed to 11-12 hour photoperiods, whereas long-day cultivars require 14-16 hour day lengths to bulb. Intermediate-day plants require day lengths near 13 hours to bulb (Brewster, 1990).

Unlike many documented photoperiodic responses in plants, bulbing in onions requires a sustained exposure to a critical day-length. A brief exposure to a critical day-length is not sufficient for bulbs to form. Bulbing can be reversed, if plants are moved to a non-inductive photoperiod for a length of time after bulbing has initiated; however, as plants age, this becomes increasingly difficult. (Brewster, 1990). Light intensity also plays a role in bulbing. It has been reported that bulb scales will be initiated earlier with increasing light intensity (Brewster, 1990). In addition to light intensity, spectral quality also plays a role in onion bulb development. The lower the ratio of red (660 nm) to far-

red (730 nm) light, the more bulbing will be enhanced. Furthermore, when plants were exposed to periods of red light during an inductive photoperiod, bulbing has been reported to be delayed, or even reversed (Mondal et al., 1986).

Temperature is another factor that influences the rate of bulbing in onion. Plants grown at temperatures below 10^{o}C will tend to have unreliable bulbing, even when exposed to inductive photoperiods (Brewster, 1977). The rate of bulbing generally increases with temperature; however, bulb yields tend to decline at temperatures nearing 30^{o}C (Brewster, 1979; Steer, 1982). Cultural practices, such as N fertility will also affect bulb development in onion. Brewster and Butler (1989) reported that applications of N late in the growing season may delay onion bulbing. Additionally, emperical observations have shown that high levels of N late in the season may increase the rate bulb splitting.

When bulbs near maturity, the leaf sheaths (pseudostem) weaken due to leaf senescence, predicated by the loss of photsynthate from the leaves during bulbing (Jones and Mann, 1963). Eventually the pseudostem can no longer support the weight of the leaf blades, and the foliage falls. When the foliage of an onion lodges at maturity, the plants are said to go "tops down." Generally onions are harvested in commercial production when approximately 50% of the plants in a field have gone tops down (Peirce, 1987). To harvest, onion bulbs are undercut, and left in the field to cure under direct sunlight, or into containers to be cured using forced air (Peirce, 1987).

Georgia onion production

The Georgia sweet-onion industry, so named due to the mild flavor and high sugar content in Georgia onions, is an important component of Georgia agriculture.

Onions for fresh market are the number one vegetable in terms of farm-gate income in GA, and number four in total acreage. In 2006 10,500 acres were harvested with an estimated farm value of $82 million (Maw, 2006). The onion industry is located in the South Eastern (S.E.) portion of the state and encompasses 20 counties. “Vidalia” onions, named after the town in which they were first grown, have become synonymous in the marketplace with a mild sweet flavor and high quality. This emphasis on high quality has allowed GA growers to command a premium price for their onions, often charging more than $20 for a 50 lb bag early in the season (Bill Randle personal communication).

Vidalia onions owe their mild flavor to a lack of sulfur (S) compounds in the bulbs. Typically S-containing amino acids, called the S-alk(e)nyl cysteine sulfoxides (ACSO)s are hydrolyzed via the enzyme alliinase during maceration of the bulb (Block 1992; Randle and Lancaster, 2002). Low levels of ACSOs result in mildly flavored bulbs. Because Vidalia onions are grown on sandy soils which are slightly deficient in S fertility, growers are able to produce bulbs with low ACSO levels that possess a mild flavor (Freeman and Mossadeghi, 1970; Randle et al., 1995). A mild, moist climate in the S.E. region of the state also improves mild onion production (Coolong and Randle 2003; Freeman and Mossadeghi, 1973; Platenius 1941).

Sulfur compounds in onion

For a detailed summary of the synthesis of the *S*-alkenyl cysteine sulfoxides ACSOs (flavor precursors) in *Allium sp.* the reader is directed to a number or excellent reviews (Block 1992; Breu 1996; Granroth, 1970; Jones et al., 2004; Randle and Lancaster, 2002; Whitaker 1976).

In the flavor biosynthetic pathway of onion, a glycine residue may be cleaved from glutathione (GSH) by a carboxypeptidase leaving γ-glutamyl-L-cysteine. Gamma-glutamyl-L-cysteine is the building block for a number of γ-glutamyl peptides (γGP) in onion, which ultimately are utilized in the formation of the ACSOs (Jones et al., 2004). The three principal ACSOs found in onion are: *trans*-(+)-*S*-(1-propenyl)-L-cysteine sulfoxide (1-PRENCSO), (+)-*S*-methyl-L-cysteine sulfoxide (MCSO), and (+)-*S*-propyl-L-cysteine sulfoxide (PCSO) (Jones et al, 2004; Randle and Lancaster, 2002).

When an onion cell is lysed either during maceration or due to penetration by an invading pathogen, the enzyme alliinase (alliin alkyl-sulfate-lyase) (E.C. 4.4.1.4) is released from the vacuole. Alliinase catalyses the cleavage of the *S*-alk(en)yl sulfoxide group from individual ACSOs, with pyridoxal-5'-phosphate acting as a co-factor. The products of the reaction are α-iminoproprionic acid and a sulfenic acid. The α-iminoproprionic acid is highly unstable and is immediately converted to pyruvate and ammonia (Block 1992). The sulfenic acids that are produced immediately condense with each other to form a number active S compounds which include the thiosulfinates (flavor compounds), (Z)-Propanthiol-S-oxide (lachrymatory factor), and lesser studied thiosulfonates. (Block 1992; Breu 1996; Whitaker 1976).

Antiseptic properties of *Allium spp.* have been ascribed to the organosulfur compounds that arise from the hydrolysis of the ACSOs with alliinase. Most studies reporting the antimicrobial effects of *Allium spp.* consider the effects of gross extracts instead of individual compounds (Harris et al., 2001; Kim et al., 2004; Kumar and Berwal, 1998; Kyung and Lee, 2001). This is likely due to the fact that breakdown products of the ACSOs are in a continuous state of flux. The initial products of the

reaction with alliinase (after the spontaneous recombination of various sulfenic acids) are typically thiosulfinates, capaenes or the lachrymatory factor (Breu 1996). These compounds are transient in nature however; and will quickly form thiosulfonates, volatilize, and/or form a large number of mono, di and tri sulfides (Block 1992; Breu 1996; Kopsell et al., 2002) The reactions are temperature, light, and pH dependent (Block et al., 1992; Breu 1996; Whitaker 1976). With dozens of S compounds in the same reaction mixture it would be difficult to determine the effects of an individual compound *in vivo*. There is a strong consensus however, that it is the S compounds in *Allium spp.* that give them their antimicrobial properties (Block 1986; Block 1992; Dorsch 1996; Griffiths et al., 2002; Kim et al, 2004; Kyung and Lee 2001)

Non-sulfur compounds in onion

Despite the presence of antioxidant flavor compounds, onions are considered nutritionally poor. A typical (110 g) bulb will yield about 40 calories (Nonnecke, 1989). An average 100 g fresh sample will consist of 90% water, 1.5 g of soluble proteins, 0.1 g of fat, and about 8.7 g of carbohydrate (Peirce, 1987). Fresh bulbs contain approximately 10 mg of ascorbic acid (vitamin C), 0.03 mg of thiamine, 0.04 g riboflavin, and 0.2 mg of niacin per 100 g sample (Peirce, 1987). Mineral content in bulbs is also very low. On average, 100 g of dry bulb tissue contains about 363 mg calcium, 3 mg iron, 122 mg magnesium, 340 mg phosphorous, 943 mg potassium, 54 mg sodium and 2 mg zinc (Fenwick and Hanley, 1990).

Various flavonoids have been isolated from onion tissue. Quercetin was the first flavonoid isolated from dry onion scales by Hummel and Perkin in 1896. Several other flavonoids have since been isolated, including quercitin-4'-glucoside, quercitin-3,4'-

glucoside and quercitin,4-7'-glucoside (Breu, 1996). Flavonol content varies between bulb color and cultivar, with white bulbs generally having least amounts and red or yellow bulbs having the highest. Trammel and Peterson (1976) reported that flavonol distribution within the bulb varies, decreasing from the outside and top of the bulb.

Much of the non-structural carbohydrate content in onion bulbs can be attributed to glucose, fructose, and sucrose, which contribute 2.0, 0.9 and 3.2% respectively of the total fresh weight (Breu, 1996). Fructans are also important in high dry matter cultivars, contributing up to 5% fresh weight. Sugar content can vary with cultivar, location within the bulb and growing conditions (Breu, 1996; Darbyshire, 1978).

Structural carbohydrates include the pectins, celluloses and hemicelluloses that make up the cell wall of onion. Though they are considered a source of dietary fiber, along with fructans, most interest lies in the role they play in cell wall architecture and structural integrity in the bulb.

Structural carbohydrates/cell wall

The plant cell wall is composed of the primary cell wall, middle lamella and in some cases a secondary cell wall. Primary cell walls primarily consist of the polysaccharides, cellulose, hemicellulose and pectin (Reiter, 2002). The middle lamella is the gel-like structure between adjacent cells that is a pectin rich region (Vorwerk et al., 2004). Calcium in the cell wall is associated with the pectins in the primary cell wall and the pectin rich middle lamella.

Pectin is a generic term that covers a number of polysaccharides rich in galacturonic acid (Ridley et al., 2001). Specifically there are three classes of pectins: homgalacturonan (HGA), rhamnogalacturonan-I (RG-I), and rhamnogalacturonan-II

(RG-II) (Carpita and Gilbeaut, 1993). Homogalacturonan is composed of a linear chain of β-1,4 linked α-D-galactopyranosyluronic acid that can form a number of methyl and acetyl esters with a carboxyl group bound to the number six carbon (Ridley et al., 2001). In contrast RG-II is made up of a galactopyranosyluronic acid backbone, but has a number of complex side chains and does not display a linear structure like HGA (Willats et al., 2001). Classified as a pectin, little is known about the structure of RG-I. It is composed of a disaccharide backbone of (-4-α-D-galactopyranosyluronic acid-{1-2}α-L-rhamnose) (Ridley et al., 2001). The tertiary structure is highly variable with a number of complex side chains and suspected methyl and acetyl esterification (Ridley et al., 2001). HGA, however, is the only pectin in which $Ca^{2+}$is known to interact. Another essential plant nutrient, boron, is involved in linking RG-II chains (Carpita and McCann, 2000).

In HGA, methyl esters can become cleaved in the presence of pectin methyl esterase (PME). This leaves free carboxylic acid residues on the HGA backbone that can bind to Ca^{2+} if present (Kunzek et al., 1999; Ridley et al., 2001; Thakur et al., 1997). Calcium can form an ionic bond between two HGA backbones thereby linking them. Because of the proposed structure this gives the polygalacturonate chains, it is often called the "egg-box" model (Carpita and McCann, 2000). The presence more Ca^{2+} can lead to an increased number of cross linkages in HGA thus strengthening the middle lamella and increasing fruit firmness (Kunzek et al., 1999). Polygalacturonases can also act to cleave the homogalacturonic acid pectin chains once they have been de-esterified by PME. If this happens, long chain pectins are degraded and cell wall loosening occurs leading to possible a loss of structural integrity during storage.

Storage of onion

The successful storage of onion bulbs is essential to the onion industry for two reasons. One, it allows for onions to be available at all times of the year, and two, onions are a biennial crop, therefore, bulbs for seed production must be able to be stored until the following growing season.

Bulbs are storage organs by nature and therefore are better suited for storage than other vegetables. Onion dormancy begins with a decrease in respiration and levels of abscisic acid in the bulb (Matsubara and Kimura, 1991) and is generally initiated prior to foliar lodging. Typically long day and high dry matter type cultivars store better than short-day low dry matter types (Gubb and MacTavish, 2002; Suzuki and Cutcliff, 1989). Short day onions are adapted to lower latitudes they typically do not have to contend with long overwintering periods. Therefore, they tend to have short periods of dormancy and will often break dormancy quickly if stored at higher temperatures (Miedema 1994). To decrease sprouting in storage, growers harvest onions for storage at a later date than fresh market bulbs, store onions under cool temperatures (0-4^{o}C) and apply maleic hydrazide, a sprouting inhibitor (Brewster 1987; Salama and Hicks, 1987; Smittle and Maw 1988; Wall and Corgan 1994). Water loss through the neck of the bulbs is also a significant factor affecting onion storage (Rajapakse et al., 1992). This is counteracted using controlled atmosphere storage (5% O_2, 75% relative humidity) (Smittle 1988; Smittle 1991). All of the above mentioned storage methods are employed to decrease respiration in bulbs, thereby reducing dry matter losses in storage (Hong and Kim, 2001; Ladeinde and Hicks 1988; Salama and Hicks, 1987).

Although water loss and sprouting represent significant problems for storing short day onions, disease accounts for most postharvest losses (Ko et al., 2002; Rajapakse et al., 1992; Tietjan and Ceponis 1981). The most important disease in onion storage is neck rot, caused by the fungus, *Botrytis allii*. In some years storage losses due to neck rot can reach 35% of stored product (Williams Woodward, 2001).

Botrytis allii

Botrytis allii can infect bulbs in a number of ways. Seed are often the primary source of infection in European countries, and are occasionally a source of inoculum in the U.S. (Ellerbrock and Lorbeer 1977; Maude, 1990; Maude and Presly, 1977a; Walcott et al., 2004). Other potential sources of *B. allii* inoculum include mycelia and sclerotia in infected onion debris and overwintering crops (Maude, 1976; Walker, 1952). Infection of the seedling occurs when mycelia, growing on or under the seed coat, penetrate the tip of the developing cotyledon during germination. Then, as the older, infected leaves begin to senesce, conidiophores, which are produced only on necrotic tissue, release their conidia and subsequently infect other leaves (Maude, 1990). The crop can then sustain the infection during the growing season, sometimes without displaying additional symptoms, suggesting that the fungus may be capable of biotrophic growth (Maude, 1990). As leaves senesce prior to harvest, the infection becomes active, spreading rapidly on necrotized leaves. During harvest mycelia from germinating conidia can infect bulbs through open wounds in the neck area when the foliage is cut and removed (Bochow and Mosallamy, 1979; Maude and Presly, 1977b; Tichelaar, 1967). Evidence suggests that the fungus will not invade dry bulb or external neck tissues (Maude and Presly, 1977b; Mumm, 1917; Walker, 1926). It is likely then that Botrytis neck rot does

not spread through bulb-to-bulb contact in storage (when outer scales are dry). Instead, the appearance of *B. allii* in storage is more likely due to the progressive deterioration of bulbs already infected from the field (Maude, 1983; Maude, 1990; Maude and Presly, 1977a).

Other onion storage pathogens

In addition to fungal diseases, several bacterial pathogens have been shown to cause significant field and storage losses for onion growers (Mark et al., 2002). Sour Skin, *Burkholderia cepacia,* (formerly *Pseudomonas cepacia*) is a gram-negative aerobe that can enter the onion through the leaf axils, resulting in bacterial lesions on the leaves (Mark et al., 2002). The disease can then progress to the bulb leading to water soaked and macerated scales, producing the typical sour skin symptoms. *Burkholderia cepacia* often infects the plant though a wound, generally in a young leaf (Kawamoto and Lorbeer, 1972a; Klement and Lovrekovich, 1962). Once the pathogen enters the plant, it spreads through the intercellular spaces releasing pectolytic enzymes which produce a soft rot and maintain a water congested environment necessary for *B. cepacia* to survive (Rudolph et al., 1994). Kawamoto and Lorbeer (1972b) reported that *B. cepacia* could spread throughout intercellular spaces, suggesting that infection in several scales may result from a single infection site. Pectolytic enzymes (polygalacturonases) are necessary for the degradation of polysaccharides in the intercellular space and middle lamella enabling *B. cepacia* to spread (Gonzalez et al., 1997; Gross and Cody, 1985). Because increased Ca^{2+} has been reported to decrease fungal polygalacturonase activity and strengthen the middle lamella (Cabbone and Doneche, 2002; Kunzek et al., 1999; Volpin and Elad, 1991), the same effect may occur with bacterial disease suppression and

polygalacturonase inhibition. We therefore feel that research with supplemental $CaCl_2$, supplied through either a foliar spray or soil applied is warranted and could decrease the severity of sour skin in onion.

Another economically important onion bacterial disease is center rot caused by *Pantoea ananatis.* It is a gram-negative bacterium that can cause yield losses of up to 100% (Gitaitis and Gay, 1997; Mergaert et al., 1993). Symptoms of center rot first appear on the center leaves of the plant and display a water-soaked appearance eventually taking on a bleached appearance as the disease progresses (Mark et al., 2002). As the disease progresses, interior bulb scales become watery and attempts to lift bulbs by pulling on the foliage can result in the outer scales separating from the bulb.

The disease was first thought to be introduced through infected seed; however, additional evidence found that the bacterium was endemic and colonized a number of asymptomatic weed species in the onion growing regions (Gitaitis et al., 2002; Walcott et al., 2002). *Pantoea ananatis* may move from weed species to neighboring onions via tobacco thrips (*Frankliniella fusca*), a common insect pest in onion fields (Gitaitis et al., 2003). *Pantoea ananatis* was first observed on onion in the USA in 1997 and little is known about the mechanisms of action of this bacterium once inside the plant (Gitaitis and Gay; 1997). Strains of *P. ananatis* utilize cellobiose, sucrose, glycerol, and inositol, but not pectins or gelatins as carbon sources *in vitro* (Bradbury, 1986).

Alternaria porri, the causal agent of purple blotch in onion is another fungal disease that can cause significant losses. *Alternaria porri* infects the leaves through the stomatal opening or via direct penetration of the leaf surface. Upon infection, secondary hyphae develop and move through intercellular spaces until uninfected mesophyll cells

are reached and subsequently penetrated (Aveling et al., 1993). Like *Botrytis spp., A. porri* secretes pectolytic enzymes facilitating cell wall penetration. Virulence is associated with pectolytic activity. Wasfy et al., (1977) reported a large (~25x) increase in polymethylgalacturonase activity on virulent *A. porri* isolates when compared to avirulent strains.

Disease management in storage

Currently no onion varieties are marketed with resistance to *B. allii*. In 2004, scientists at Cornell University reported that they had successfully bred onions with resistance to *Botrytis* leaf blight (*Botrytis squamosa*), but there is no evidence that these bulbs are also resistant to *B. allii* infection. In addition, compounds registered for use pre-harvest in the field or during bulb storage are largely ineffective against *Botrytis* neck rot. For example, fungicidal dips and sprays applied to the bulbs immediately before storage were found to be ineffective in controlling neck rot (Lorbeer and Kawamoto, 1963). Furthermore, the application of fungicidal dust or salts to the bulb neck immediately after harvest, were only marginally effective in controlling *B. allii* infection (Lorbeer, 1972). Other tactics used to attempt post harvest disease control have included gamma irradiation of the bulbs, and exposing bulbs to ozone prior to and during storage. Both approaches have exhibited limited success in controlling neck rot (Ceci et al., 1991; Curzio and Croci, 1983; Smittle, 1988).

As an alternative to late season sprays, the most commonly accepted practice for preventing the occurrence of *B. allii* in storage is forced air curing of onion bulbs at high temperatures immediately after harvest. Since 1925, curing onions immediately after harvest has been an accepted practice to decrease neck rot incidence in storage (Walker,

1925). High temperature bulb curing works because; 1: dried tissues do not support hyphal growth and 2: higher temperatures help to kill the fungus (Maude, 1990). In New Zealand, forced air ventilation (36-38^{o}C) for up to 7 days prior to storage is used to limit the spread of *B. allii* (Harrow and Harris, 1969). Although recommendations call for post harvest curing for at least 48 hours at 30-34 ^{o}C, time constraints force shorter curing periods in commercial applications (Maude et al., 1984). Curing bulbs for shorter time periods at higher temperatures (>40 ^{o}C) has not worked well, as significant bulb damage has been observed in several tests (Guzman and Haslip, 1962; Maude 1990; Maude et al., 1985).

While high temperature curing has prevented Botrytis neck rot losses in certain situations, the elevated temperatures can increase problems with other storage fungi such as the black mold caused by *Aspergillus niger* (Sumner, 1995), or cause of a number of bacterial bulb pathogens to proliferate (Maude 1990). *Burkholderia cepacia*, which has a growth optimum between 30-35 ^{o}C, spreads rapidly among bulbs maintained at these temperatures. Curing bulbs at high temperatures (>40 ^{o}C) is lethal to both fungal and bacterial pathogens, but would cause significant bulb damage (Guzman and Haslip, 1962; Maude 1990). Curing bulbs for longer periods but at lower temperatures has been considered, but is logistically unfeasible due to commercial constraints at harvest. The emergence of bacterial diseases in onion may force some growers to rethink or abandon curing, which may further exacerbate problems with *B. allii*.

Calcium application and storage

Calcium is a plant macro-nutrient, with leaf values typically ranging from 0.1 to 0.5 % dry weight (Marschner 1995). Although Ca^{2+} deficiencies rarely occur in nature,

they often occur in agriculture. Examples of Ca^{2+}disorders include blossom end rot in S*olanaceous* crops, tip burn in lettuce and bitter pit in pome fruits (Rubatzky and Yamaguchi, 1997). Calcium is taken up by the roots as part of the soil solution. Calcium is thought to move both apoplastically and symplastically through the root until it is delivered to the xylem (Cholewa and Peterson, 2004; White and Broadley, 2003). After traveling in the xylem, Ca^{2+} is unloaded into cells where it can be sequestered in the vacuole, bound to proteins in the cytosol, or incorporated into the cell wall. In addition Ca^{2+} in the cell and vacuole is often involved in a complex second messenger system that plays a vital role for a plant's response to changing environmental conditions (White and Broadley, 2003).

Numerous studies have reported the beneficial effects of $CaCl_2$ on storage characteristics of fruits. Researchers have shown that both postharvest dips and preharvest sprays of $CaCl_2$ have enhanced the postharvest quality of apples, peppers and cantaloupes (Luna-Guzman and Barrett, 2000; Sams and Conway, 1984; Toivonen and Bowen, 1999). Much work has been done with postharvest dips of apple in solutions of $CaCl_2$. Because of the thick pericarp on apple, fruit are often treated under vacuum, to enhance infiltration of Ca^{2+} into the fruit (Siddiqui and Bangerth, 1996). The effects of $CaCl_2$ dips on apple are multiple. Sams and Conway (1984) demonstrated that Ca^{2+}infiltration reduced respiration and ethylene formation during storage, as well as increasing fruit firmness in apple. The same authors also reported a decrease in disease of apple treated with $CaCl_2$, which they attributed to enhanced formation of cell wall components which increased resistance to disease (Conway and Sams, 1984; Conway et al., 1991).

Calcium influences the activity of pathogenic organisms through two documented mechanisms (Elad, 1997). First, is the indirect effect of Ca^{2+} on strengthening the cell wall, thereby creating a mechanical barrier to pathogenic attack. The second involves excess unbound Ca^{2+} inhibition of endo- and exo-polygalacturonase activity. This limits or restricts the ability of the pathogen to cleave the pectin backbone of the middle lamella, thereby preventing the spread of the disease through the plant.

Calcium applications directly affect *Botrytis* disease in plants. Botrytis leaf blight and neck rot, caused by *Botrytis cinerea* and *B. allii*, respectively, are major onion pathogens in the field and storage. *Botrytis spp.* infect plant tissues by secreting polygalacturonases which cleave the homogalacturonic backbone of cell wall and middle lamella pectins, allowing penetration into the host (Elad, 1997; Kritzman et al., 1981). Calcium applications were shown to be effective in preventing or reducing *B. cinerea* infection in a number of plant systems. Moreover, Chardonnet and Donceche (1995a,b) reported that Ca^{2+} treatments inhibited endo-polygalacturonase activity in grape through altering the chelation of pectin and reducing its susceptibility to pectolytic enzymes. Several other studies reported that free Ca^{2+} supplied via $CaCl_2$ inhibited polygalacturonase activity at Ca^{2+} concentrations typically found in plant cells (Cabbone and Doneche, 2002; Jauneau et al., 1994; Volpin and Elad, 1991).

Calcium, delivered via $CaCl_2$, has been shown to reduce fungal disease incidence in a number of plant hosts. Pathogenic polygalacturonase activity has been directly or indirectly affected by improving the resistance of homogalacturonic acid to enzymatic degradation.

Testing for disease

In addition to spraying traditional fungicides, $CaCl_2$, or curing onion bulbs other options exist to help growers manage neck rot in storage. One such option is to quantify the amount of a specific disease in bulbs at harvest and then make a decision on whether to store or sell those bulbs. This involves testing bulbs for the presence of a pathogen. Currently, there are only three methods available for testing bulbs for *B. allii.* First, samples could be taken to a well-equipped diagnostic or research laboratory where tissue samples would be cultured on semi-selective agar media for 10-14 days at 25 ^{o}C. After 14 days the plates would be examined for dark brown staining which would be indicative of polyphenol oxidase activity (Kritzman et al., 1978). Positive plates must be examined microscopically by trained personnel to distinguish *B. allii* from other fungal species. Second, samples could be tested for *B. allii* using an indirect enzyme linked immunosorbent assay (ELISA), which relies on polyclonal antisera raised against cell wall and cytoplasmic antigens from *B. allii* (Linfield et al., 1995). This test is rapid, however, results can be difficult to quantitate. Although ELISA tests can sometimes be quantitative when used in conjunction with an optical plate reader, problems with selectivity and sensitivity have been reported (Lopez et al., 2003). Thirdly, samples could be assayed for the presence of *B. allii* DNA by using the polymerase chain reaction (PCR). PCR can be extremely specific and sensitive, and oligonucleotide primers have been developed that can detect low levels of inoculum (1 pg fungal DNA). Additionally these primers differentiate between the five groups of *Botrytis*: *B. aclada* AI and AII (*B. allii*), *B. byssoidea, B. squamosa*, and *B. cinerea* (Nielsen et al., 2002). As with the ELISA test, conventional PCR is rapid and relatively inexpensive, but cannot be used to

quantify inoculum levels. The usefulness of qualitative tests for bulbs going into storage is not good because *B. allii* is ubiquitous in commercial onion fields. Just testing for the presence of *B*. *allii* on a large scale would likely yield near 100% positive results (particularly for very sensitive PCR tests), which would be useless as a decision-making tool. What is needed is a quantitative test that correlates inoculum level with levels post-harvest bulb rot.

While it is technically feasible to quantify levels of fungal pathogens using variants of conventional PCR (competitive PCR), these methods are laborious, prone to error and require advanced analytical skills (Mahuku and Platt, 2002, Schena et al., 2004). Recently, real time quantitative PCR has made it possible for researchers to easily conduct qualitative and quantitative PCR analyses.

Quantitative real-time PCR

Like conventional PCR, real time PCR uses a primer pair, plus a fluorescently labeled oligonucleotide probe which emits light energy at a specific and detectable wavelength once excited (Wilhelm and Pingoud, 2003). In the TaqMaq® PCR system a probe is designed to anneal to the template DNA internal to the two flanking primers. A fluorescent dye is bound to the 5' end of the probe while a "quencher" molecule that prevents fluorescence by absorbing emitted light energy is bound to the 3' end (Wilhelm and Pingoud, 2003). When both fluorescent dye and quencher molecules are bound to the probe, there is no fluorescence. After annealing to the target DNA sequence, the exonuclease activity of Taq DNA polymerase digests the probe, which releases the flourophore from the quencher molecule leading to fluorescence (Schena et al., 2004). The amount of fluorescence is proportional to the amount of target (pathogen) DNA is

recorded in "real-time" by a specially designed thermocycler allowing for quantification. Typically the amount of fluorescence is related to a cycle threshold (Ct) value, which is the cycle number at which the fluorescence from the desired sample surpasses background fluorescence. The lower the Ct value, the fewer cycles required for the sample to reach the threshold, and the *more* target DNA is in the sample. Conversely, a very high Ct value would mean that more amplification cycles were required to reach the threshold, and that less template DNA is in the sample. Inherently, the quantity of nucleic acid present will be directly proportional to the mass of mycelial cells of the target fungus. Thus one can develop a standard curve for mycelial mass and the corresponding Ct values.

In addition to being quantitative, real time PCR has another advantage over traditional PCR in that by detecting the presence of a PCR product (i.e., fluorescence) in the reaction tube, one does not need to run a gel after the reaction to determine if amplification occurred. This saves significant time and decreases cross-contamination error as the sample is handled less.

Real time quantitative PCR has been used successfully in a number of plant-pathogen systems to estimate pathogen levels *in planta*. Suarez et al. (2005) demonstrated a linear relationship between levels of *B. cinerea* infection in *Pelargonium sp.* Ct value indicated the ability to quantitatively predict infection levels in the plant with real time PCR. Levels of *Alternaria brassiciciola* and apple proliferation phytoplasma have also been successfully detected *in planta* using real time PCR in *Arabidopsis thaliana* and *Malus domestica*, respectively (Baric and Dalla-Via, 2004; Gachon and Saindrenan, 2004).

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CHAPTER 3

THE EFFECTS OF CALCIUM CHLORIDE AND AMMONIUM SULFATE ON ONION (*ALLIUM CEPA* L.) BULB QUALITY DURING STORAGE[1]

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Abstract

Sandy soils in the onion (*Allium cepa* L.) growing region of S.E. Georgia are low in calcium (Ca). The bulbs produced here are typically soft and susceptible to postharvest pathogens. Preliminary greenhouse studies have indicated that supplemental calcium chloride ($CaCl_2$) improved bulb firmness. The effects of supplemental $CaCl_2$ on the quality of field grown onions were therefore investigated. Additionally, preliminary studies had indicated that $CaCl_2$ may inhibit sulfur (S) in onion and improve flavor. Thus ammonium sulfate $(NH_4)_2SO_4$ and $CaCl_2$ levels were altered together to determine if $CaCl_2$ could improve flavor at different levels of nitrogen (N) and S fertility. Onions cv. 'Georgia Boy' were grown with 0, 250, and 500 $kg \cdot ha^{-1}$ $(NH_4)_2SO_4$ and 0, 115, and 230 $kg \cdot ha^{-1}$ $CaCl_2$ in a factorial combination in 2005 and 2006. Total bulb yield increased with increasing $(NH_4)_2SO_4$, but was unaffected by $CaCl_2$. The percentage of diseased bulbs increased during storage in both years, and was affected by $(NH_4)_2SO_4$ fertility in 2006. Bulb scale firmness increased with supplemental $CaCl_2$ fertility and decreased significantly during storage in both years. Total pectin concentrations increased during storage in 2005, but fell during storage in 2006. Water soluble pectin (WSP) and chelator soluble pectin (CSP) concentrations increased during storage during 2005. Calcium chloride did not affect bulb pectin composition, but $(NH_4)_2SO_4$ treatments lead to an increase in bulb CSP in 2005 and 2006. Onion pungency decreased with additional $CaCl_2$ in 2006. Calcium chloride had a limited effect on flavor precursor concentrations, however. There were no interactions between fertility treatments, though a few instances

there were $(NH_4)_2SO_4$ by storage time interactions. With the exception of yield differences among years in the parameters measured were generally small.

Introduction

Onions (*Allium cepa* L.) are grown worldwide for their flavor attributes. In some regions niche markets have developed for producing sweet, mild flavored bulbs. Onions grown in S.E. Georgia are sold under the trademark of Vidalia onions and account for nearly $100 million in farm-gate income annually (Maw, 2006). Valued for their flavor, these bulbs are typically soft and store poorly (Kopsell and Randle, 1997). Although genetic differences are important in determining bulb qualities such as flavor, firmness and postharvest disease susceptibility, several studies have indicated that the environment also can affect these quality characteristics (Kopsell and Randle, 1997; Randle, 2000; Uzo and Currah, 1990, Yoo et al., 2006). Empirical observations suggest that bulbs grown on the sandy, low-calcium (Ca) soils such as those found in S.E. Georgia are softer and more susceptible to disease than similar cultivars grown on higher Ca soils. Furthermore greenhouse studies (Randle, 2005) have suggested that the Ca status of bulbs can affect bulb firmness and postharvest quality.

Calcium has a myriad of roles in the plant. A high proportion of Ca in plant cells is often found in the cell wall/middle lamella region (Marschner, 1995). Here it is bound to carboxyl groups of polygalacturonic acids (pectin) serving to link adjacent chains through ionic bonds (Carpita and Gibeaut, 1993). Due to its role in cell wall architecture, Ca has been considered important in determining cell wall strength and firmness of fruits and vegetables (DeEll et al., 2001; Sams and Conway, 1984; Van Buren, 1979). Numerous studies have reported an improvement in firmness of fruits and vegetables

when supplemental Ca is applied during growth (DeEll et al., 2001; Manganaris et al., 2006; Toivonen and Bowen, 1999). In addition to improvements in firmness, Ca applications have also been reported to enhance disease resistance in some crops (Conway et al., 1991; Volpin and Elad, 1991). Because mild flavored, sweet onions are often soft and highly susceptible to disease during storage, they may be suitable candidates for supplemental Ca fertility. Furthermore, Somers (1973) reported that onion cell walls had a high affinity for Ca ions. Earlier studies on the effects of Ca fertility on onion have focused on nutrient balancing or yield (Boyhan et al., 2002, Coolong et al., 2004; Fenn and Feagley 1999). Hence, little is known regarding the effects of Ca on bulb quality attributes.

In addition improving bulb firmness, applications of $CaCl_2$ have been correlated to decreases in bulb pungency in greenhouse studies (Randle, 2005). Previous studies have indicated that enhanced N and S fertility can lead to increases in pungency (Coolong and Randle, 2003, Randle and Lancaster 2002). The lower pungency observed when $CaCl_2$ is applied may be the result two mechanisms. One, chloride may compete with nitrate or sulfate for uptake by the plant, therefore reducing pungency (Barbier-Brygoo et al., 2000). Two, the application of Ca to the soil may lead to the formation of calcium sulfate, which is much less soluble than other sulfate containing minerals in the soil (Doner and Lynn, 1989). This could lead to a removal of available S to the onion, thus reducing pungency. Supplemental $CaCl_2$ therefore may have several benefits to sweet onion growers. In addition to improving firmness and postharvest quality it may also decrease N or S availability to the plant thus reducing flavor intensity. However, if N or

S uptake or availability is substantially inhibited, other parameters such as yield could be negatively influenced.

Therefore the objectives of this research were to determine suitability of $CaCl_2$ as an amendment to improve onion bulb firmness and postharvest quality in field grown bulbs. To test the possible inhibitory effects of $CaCl_2$ on bulb flavor, N, and S uptake, $CaCl_2$ treatments were combined with different levels $(NH_4)_2SO_4$, a common form of N and S employed by onion growers. To understand possible mechanisms by which $CaCl_2$ affects bulb firmness total pectin and pectin fractions were measured. In addition, bulb S-flavor compounds were measured to determine if $CaCl_2$ applications would affect flavor in field grown onions.

Materials and Methods

Plant material. All plants used in this study were grown at the Vidalia Onion and Vegetable Research Center, Lyons, GA (~32 ^{o}N). Soils at the farm are an Irvington Loamy Sand pH 6.2. Seeds for the onion cultivar 'Georgia Boy' were sown into seed beds at a rate of 200 seeds per linear meter on 19 Sept. 2004 and 19 Sept. 2005. Seedlings were grown for eight weeks following the guidelines of The University Georgia Cooperative Extension Service (Boyhan et al., 2001). On 23 Nov. 2004 and 1 Dec. 2005 seedlings were pulled and cut to a length of approximately 15 cm. The transplants were set into raised beds spaced 1.8 m on center. Transplants had an in-row spacing of 14 cm and a between-row spacing of 30.5 cm with four rows per raised bed. Each experimental plot was 9.15 m in length with a 1.8 m space between plots in each row for a total of 260 plants per plot. The study was arranged as a 3 x 3 factorial completely randomized design with three treatment levels each of $(NH_4)_2SO_4$ and $CaCl_2$.

Each treatment was replicated four times, for a total of 36 experimental plots. Border plots were planted with 'Georgia Boy' and received no supplemental $CaCl_2$ or $(NH_4)_2SO_4$. With the exception of fertility treatments, onions were grown according to Cooperative Extension Guidelines (Boyhan et al., 2001). In addition, soil samples for both growing seasons were obtained from each test plot prior to planting and after harvest and mineral concentrations determined (The University of Georgia Cooperative Extension Service Soils Testing Laboratory).

Raised beds received 455 $kg \cdot ha^{-1}$ 5N-10P_2O_5-15K_2O with 9% S three weeks prior to transplant. At six and eight weeks post-transplant all plants received KNO_3 and KH_2PO_4 at a rates of 145 and 75 $kg \cdot ha^{-1}$, respectively. At 12 weeks post-transplant all plants received $CaNO_3$ (15.5N-0-0) at a rate of 225 $kg \cdot ha^{-1}$. Calcium fertility treatments consisted of three levels of $CaCl_2$ (0, 28.75, 57.5 $kg \cdot ha^{-1}$ $CaCl_2$) applied as a liquid band at the base of the plants at 8,12,16, and 20 weeks after transplant to ensure an even distribution throughout the growing season. This resulted in a total season application of 0, 115, and 230 $kg \cdot ha^{-1}$ $CaCl_2$. The $(NH_4)_2SO_4$ treatments consisted of two applications of 0, 250, and 500 $kg \cdot ha^{-1}$ $(NH_4)_2SO_4$ applied in granular bands at the base of the plants at six and ten weeks post-transplant resulting in a total application of 0, 250 and 500 $kg \cdot ha^{-1}$ $(NH_4)_2SO_4$ for the growing season. These fertility treatments were chosen based on low, medium and high levels of S fertility and levels of $CaCl_2$ typically employed by growers in the region.

Plants were undercut and hand harvested on 11 May 2005 and 10 May 2006. Bulbs were cured with forced air at 36 ^oC for 48 h. Bulbs were weighed to get cured yields and sorted into 20-bulb nylon mesh bags for immediate analysis or refrigerated

storage. Bulbs were stored for 10 and 20 weeks with set points of -15.5 ^{o}C and 70% RH. Upon removal from storage bags, bulbs were weighed, cut longitudinally and disease symptoms visually assessed (Schwartz and Mohan, 1995). Subsequent analyses were performed on the combined tissue from each 20 bulb replication. Heavily diseased bulbs were excluded from analysis.

Mineral Analysis. A 5 mm longitudinal slice was taken from each bulb, weighed and oven dried for 7 d at 70 ^{o}C. After dry weights (DW) were determined, tissue was ground to a fine powder using a coffee grinder. Total bulb S was determined by combining 0.2 g of dry tissue with 0.1 g of vanadium pentoxide accelerant and analyzing using a Leco 232 S analyzer (Leco Corp., St. Joseph Mich.). Bulb total N was determined with approximately 0.25 g of dried bulb tissue using a Leco CNS 2000 (Leco corp.). Calcium concentrations were determined using the wet acid digestion method and a Perkin Elmer AAnalyst 300 (Perkin Elmer, Norwalk, Conn.) (Mills and Jones, 1996).

Firmness. Bulb scale firmness was measured by cutting a 2 x 4 cm rectangular piece from the first fully fleshy scale (usually the second or third scale from the outside of the bulb) at the equatorial region of each bulb. Firmness was measured as the force in Newtons (N) required to penetrate the scale using a 1 mm diameter probe coupled to a fruit penetrometer mounted to a motorized press operated at a speed of 1.5 mm·s^{-1} (Model 327, McCormick Fruit Tech, Yakima, Wash.). Firmness for each 2 x 4 cm slice was measured three times and averaged.

Alcohol-insoluble residue, pectin fractioning and total pectin determination. The alcohol insoluble residue (AIR) was prepared from onion tissue according to a modification of the method of Huber and Lee (1986). Longitudinal slices 5-10 mm in

thickness were cut from bulbs. Slices were homogenized in a blender for 60 s with four volumes (w/v) of 95% ethanol. Two more volumes of 95% ethanol were added and the homogenate was boiled at 100 ^{o}C for 20 min with slow stirring. The homogenate was cooled in an ice-water bath for 30 min. The residue was filtered under vacuum through glass fiber filters (APFF, 0.7 µm, Millipore, Billerica, Mass.). Based on the initial sample weight the residue was sequentially washed with six volumes of 95% ethanol, with four volumes of 100% ethanol, and four volumes of acetone. The residue was dried overnight in a fume hood. The dried AIR was weighed and ground to a fine powder using a coffee grinder and stored at -20 ^{o}C until analysis.

The pectin in the AIR was fractionated into water, chelator, acid, and alkali soluble pectins according to a modification of the method of DeVries et al. (1981). Approximately 30 mg of onion AIS was extracted at 60 ^{o}C for 90 min in 40 mL of 0.05 M sodium acetate buffer, pH 5.2 to obtain water soluble pectin (WSP). The WSP was obtained by centrifuging the extract at 30,000 *g* for 15 min and filtering the supernatant through one layer of Miracloth (CalBiochem, EMD Biosciences, San Diego, Calif.). The remaining pellet was resuspended in 40 mL of 0.05 M sodium oxalate, 0.05 M ammonium oxalate, and 0.05 M sodium acetate pH 5.2 and incubated for 90 min at 60 ^{o}C to obtain chelator soluble pectin (CSP). The extract was again centrifuged at 30,000 *g* for 15 min and supernatant filtered through one layer of Miracloth to obtain the CSP. The remaining pellet was again resuspended in 40 mL of HCl pH 2.5 and incubated for 90 min at 60 ^{o}C. The extract was centrifuged and filtered as previously to obtain the acid soluble pectin (ASP). The remaining pellet was resuspended in 40 mL of 0.05 M NaOH and incubated for 90 min at 60 ^{o}C, centrifuged and filtered as previously in order to

obtain the alkaline soluble pectin (ALSP). The pectin (uronic acid) content of each fraction was determined with the *m*-hydroxydiphenol method (Blumenkrantz and Asboe-Hansen, 1977). Total pectin was determined with the method of Ahmed and Labavitch (1977). Approximately 5 mg of AIR was weighed into a 50 mL beaker to which 5 mL of concentrated cold sulfuric acid was slowly added to minimize charring. The beaker was stirred in an ice bath for approximately 10 min until nearly all of the AIR dissolved and 5 mL of ice cold deionized water was added in 1 mL increments. After 10 min cold deionized water was added to bring the solution to a volume of 25 mL in a volumetric flask. An aliquot of the solution was analyzed for galacturonic acid (Blumenkrantz and Asboe-Hansen, 1977). Galacturonic acid content of samples was estimated from a linear regression using galacturonic acid as a standard at concentrations of 0 - 20 μg.

Soluble Solids, Pungency and Lachrymatory Factor. The soluble solids content (SSC) and pyruvic acid concentration (pungency) were determined according to Randle and Bussard (1993a). In brief, a 1 cm diameter core was taken from the equatorial region of each bulb and crushed in a pneumatic press. The juice was collected and 0.2 mL was analyzed for SSC using a hand-held refractometer (Kernco, Tokyo, Japan). A 0.5 mL portion of juice was used for pyruvic acid analysis using the *2,4*-Dinitrophenylhydrazine method (Randle and Bussard, 1993a). The concentration of propanethial S-oxide (lachrymatory factor) (LF) was determined according to a slight modification of Schmidt et al. (1996). In brief, 2.0 mL of juice was added to 2.0 mL of chilled methylene chloride containing 0.4% m-xylene (internal standard). The mix was inverted several times and centrifuged at 1,000 g for 5 min. The methylene chloride fraction was analyzed using

GC analysis. The LF was analyzed on a Shimadzu GC-17A GC with a flame ionization detector (Shimadzu Corp., Kyoto, Japan). A 5 m x 0.53 mm fused silica 2.65 μm film thickness capillary column was used (DB-1, J&W Scientific, Agilent Technologies, Santa Clara, CA. Injector and detector temperatures were 210 ^{o}C and 250 ^{o}C, respectively. The temperature program was as follows: initial temperature was 60 ^{o}C held for 20 s, increased at

15 ^{o}C·min^{-1} to100 ^{o}C and held for 30 s. Helium was used as the carrier gas and column flow rates were 8.2 mL·min^{-1}. A 1 μL sample injection was made with a 10:1 split ratio. Identity of the LF was confirmed using gas chromatography-mass sprectrometry (GC-MS) (The University of Georgia Chemical and Biological Sciences Mass Spectrometry Facility).

Flavor Precursors. The S-alkenyl cysteine sulfoxides (ASCO) were determined according a modification of the GC method of Kubec et al., (1999). First, 2-5 mm thick longitudinal slices were taken from bulbs and extracted (10:1, w/v) in 80% methanol at -20 ^{o}C for ten days. To each 10 mL extract, 0.5 mg of ethyl cysteine sulfoxide (ECSO), synthesized according to Lancaster and Kelly (1983), was added dried using forced air (Evap-o-Rac; Cole Parmer, Vernon Hills, Ill.). The dry samples were redissolved in 1mL of high performance liquid chromatography (HPLC) water and passed through a column (1.5 x 12 cm) containing 1 x 5 cm of cation exchange resin (Dowex 50W-X8, 200-400 mesh) Bio-Rad, Richmond, Calif.) pre-treated with 20 mL of 3% HCl. Interfering substances were removed with 10 mL of 3% HCl followed by 15 mL of HPLC water. The ACSOs were eluted from the column with 15 mL of 2 M ammonium hydroxide. The eluate was evaporated using forced air. The dry residue was dissolved in 1 mL of

32:60:8 ethanol:water:pyridine; 0.4 mL of which was derivatized with 0.1 mL of ethyl chloroformate. After 1 h, derivatized ACSOs were reduced to alkenyl cysteines by the addition of 0.2 mL of sodium iodide solution (0.5 $g \cdot mL^{-1}$) and 50 µL of acetyl chloride. To complete the reduction of the ACSOs, samples were allowed to stay at room temperature for 8 h. The derivatized alkenyl cysteines were extracted with the addition of 0.3 mL of methylene chloride. The organic phase was separated and analyzed using GC analysis.

Samples were analyzed on a Shimadzu GC-17A GC with flame ionization detector (Shimadzu Corp.). A 30 m x 0.32 mm fused silica 0.25 µm film thickness capillary column was used (HP-5, J&W Scientific, Agilent Technologies, Santa Clara, Calif.). Injector and detector temperatures were 180 ^{o}C and 250 ^{o}C, respectively. The temperature program was as follows: initial temperature was 120 ^{o}C held for 0 s, increased at 2 $^{o}C \cdot min^{-1}$ to160 ^{o}C held for 30 s, then to 280 ^{o}C at a rate of 10 $^{o}C \cdot min^{-1}$ and held for 5 min. Helium was used as the carrier gas with a column flow rate of 8.2 $mL \cdot min^{-1}$. A 1 µL injection was made with a split ratio of 10:1. Response factors for the derivatized alkenyl cysteines were determined using standards. The response factor for *S*-1-propenyl-L-cysteine sulfoxide [isoalliin(PECSO)], which is not commercially available and very difficult to synthesize, was estimated to be the same as that of *S*-2-propenyl-cysteine sulfoxide (alliin) (Kubec, 1999). Propyl cysteine was prepared according to the method of Lancaster and Kelly (1983), while methyl cysteine, ethyl cysteine (Sigma, St. Louis, MO), and allyl cysteine (TCI America, Portland, OR) were purchased. The identities of individual derivatized alkenyl cysteines, and cycloalliin were confirmed using GC-MS. Because PECSO will easily cyclize when subjected to alkaline conditions

such as those incurred during cation exchange chromatography, it was necessary to include cycloalliin in our measurements of PECSO (Virtanen and Matikkala, 1959). Kubec et al. (1999) found almost complete conversion PECSO to cycloalliin using a similar method of sample preparation. Therefore the areas of both isomers of cycloalliin were added to the area found for PECSO to get an estimate of PECSO concentration in the samples.

An Agilent 6870N GC coupled to an Agilent 5973 MS (Agilent Tech.) was used for GC-MS analysis of derivatized alkenyl cysteines and cycloalliin. A 30 m x 0.25 mm capillary column with a 0.25 μm film thickness was used for compound separation (HP-5MS, Agilent Tech.). The temperature program was as mentioned previously for GC analysis. One microliter of sample was injected into a split injector (10:1) ratio with He carrier gas and an injector temperature of 180 ^{o}C. Column flow was 1.0 mL/min. The MS ion source was held at 230 ^{o}C and mass spectra were obtained over the range of 50-550 mass units.

Statistical Analysis. All data were subjected to the GLM procedure testing the significance of main effects and interactions SAS statistical software (version 9.1.3, SAS Institute, Cary, NC). Mean separations were performed using Duncan's test of mean separations when appropriate. Percentage data were subjected to the arcsin transformation prior to analysis. Interactions among treatments and/or sampling time were uncommon and generally did not affect the interpretation of the results. Therefore main effects are primarily described in the proceeding sections, though interactions are discussed when significant.

Results and Discussion

Yield and mineral nutrients. In 2005 and 2006 $(NH_4)_2SO_4$ applications lead to an increase in bulb yield, while $CaCl_2$ treatments had no affect (Table 1). No treatment interactions were observed. The yield increase resulting from $(NH_4)_2SO_4$ could be expected as N and S fertility have been shown to increase yields in onion (Coolong and Randle, 2003; Hussaini et al., 2000). The addition of 250 and 500 $kg \cdot ha^{-1}$ $(NH_4)_2SO_4$, resulted in similar yield increases, indicating that the baseline fertility applied may have been limiting growth, but the application of 250 $kg \cdot ha^{-1}$ $(NH_4)_2SO_4$ was sufficient to increase yields. In addition yields were significantly greater in 2006 than in 2005. In 2005, environmental conditions were conducive for onions to flower (bolt) during maturation. During 2005 up to 20% of the bulbs in a given plot bolted, though no treatment differences were observed. In 2006 however, only a very small number of plants (<0.1%) formed inflorescences. Because bulbs that have already formed an inflorescence stalk were not harvested, their prevalence in the 2005 growing season may be responsible for the low yields observed that year.

In order to confirm the efficacy of the fertility treatments, bulb N, S and Ca concentrations were measured at harvest. In both years bulb N concentrations increased with $(NH_4)_2SO_4$ fertility indicating that the additional supply of N was taken up by the plants (Table 2). Bulb S increased with the addition $(NH_4)_2SO_4$ in 2006 but not in 2005. The increase in bulb N in 2005 indicates that the $(NH_4)_2SO_4$ treatment did affect the mineral concentration in bulbs, despite bulb S remaining unchanged. Supplemental $CaCl_2$ increased bulb Ca concentrations in 2005 and 2006 (Table 2). This indicates that $CaCl_2$ treatments were successfully applied. Additional $CaCl_2$ also lead to a significant

decrease in bulb S in 2006. This is confirms earlier results observed in greenhouse studies (Randle, 2005). No interactions were observed between $(NH_4)_2SO_4$ and $CaCl_2$ for N, S or Ca concentrations in the bulb. This indicates that $CaCl_2$ could be employed to reduce S uptake by onion, over a large range of S levels in the soil, potentially improving flavor. The mechanism for $CaCl_2$ to decrease bulb levels of S is not clear. However it is plausible that chloride and sulfate compete for availability and uptake by anion channels (Barbier-Brygoo et al., 2000). It is also possible that the additional Ca could combine with available sulfate to form gypsum, therefore reducing availability of S to the plant.

In addition, bulb concentrations of N, S, and Ca were lower in 2006 than in 2005. The higher yields in 2006 may have resulted in diluting the pool of available nutrients in the root zone, leading to lower bulb concentrations of N, S and Ca (Zink, 1966).

Weight loss and dry matter. Bulb weight loss during storage was not affected by $CaCl_2$ or $(NH_4)SO_4$ in either year. Bulb weight loss increased significantly during storage (Table 3). The percentage %DW was not affect by treatment or storage time in 2006, averaging 10.2%. In 2005 however bulb %DW decreased when 500 $kg \cdot ha^{-1}$ $(NH_4)_2SO_4$ was supplied. This would be expected as the excessive growth associated with plants grown under high N would lead to a decrease in dry matter concentration. In addition, bulb %DW decreased during storage in 2005. This is interesting, because it suggests that there was significant loss of dry matter in the bulb in first 10 weeks of storage in 2005. Generally water is believed to account for more than 90% of weight loss in onion bulbs during storage (Komochi, 1990). Bulb SSC, a measure of gross soluble carbohydrates was not affected by $CaCl_2$ or $(NH_4)_2SO_4$ in either year, and was only affected by storage

time in 2006 (Table 3). Bulb SSC increased slightly between harvest and 10 weeks of storage in 2006. This was most likely due to water loss leading to an increase in SSC.

Disease status. One of our primary objectives in this study was to determine if $CaCl_2$ affected disease incidence during storage. Onion neck rot caused by *Botrytis allii* was the most prevalent disease observed, though some bulbs displayed symptoms of Sour Skin and Center Rot, caused by *Burkholderia cepacia* and *Pantoea ananatis*, respectively. Calcium chloride did not affect disease in 2005 or 2006. In 2005 $(NH_4)_2SO_4$ applications had no effect on disease incidence, but in 2006 interacted with storage time to affect the number of diseased bulbs at 10 and 20 weeks of storage (Table 4). In 2006 applications of 250 $kg \cdot ha^{-1}$ $(NH_4)_2SO_4$ lead to a decrease in disease incidence during storage. Bulbs receiving 500 $kg \cdot ha^{-1}$ of $(NH_4)_2SO_4$ had a decrease in visible disease symptoms when compared to the 0 $kg \cdot ha^{-1}$ $(NH_4)_2SO_4$ at 20 weeks of storage, but had a greater incidence of disease symptoms than the 250 $kg \cdot ha^{-1}$ $(NH_4)_2SO_4$ treatment. The reason for the decrease in storage disease observed with the application 250 $kg \cdot ha^{-1}$ $(NH_4)_2SO_4$ compared to the 0 $kg \cdot ha^{-1}$ $(NH_4)_2SO_4$ treatment could be that bulbs grown with no additional $(NH_4)_2SO_4$ in 2006 may have been slightly deficient in S (1416 $mg \cdot kg^{-1}$ DW) (Table 2). However the application of 500 $kg \cdot ha^{-1}$ $(NH_4)_2SO_4$, while providing sufficient S, may have provided excessive N which can lead to an increase in onion storage rot (Batal et al., 1994). This may be why the application of 500 $kg \cdot ha^{-1}$ $(NH_4)_2SO_4$ had less disease incidence than the 0 $kg \cdot ha^{-1}$ $(NH_4)_2SO_4$, but more than the 250 $kg \cdot ha^{-1}$ $(NH_4)_2SO_4$ at 20 weeks of storage in 2006.

Disease incidence in bulbs increased during storage for both years (Table 4). At harvest about 3-4% of bulbs displayed visible disease symptoms, however this increased

to 25-30% of bulbs after 20 weeks of storage. Large increases in the presence of disease, particularly neck rot, during storage are typical during onion storage (Williams-Woodward, 2001)

Bulb scale firmness. In 2005 and 2006 supplemental $CaCl_2$ interacted with storage time to affect onion bulb firmness (Table 5). Additional $CaCl_2$ increased scale firmness by 5 and 8% in 2005 and 2006, respectively at harvest. However, after 10 weeks of storage there were no differences in firmness among the $CaCl_2$ treatments. This indicates that at the levels used in this experiment, additional $CaCl_2$ will improve firmness at harvest, but the increase in firmness would not be enough to compensate for the softening that occurs during storage. Because the effects of $CaCl_2$ disappeared during storage this may indicate that the mechanism for the increase in bulb firmness when additional $CaCl_2$ is applied may be different than the mechanism by which softening occurs. In addition the application of 500 $kg \cdot ha^{-1}$ $(NH_4)_2SO_4$ lead to a decrease in firmness in 2006. Mean bulb firmness decreased from 2.97 N in the 0 and 250 $kg \cdot ha^{-1}$ $(NH_4)_2SO_4$ to 2.88 N in the 500 $kg \cdot ha^{-1}$ $(NH_4)_2SO_4$ treatments. There was no interaction between storage time and $(NH_4)_2SO_4$ as the differences in firmness remained throughout storage. A decrease in firmness with additional $(NH_4)_2SO_4$ may be expected as additional N has been reported to decrease firmness the firmness of hydroponically grown bulbs at harvest (Randle, 2000).

Although storage time interacted with $CaCl_2$ applications to affect firmness, it is worth discussing the degree of softening that occurred during storage. After 10 weeks of storage, bulb firmness decreased 8% and 14% in 2005 and 2006, respectively. Declines in firmness are often associated with a decrease in cell turgor that accompanies water loss in fruits and vegetables (Gomez-Galindo et al., 2004). Water loss is generally considered to

be responsible for most of the weight loss of bulbs in storage (Komochi, 1990). Bulb weight loss from harvest in 2005 and 2006 was not correlated (P=0.22, r=0.1) to changes in bulb scale firmness during storage. This suggests that water loss may not be the primary mechanism for softening of onion scales during storage. Recent findings in our laboratory suggest that softening in onion bulbs may be related to changes in the middle lamella region resulting in cell slippage (unpublished data).

Pectin Supplemental Ca fertility is thought to improve the firmness of fruits and vegetables by increasing the Ca available to interact with carboxyl groups in adjacent polygalacturonic acid (pectin) chains strengthening the primary cell wall (Micheli, 2001). Therefore we chose to measure total pectin and individual pectin fractions in an effort to determine if supplemental $CaCl_2$ or $(NH_4)_2SO_4$ affected the composition of structural carbohydrates in onion. Total pectin concentrations were not affected by $CaCl_2$ or $(NH_4)_2SO_4$ treatments in 2005 or 2006 (Table 6). Total pectin concentrations decreased during storage in 2005 and 2006. The decrease in uronic acid concentrations can be explained by changes in the amount of extractable AIR. In both years total uronic acid concentrations expressed per unit of AIR did not change during storage. However in 2005 and 2006 the amount of AIR extracted per unit of fresh tissue decreased during storage. These differences in extractable AIR resulted in changes in total pectin concentrations when expressed in $mg \cdot g^{-1}$ DW. Significant decreases in extractable AIR have been observed in apple fruit during development and postharvest ripening (Fischer and Amado, 1994). The decrease in AIR in apple was attributed to changes in starch content during storage (Fischer and Amado, 1994). However, onions contain very little starch (Darbyshire and Steer, 1990) suggesting that the decrease in extractable AIR is the

result of the metabolism of other alcohol insoluble compounds in the bulb. There were no significant interactions between treatments or storage time and treatments for total pectin or pectin fractions in 2005 or 2006.

The water soluble pectin (WSP) fraction is characterized by highly esterified, unbranched polymers that have little interaction with surrounding components of the cell wall (Heredia et al., 1995). The concentration of WSP was not affected by $CaCl_2$ or $(NH_4)_2SO_4$ applications in 2005 and 2006 (Table 6). The WSP was affected by storage time though. The WSP increased during storage in 2005, but fell during storage in 2006 (Table 6). The small increase in WSP during storage in 2005 would be expected as increases in WSP have been correlated to softening in many fruits and vegetables (Brummell, 2006). In 2006 WSP decreased slightly between 10 and 20 weeks of storage. This change mirrored a small increase in alkaline soluble pectin (AKSP) suggesting changes in the solubility of the pectin during storage. Cantor et. al (1992) also found changes in the solubility of pectin during cold storage of peach.

The chelator soluble pectin (CSP) generally represents low to medium esterified pectin chains that are stabilized by forming ionic bonds with Ca ions, and as such are solubilised by chelating agents (Heredia et al., 1995). It was thought that supplemental $CaCl_2$ would increase the concentration of CSP. However, in this study $CaCl_2$ did not affect the amount of CSP in the bulbs (Table 6). Additional $(NH_4)_2SO_4$ applications lead to an increase in CSP concentrations in 2005 and 2006. O' Donoghue et al. (2004) reported that additional S fertility did not affect onion CSP. This suggests that the increase in CSP observed in bulbs grown with additional $(NH_4)_2SO_4$ may be due to N or the combined effects of N and S together. In 2005 there was an increase in CSP during

storage, though in 2006 CSP concentrations simply fluctuated during storage (Table 6). This indicates that pectin methyl esterase (PME) may be active in bulbs during storage as WSP pectin chains must be first de-esterified prior to forming CSP. Garcia et al. (2002) reported PME activity in fresh onion tissue, though did not investigate PME activity during storage. Because PME activity is related to softening in some fruits and vegetables in future studies it may be appropriate to investigate the role of PME in softening of onion during storage.

The acid soluble pectin fraction (ACSP) consists of covalently bound pectins that are solubilised by weak acids (Heredia et al., 1995). As expected the ACSP made up the smallest portion of pectin fractions measured (Table 6). The concentration of ACSP was not affected by either fertility treatment or storage time in either year. The AKSP, which primarily consists of highly branched pectin polysaccharides in onion (Ng et al., 1998), was unaffected $(NH_4)_2SO_4$ and $CaCl_2$ in 2005 and 2006. The AKSP was affected by storage time in both years, however. In 2005 the AKSP decreased during storage, suggesting an increase in solubility of long chain pectins during storage. In 2006 AKSP concentrations declined at 10 weeks of storage and then increased to harvest levels after 20 weeks, mirroring changes in CSP that occurred during storage in 2006. The results obtained here indicate that increase in firmness observed with supplemental $CaCl_2$ fertility may not be realized through changes in pectin concentrations suggesting an different mechanism by which firmness is enhanced with $CaCl_2$. In addition, although the composition of onion cell wall pectin has previously been investigated (Ng et al., 1998), to our knowledge this is the first attempt to quantify changes in pectin

composition in onion during storage. The results obtained here suggest that pectin metabolism in onion is a dynamic process that may affect quality of stored bulbs.

Pungency, LF and Flavor precursors Although sugars contribute to onion flavor, S-containing compounds dominate the flavor profile of freshly consumed bulbs (Block, 1992). Additional S fertility has been shown to lead to an increase in flavor intensity in numerous greenhouse studies (Coolong and Randle, 2003; Randle et al., 1995; Randle and Bussard, 1993b). Recently S and N applications have been shown to affect flavor potential in field-grown onions as well (McCallum et al., 2005). Calcium chloride has also been reported to decrease bulb S concentrations and reduce flavor potential in greenhouse-grown onions, thus improving palatability of bulbs for fresh consumption (Randle, 2005). In order to determine the effects of $CaCl_2$ on onion flavor potential at a variety of N and S levels in field-grown onions, pungency, flavor precursor and lachrymatory factor (LF) concentrations were measured.

Pyruvic acid is a bi-product that is formed during the enzymatic hydrolysis of the *S*-alkenyl cysteine sulfoxides (ACSO) (flavor precursors) by the enzyme alliinase (EC 4.4.1.4) when bulb tissue is disrupted (Block, 1992). Because the amount of pyruvic acid that is generated is proportional to the gross flavor intensity of bulbs it has been utilized to estimate the overall flavor intensity, or pungency of bulbs (Schwimmer and Weston, 1961; Wall and Corgan, 1992). Total pyruvic acid (TPY) increased with additional $(NH_4)_2SO_4$ fertility in 2005 and 2006 (Table 7). Additionally, in 2006 TPY levels decreased when additional $CaCl_2$ was supplied. Though not observed in 2005, these results indicate that additional $CaCl_2$ could lead to a decrease in bulb pungency. Accordingly, additional $CaCl_2$ also reduced bulb S levels in 2006 (Table 2). Bulb TPY

concentrations increased during storage in 2005 and 2006. Increases in pungency during storage have been observed previously as additional ACSOs are believed to be synthesized during storage leading to an increase in pungency (Kopsell and Randle, 1997 Kopsell et al., 1999). No treatment or storage time interactions were observed with bulb TPY.

The lachrymatory factor [LF, (*Z,E*) propanethial *S*-oxide] is a direct product of the hydrolysis of 1-propenyl cysteine sulfoxide (PECSO) and can dominate the onion flavor profile when present in high levels. As the name implies, it is responsible for the tearing sensation associated with chopping onions. Though not affected by $CaCl_2$, LF concentrations increased with additional increasing $(NH_4)_2SO_4$ (Table 7). Although McCallum et al. (2005) reported that N and S fertility affected LF concentrations in a field-grown a pungent cultivar 'Kojak', to our knowledge this is the first time that N and S supply have been shown to affect LF concentrations in field-grown mild onion cultivar. Onion LF concentrations were significantly affected during storage in both years (Table 7). However, while LF concentrations generally increased after 20 weeks of storage in 2005, in 2006 LF concentrations decreased during storage. The LF is the product of the hydrolysis of the flavor precursor, PECSO. However during storage, changes in the LF did not correspond to changes in PECSO. In 2005 concentrations of PECSO decreased in storage, while LF levels increased. In 2006 PECSO concentrations decreased by 47% after 20 weeks in storage, but LF concentrations only decreased by 14%. The reason for the poor correlation between LF and PECSO concentrations in storage could due to differences in alliinase activity in the onion macerate at different storage times in 2005 and 2006 (Uddin and MacTavish, 2003). In 2005 and 2006 PECSO concentrations were

not affected by $CaCl_2$, but increased with additional $(NH_4)_2SO_4$ as was the case with the LF (Table 7). No treatment or storage interactions were observed with LF or PECSO concentrations.

Two additionally ACSOs, methyl cystiene sulfoxide (MCSO) and propyl cysteine sulfoxide (PCSO) were measured. Found in majority *Allium* and some *Brassica* species, MCSO when hydrolyzed by alliinase, is thought to impart a cabbage-like flavor to onion (Lancaster and Boland, 1990; Randle et al., 1994). In 2005 and 2006 bulb MCSO concentrations were not affected by $CaCl_2$, but increased when grown under additional $(NH_4)_2SO_4$ (Table 7). Of the three ACSOs measured MCSO responded the most to applications of $(NH_4)_2SO_4$. McCallum et al., (2005) reported similar increases in MCSO concentrations in field-grown bulbs subjected to an additional 200 $kg \cdot ha^{-1}$ supply of S. Bulb MCSO concentrations increased in storage in 2005 and 2006, indicating an active synthesis of MCSO during storage. Propyl cysteine sulfoxide is typically found in the lowest concentrations among the three flavor precursors routinely detected in onion. Volatiles generated from the hydrolysis of PCSO lend a chive-like flavor to bulbs (Randle et al., 1994). As expected, PCSO was present in the lowest concentration of the three ACSOs measured (Table 7). Onion PCSO concentrations increased with additional $(NH_4)_2SO_4$ in 2005 and 2006. Interestingly, PCSO concentrations decreased with additional $CaCl_2$ in 2006. It is likely that this decrease in PCSO is responsible for the decrease in TPY observed with supplemental $CaCl_2$ in 2006. Bulb PCSO concentrations also increased during storage in 2005 and 2006. No treatment or storage interactions were evident when measuring bulb MCSO and PCSO concentrations.

As expected supplemental $(NH_4)_2SO_4$ increased bulb flavor potential. To our knowledge this is only the second time that supplemental N and S have been reported to affect flavor precursors and the LF in field grown onions (McCallum et al., 2005) Additionally $CaCl_2$ applications did lead to a small decrease in TPY and PCSO concentrations in 2006. Although the effects were small they did support earlier findings (Randle 2005). The lack of interactions between $CaCl_2$ and $(NH_4)_2SO_4$ suggest that $CaCl_2$ could be used over a wide range of N and S levels. Higher levels of $CaCl_2$ may be necessary however, to receive substantial benefits in reducing onion flavor potential.

Conclusion

The results of this experiment indicate that supplemental $CaCl_2$ could be used to improve onion bulb firmness at harvest on low Ca soils. However, the effects of $CaCl_2$ decline with storage due to the high degree of bulb softening occurring during storage. Larger applications of $CaCl_2$ may be necessary to realize improvements in firmness during storage. Although changes in pectin fractions were not affected by $CaCl_2$, the results obtained here indicate that there are significant changes in the pectin composition of onion bulbs during storage. These changes may provide insight into the mechanisms involved in onion bulb softening and degradation during storage. The effects of $CaCl_2$ on flavor were also measured. Though applications of $CaCl_2$ did not affect flavor potential in 2005, they did lead to a decrease in pungency and PCSO in 2006, regardless of the level of $(NH_4)_2SO_4$ present. Further research with higher levels of $CaCl_2$ may result in further reductions in onion flavor potential. If successful this may be a tool that growers could use to produce firmer and milder bulbs in regions high in soil S.

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Table 1. Main effect means for ammonium sulfate [$(NH_4)_2SO_4$] and calcium chloride ($CaCl_2$) fertility treatments for cured yield of 'Georgia Boy' onion (*Allium cepa* L.) in 2005 and 2006. No treatment interactions were observed.

	2005	**2006**
$(NH_4)_2SO_4$ ($kg \cdot ha^{-1}$)	Yield ($kg \cdot ha^{-1}$)	
0	56,170 b*	95,843 b
250	61,081 a	105,589 a
500	61,389 a	107,697 a
$CaCl_2$ ($kg \cdot ha^{-1}$)		
0	60,388 a	103,065 a
115	58,540 a	102,048 a
230	59,429 a	104,016 a

* Any means within the same treatment and year not followed by the same letter are significantly different at $P < 0.05$.

Table 2. Main effect means for ammonium sulfate [$(NH_4)_2SO_4$] and calcium chloride ($CaCl_2$) fertility levels on for total bulb nitrogen (N), sulfur (S) and calcium (Ca) in field grown 'Georgia Boy' onions (*Allium cepa* L.) in 2005 and 2006. No treatment interactions were observed.

	2005			2006		
	N	S	Ca	N	S	Ca
$(NH_4)_2SO_4$ ($kg \cdot ha^{-1}$)	($mg \cdot kg^{-1}$ dry wieght)			($mg \cdot kg^{-1}$ dry weight)		
0	9076 b*	2923 a	1747 a	8079 b	1416 c	1304 a
250	10048 b	2682 a	1677 a	8785 b	2363 b	1332 a
500	11908 a	2869 a	1742 a	11159 a	2973 a	1272 a
$CaCl_2$ ($kg \cdot ha^{-1}$)						
0	10318 a	2829 a	1624 a	9469 a	2421 a	1214 b
115	10512 a	2676 a	1640 a	9310 a	2198 ab	1306 ab
230	10139 a	2975 a	1890 b	9243 a	2057 b	1376 a

* Any means within the same treatment and year not followed by the same letter are significantly different at $P < 0.05$.

Table 3. Main effect means for ammonium sulfate [$(NH_4)_2SO_4$], calcium chloride ($CaCl_2$), and storage time for the percentage weight loss (% loss) from harvest, percentage dry weight (%DW) and soluble solids content (%SSC) for 'Georgia Boy' onion (*Allium cepa* L.) in 2005 and 2006. No interactions were observed

	2005			2006		
	% Loss	% DW	%SSC	% Loss	% DW	%SSC
$(NH_4)_2SO_4$ ($kg \cdot ha^{-1}$)						
0	6.1 a*	11.3 a	9.8 a	5.1 a	10.3 a	9.9 a
250	6.6 a	11.3 a	9.8 a	4.5 a	10.2 a	10.1 a
500	6.6 a	10.7 b	12.8 a	5.5 a	10.0 a	10.1 a
$CaCl_2$ ($kg \cdot ha^{-1}$)						
0	5.7 a	11.1 a	12.8 a	5.2 a	10.2 a	10.0 a
115	7.0 a	11.1 a	9.7 a	4.7 a	10.0 a	9.9 a
230	6.6 a	11.1 a	9.7 a	5.2 a	10.2 a	10.0 a
Storage Time						
Harvest	-	12.3 a	9.4 a	-	10.2 a	9.7 b
10 weeks	4.9 b	10.5 b	13.3 a	4.5 b	10.2 a	10.0 a
20 weeks	7.9 a	10.4 b	9.1 a	6.6 a	10.1 a	10.1 a

* Any means within the same treatment and year not followed by the same letter are significantly different at $P < 0.05$.

Table 4 Means for ammonium sulfate [$(NH_4)_2SO_4$] and storage time for the percentage of diseased 'Georgia Boy' onion (*Allium cepa* L.) bulbs displaying visual symptoms of neck rot, center rot or sour skin caused by *Botrytis allii, Pantoea ananatis,* and *Burkholderia cepacia*, respectively at harvest, 10 and 20 weeks of storage.

	2005	**2006**
	% Diseased Bulbs	
Harvest $(NH_4)_2SO_4$ ($kg \cdot ha^{-1}$)		
0	0 b*	1.7 e
250	4.2 b	3.3 e
500	1.9 b	3.8 e
10 weeks $(NH_4)_2SO_4$		
0	8.9 b	22.5 bc
250	7.3 b	7.1 de
500	6.7 b	12.5 cde
20 weeks $(NH_4)_2SO_4$		
0	29.6 a	40.4 a
125	26.7 a	17.1 cd
250	28.5 a	29.6 b

* Any means within the same column not followed by the same letter are significantly different at $P < 0.05$

Table 5. Mean onion (*Allium cepa* L.) scale firmness for calcium chloride ($CaCl_2$) and storage time from harvest for 'Georgia Boy' onion bulbs in 2005 and 2006.

	2005	**2006**
	Firmness (N)	Firmness (N)
Harvest $CaCl_2$ ($kg \cdot ha^{-1}$)		
0	3.36 b*	3.13 b
115	3.50 a	3.30 a
230	3.54 a	3.38 a
10 weeks $CaCl_2$		
0	3.09 cd	2.81 cd
115	3.07 cd	2.78 cd
230	3.17 c	2.85 c
20 weeks $CaCl_2$		
0	3.00 d	2.72 d
115	2.99 d	2.72 d
230	3.00 d	2.76 cd

* Any means within the same column not followed by the same letter are significantly different at $P < 0.05$

Table 6. The main effect means of ammonium sulfate [$(NH_4)_2SO_4$], calcium chloride ($CaCl_2$), and storage for uronic acid [total pectin, (TP)], water soluble pectin (WSP), chelator soluble pectin (CSP), acid soluble pectin (ACSP), and alkaline soluble pectin (AKSP) in $mg \cdot g^{-1}$ dry weight (DW) of field grown 'Georgia Boy' onions (*Allium cepa* L.) in 2005 and 2006. No treatment interactions were present.

	2005					2006				
	TP	WSP	CSP	ACSP	AKSP	TP	WSP	CSP	ACSP	AKSP
	$mg \cdot g^{-1}$ DW					$mg \cdot g^{-1}$ DW				
$(NH_4)_2SO_4$ ($kg \cdot ha^{-1}$)										
0	48.0 a*	11.5 a	13.5 b	4.1 a	19.5 a	43.9 a	12.4 a	7.1 b	1.3 a	18.8 a
250	48.6 a	10.9 a	13.3 b	3.9 a	19.1 a	43.8 a	12.3 a	8.1 a	1.5 a	19.6 a
500	50.4 a	11.7 a	15.1 a	4.5 a	19.6 a	46.3 a	12.1 a	8.2 a	1.4 a	19.7 a
$CaCl_2$ ($kg \cdot ha^{-1}$)										
0	49.5 a	11.7 a	14.1 a	4.2 a	19.8 a	45.8 a	12.2 a	7.6 a	1.2 a	19.4 a
115	49.0 a	11.2 a	13.6 a	4.1 a	19.1 a	44.9 a	12.5 a	8.0 a	1.5 a	19.9 a
230	48.5 a	11.1 a	14.2 a	4.1 a	19.2 a	43.4 a	12.2 a	7.8 a	1.5 a	18.9 a
Storage Time										
Harvest	55.5 a	10.2 b	9.6 c	4.5 a	21.6 a	48.1 a	12.6 ab	8.7 a	1.1 a	19.4 ab
10 weeks	46.9 b	11.1 b	11.9 b	3.9 a	19.0 b	43.7 ab	12.9 a	6.4 b	1.4 a	18.1 b
20 weeks	44.4 b	12.8 a	20.5 a	4.1 a	17.6 b	42.3 b	11.3 b	8.3 a	1.7 a	20.7 a

* Any means within the same treatment and year not followed by the same letter are significantly different at $P < 0.05$.

Table 7. The main effect means for ammonium sulfate [$(NH_4)_2SO_4$], calcium chloride ($CaCl_2$), and storage time for total pyruvic acid (TPY), and lachrymatory factor (LF) in µmol·mL^{-1} juice, and *S*-methyl cysteine sulfoxide (MCSO), *S*-propyl cysteine sulfoxide (PCSO) and *S*-1-propenyl-cysteine sulfoxide (PECSO) in µmol·g^{-1} dry weight (DW) for 'Georgia Boy' onions (*Allium cepa* L.) in 2005 and 2006.

	2005					**2006**				
	TPY	LF	PECSO	PCSO	MCSO	TPY	LF	PECSO	PCSO	MCSO
	(µmol·mL^{-1} juice)		(µmol·mg^{-1} DW)			(µmol·mL^{-1} juice)		(µmol·mg^{-1} DW)		
$(NH_4)_2SO_4$ (kg·ha^{-1})										
0	4.25 b*	5.85 b	5.15 c	1.92 c	2.14 c	4.05 b	3.58 b	4.08 b	1.28 c	1.97 a
250	5.00 a	6.86 a	6.46 b	2.46 b	4.12 b	5.05 a	4.34 a	6.15 a	1.78 b	4.89 b
500	5.04 a	7.08 a	7.71 a	2.88 a	5.11 a	5.25 a	4.23 a	6.82 a	2.34 a	6.71 a
$CaCl_2$ (kg·ha^{-1})										
0	4.75 a	6.47 a	6.25 a	2.34 a	3.68 a	5.03 a	4.06 a	5.92 a	1.97 a	4.72 a
115	4.82 a	6.59 a	6.60 a	2.37 a	3.77 a	4.64 b	4.03 a	5.58 a	1.82 ab	4.34 a
230	4.68 a	6.66 a	6.37 a	2.52 a	3.82 a	4.68 b	4.07 a	5.56 a	1.62 b	4.51 a
Storage Time										
Harvest	4.08 c	6.46 b	7.03 a	2.28 b	2.54 b	4.43 b	4.48 a	8.77 a	1.79 b	2.93 b
10 weeks	4.76 b	5.63 c	6.35 a	1.77 c	4.17 a	4.62 b	3.84 b	3.61 c	1.48 c	5.07 a
20 weeks	5.41 a	7.66 a	5.21 b	3.21 a	4.57 a	5.31 a	3.84 b	4.67 b	2.13 a	5.57 a

*Any means within the same treatment and year not followed by the same letter are significantly different at $P < 0.05$.

CHAPTER 4

EVALUATION OF BULB QUALITY, CARBOHYDRATES AND RELATED ENZYMES IN THREE LINES OF ONION (*ALLIUM CEPA* L.) DURING STORAGE[2]

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Abstract

Firmness in fruits and vegetables is an important textural attribute that influences consumer attitudes toward freshness and quality. In order to determine what factors may influence onion (*Allium cepa* L.) scale firmness at harvest and during storage, structural and non-structural carbohydrates, as well as polygalacturonase (PGA), pectin methylesterase (PME), invertase, and 1-fructan exohydrolase were evaluated in three onion lines. MBL87-WOPL was the firmest (4.2 N) line at harvest and experienced the longest delay in softening during storage. This line cultivar had the highest levels of dry matter, soluble solids content, total fructans, carbon, pectins and cellulose. Additionally, it had the lowest levels of PGA and PME activity for the duration of the study. When viewed under transmission electron microscopy (TEM), MBL87-WOPL had the thickest cell wall/middle lamella regions of the lines tested. Pegasus, the softest, poorest storing cultivar had the lowest levels of structural carbohydrates measured. It also had high levels glucose and fructose as well as PGA and PME activity. TEM analysis indicated that Pegasus had the thinnest cell wall/middle lamella region. A good storing, moderately firm variety, MSU4535B typically had intermediate levels of the measured attributes. Our results indicate that bulb pectin and cellulose concentrations may determine bulb firmness and that PME and PGA activity in onion may be related to softening in storage.

Introduction

Onions (*Allium cepa L.*) are valued for their flavor and medicinal attributes worldwide. A primary reason for the popularity of onions is their ability to be stored for long periods of time. Onions are biennial plants and the bulbs formed after the first year of growth are storage organs to be utilized in the second year of growth. Once bulbs are formed the plant enters a dormant period when growth ceases and metabolism slows (Komochi, 1990). This dormancy makes onion bulbs ideal candidates for long term storage. However, there are large varietal differences in storage suitability. Traditionally, storage potential of onions has been equated to the ability of bulbs to resist disease, sprouting or dry matter loss (Gubb and Mactavish, 2002; Rutherford and Whittle, 1984). However, because many onions are consumed raw, changes in other attributes during storage such as flavor and texture are also relevant. Kopsell and Randle (1997) reported significant changes in onion flavor potential during storage. Though, little is known regarding what factors affect bulb firmness at harvest and during storage. Softening of bulbs during storage may have a negative impact on the perceived freshness of bulbs.

Recently structural and non-structural carbohydrates in two onion cultivars were evaluated (O'Donoghue et al., 2004). The authors reported high concentrations of hemicellulose and cellulose in a firm, long-storing cultivar, Pukekohe Longkeeper, and low levels of cellulose and hemicellulose in a soft, short-storing cultivar, Houston Grano. Their results suggest that cellulose and pectin concentrations may be linked to bulb firmness. In addition to structural carbohydrates, the activity cell wall modifying enzymes, polygalacturonase (PGA) (E.C. 3.2.1.15) and pectin methylesterase (PME) (E.C. 3.1.1.11) may affect onion firmness.

The activities of PME and PGA are well correlated with softening in a number fruits and vegetables during the ripening process (Gomez-Galindo, 2004; Micheli, 2001). Although neither enzyme has been thoroughly investigated in onion, PGA activity has been linked with a loss of cellular adhesion in Leek (*Allium porrum* L.), a close relative of onion (Peretto et al., 1992). In addition, Garcia et al., (2002) reported PME activity in fresh and dehydrated onion tissue. Although other enzymes such as expansin, have been linked to softening in fruits and vegetables (Brummell, 2006; Brummell and Harpster, 2004; Smith et al., 1990), the role of PME and PGA in relation to onion firmness has yet to be defined. However, PME and PGA have previously been detected in *Allium* species and it is likely that they play a role in onion pectin metabolism during storage.

The objectives of this study were to investigate if pectin, cellulose, PME, or PGA were related to changes in onion scale firmness at harvest and during refrigerated storage. Bulb dormancy was also measured to determine if firmness changes were related to breaks in dormancy. In addition, bulbs were examined using transmission electron microscopy (TEM) to determine if there were visible differences in cell wall morphology among cultivars at harvest and after 12 weeks of storage. In addition, parameters such as non-structural carbohydrates and the activities of 1-fructan exohydrolase (FEH) (E.C. 3.2.1.153) and invertase (INV) (E.C. 3.2.1.26) were measured to obtain baseline data for comparison to previous studies and to determine if they were correlated to changes in firmness as well.

Materials and Methods

Plant Material. Three onion cultivars with different storage characteristics were chosen for this study. The short-day cultivar ‘Pegasus’ (Seminis seeds, Oxnard, Calif.),

represented a low dry matter poor storing bulb grown for the sweet onion market. The long day inbred cultivar, MSU4535B, had long term storage potential. The open pollinated selection MBL 87-WOPL, is used for dehydration or long term storage. On 18 Nov. 2005 seeds from each variety were seeded into 200-cell plastic plug trays using Fafard Super Fine Germination Mix (Fafard Inc. Agawam, Mass.). Seedlings were watered daily and fertilized twice-weekly with Peter's 20-20-20 (Scotts Inc., Marysville, Ohio) at a concentration of 1 $g \cdot L^{-1}$. Seedlings were greenhouse grown under natural photoperiods and light intensities (≈34° N latitude) with day/night temperature set points of 25/20 °C. After six weeks, seedlings were transplanted into boxes (2.45 m x 1.22 m x 0.15 m) containing Fafard 52 Mix (Fafard Inc.). Plants were evenly spaced 8.75 cm on center and each box held 96 plants of each of the three lines. Four boxes were randomLy spaced throughout the greenhouse and each box was treated as a replication for a completely randomized design with four replications.

Plants were watered daily and fertilized twice-weekly with a ½ strength Hoagland's #2 nutrient solution (Hoagland and Arnon, 1950). Ten weeks after transplant supplemental overhead lighting with an average canopy light intensity of 277 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Basic Quantum Meter, Spectrum Technologies, Plainfield, Ill.) was provided until harvest to induce bulbing in the long-day onion lines. All varieties were subjected to 17 hour day lengths.

All plants were harvested at the same developmental state, which was when 50-70% of the plants displayed softened pseudostems. On 3 May 2006 bulbs from the line 'Pegasus' were harvested and placed in nylon mesh bags and cured at 36 °C for 72 h. Each bag contained 15 bulbs and four bags were harvested from each of the four

replications. Bags were placed into refrigerated storage 6.6 ± 1.4 ^{o}C and 82 ± 4.2% RH. In addition, five bulbs from each replication were placed into 3.78 L glass jars and stored under the same conditions in order to measure bulb respiration. Bulbs from the MBL 87-WOPL and MSU4535B lines were harvested on 25, May 2006 and 16, June 2006, respectively and were cured and placed into storage as described previously. Due to the presence of disease only three replications of the line MBL 87-WOPL were able to be harvested and stored. Bulbs were analyzed at harvest and after 4, 8 and 12 weeks in storage. Unless otherwise noted all subsequent analyses were conducted on 15 bulb composite samples with four replications of Pegasus and MSU4535B and three replications of MBL 87-WOPL, respectively.

Sprouting and rooting. As an indicator of the state of dormancy of bulbs the percentage of bulbs that could resume root or shoot growth during storage was measured. To do this, 2-3 cm thick cross-sections of 15 bulbs containing the basal plate and apical meristem were placed in boxes filled with Fafard Super Fine Germinating Mix (Fafard Inc.). Bulb slices were watered daily and maintained in a greenhouse at day/night temperature set points of 28/20 ^{o}C under natural light intensities and photoperiods. After 10 days the percentage of bulbs exhibiting rooting and sprouting were recorded.

Soluble solids and firmness. Total soluble solids content (SSC) was measured by taking 1-2 mm longitudinal slices from bulbs at each sampling time and crushing them in a pneumatic press. Several drops of the fresh juice were placed on a hand-held refractometer (Kernco, Tokyo, Japan) to determine SSC. Bulb scale firmness was measured by cutting a 2 x 4 cm rectangular piece from the first fully fleshy scale (usually the second or third scale from the outside of the bulb) at the equatorial region of each

bulb. Firmness was measured as the pressure required to penetrate the scale using a 1 mm diameter probe coupled to a fruit penetrometer mounted to a motorized press operated at a speed of 1.5 $mm \cdot s^{-1}$ (Model 327, McCormick Fruit Tech, Yakima, Wash.). Firmness for each 2 x 4 cm slice was measured three times and averaged.

Sugars. The sugars, fructose, glucose and sucrose were determined using a modification of the gas chromatography (GC) method of Chapman and Horvat (1989). In brief, a 2-5 mm thick longitudinal slice was, weighed and immediately frozen in liquid N_2 and lyophilized (FreeZone 6; Labconco, Kansas City, Mo.). Lyophilized tissue was ground into a fine powder with a coffee grinder. Sugars were extracted from approximately 25 mg of lyophilized tissue in 3.0 mL of 80:20 methanol:water containing 8.75 mM of phenyl-β-D-glucoside (internal standard). Samples were extracted for 24 h at 25 ^{o}C, centrifuged for 5 min at 5,000 *g* and 40 µl of supernatant was evaporated to dryness at 45 ^{o}C under vacuum using a centrifugal concentrator (Labconco). Sugars were converted to their oximes through the addition of 30 µL of hydroxylamine-HCl in pyridine (25 $g \cdot L^{-1}$) (Sigma, St. Louis, MO, USA). The oxime sugars were derivatized with the addition of 70 µL of 99:1 (N,O-bis(trimethylsilyl)triflouroacetamide): trimethylchlorosilane [BFTMS (Sylon BFT, Supelco, Bellefonte, Penn.]. The content of the vials was subjected to GC analysis.

The oxime-TMS sugar derivatives were analyzed on a Shimadzu GC-17A GC with flame ionization detector (Shimadzu Corp., Kyoto, Japan). A 30 m x 0.32 mm fused silica 0.25 µm film thickness capillary column was used (HP-5, J&W Scientific, Agilent Technologies, Santa Clara, Calif.). Injector and detector temperatures were 250 ^{o}C and 300 ^{o}C, respectively. The temperature program was as follows: initial temperature was

150 ^{o}C, increased at 4 ^{o}C·min^{-1} to 180 ^{o}C held for 30 s and increased to 193 ^{o}C at a rate of 1 ^{o}C·min^{-1} and held for 30 s, and increased at 7 ^{o}C·min^{-1} to 280 ^{o}C and held for 9 min. Helium was used as the carrier gas with a column flow rate of 1.7 mL·min^{-1}. A split injector was used with a split ratio of 12:1. Identity of the sugar oxime-TMS derivatives and response factors were determined by comparing retention times and areas of sample peaks with those of authentic compounds (Sigma).

Total fructans and carbon. Total fructans were determined in lyophilized onion tissue by the enzymatic method using a commercially available kit and following instructions provided by the manufacturer. (Megazyme International, Wicklow, Ireland) (McCleary et al., 1997). Average recovery of standards in spiked samples was 94%. Total carbon was determined by analyzing approximately 250 mg lyophilized onion tissue using a Leco CNS 2000 (Leco corp. St. Joseph, Mich.)

Alcohol-insoluble solids, pectin fractioning and total pectin determination. The alcohol insoluble solids (AIS) residue was prepared from onion tissue according to a modification of the method of Huber and Lee (1986). Longitudinal slices 5-10 mm in thickness were cut from bulbs. Slices were homogenized in a blender for 60 s with four volumes (w/v) of 95% ethanol. Two more volumes of 95% ethanol were added and the homogenate was boiled at 100 ^{o}C for 20 min with slow stirring. The homogenate was cooled in an ice-water bath for 30 min. The cooled residue was filtered under vacuum through glass fiber filters (APFF, 0.7 μm, Millipore, Billerica, Mass.). Based on the initial sample weight, the residue was sequentially washed with six volumes of 95% ethanol, with four volumes of 100% ethanol, and four volumes of acetone. The residue

was dried overnight in a fume hood. The dried AIS residue was weighed and ground to a fine powder using a coffee grinder and stored at -20 ^{o}C until analysis.

The pectin in the AIS was fractionated into water, chelator, acid, and alkali soluble pectins according to a modification of the method of DeVries et al., (1981). Approximately 30 mg of onion AIS was extracted at 60 ^{o}C for 90 min in 40 mL of 0.05 M sodium acetate buffer, pH 5.2 to obtain water soluble pectin (WSP). The WSP was obtained by centrifuging the extract at 30,000 *g* for 15 min and filtering the supernatant through one layer of Miracloth (CalBiochem, EMD Biosciences, San Diego, Calif.). The remaining pellet was resuspended in 40 mL of 0.05 M sodium oxalate, 0.05 M ammonium oxalate, and 0.05 M sodium acetate pH 5.2 and incubated for 90 min at 60 ^{o}C to obtain chelator soluble pectin (CSP). The extract was again centrifuged at 30,000 *g* for 15 min and supernatant filtered through one layer of Miracloth to obtain the CSP. The remaining pellet was again resuspended in 40 mL of HCL pH 2.5 and incubated for 90 min at 60 ^{o}C. The extract was centrifuged and filtered as previously to obtain the acid soluble pectin (ASP). The remaining pellet was resuspended in 40 mL of 0.05 M NaOH and incubated for 90 min at 60 ^{o}C, centrifuged and filtered as previously in order to obtain the alkaline soluble pectin (ALSP). The pectin (uronic acid) content of each fraction was determined with the *m*-hydroxydiphenol method (Blumenkrantz and Asboe-Hansen, 1977). Fructans with a degree of polymerization longer than three are co-extracted in the AIS. Although fructans do react with the *m*-hydroxyphenol used to determine uronic acid concentrations, their contribution to the total absorption of each fraction is minimal and did not significantly affect the results of the assay (data not shown).

Total pectin was determined with the method of Ahmed and Labavitch (1977). Approximately 5 mg of AIS was weighed into a 50 mL beaker to which 5 mL of concentrated cold sulfuric acid was slowly added to minimize charring. The beaker was stirred in an ice bath for approximately 10 min until nearly all of the AIS dissolved. Then 5 mL of cold deionized water was added in 1 mL increments. After 10 min., cold deionized water was added to bring the solution to a volume of 25 mL in a volumetric flask. An aliquot of the solution was analyzed for galacturonic acid (Blumenkrantz and Asboe-Hansen, 1977). Galacturonic acid content of samples was estimated from a linear regression using galacturonic acid as a standard at concentrations of 0 - 20 µg.

Cellulose content. Cellulose concentration in the AIS residue was determined using the method of Updegraff (1969). Approximately 30 mg of AIS residue was weighed into glass test tubes and dissolved with 3.0 mL of 10:1 (80% acetic acid:concentrated nitric acid) and heated in a boiling water bath at 100 ^{o}C for 30 min, centrifuged for 10 min at 5000 *g* and supernatant discarded. The pellet was washed with 10 mL of deionized water and centrifuged at 5000 *g* for 10 min three times. The washed pellet was dissolved in 10 mL of 67% sulfuric acid, mixed and diluted to 100 mL with deionized water. An aliquote of the solution was pipetted into a glass test tube to which 3.5 mL of deionized water and 10 mL of cold anthrone reagent (0.2 g anthrone in 100 mL concentrated sulfuric acid) (Sigma) was added. Tubes were mixed and placed in a boiling water bath for 18 min. After cooling in an ice bath for 5 min samples were read on a spectrophotometer at 620 nm and concentrations calculated based on a standard curve developed using cellulose. Spiked samples containing purified cellulose averaged 90% recovery (Sigma).

Enzyme activity and protein determination. All enzyme extractions and purification steps were performed in a cold room at 4 ^{o}C. Enzyme extracts that were boiled for 10 min prior to assaying served as blanks for each sample. Polygalacturonase was extracted from approximately 15 g fresh tissue obtained from the equatorial region of bulbs using a 6 mm cork borer and homogenized in 45 mL of cold 1 M NaCl solution pH 6.0 for 1 min using a Waring Blender at high speed (Waring Laboratory and Science, Torrington, Conn). The homogenate was extracted overnight at 4 ^{o}C. The homogenate was filtered through two layers of Miracloth and centrifuged at 4 ^{o}C and 10,000 *g* for 10 min. The supernatant was collected and a 0.5 mL aliquot was concentrated and reducing sugars removed using centrifugation at 12,000 *g* and 4 ^{o}C for 1 h (Microcon YM-10, Millipore). Then 0.1 mL of 50 mM sodium acetate pH 4.4 was added to the retentate and the solution was re-concentrated by centrifugation at 12,000 *g* and 4 ^{o}C for 40 min. The retentate was redissolved in 0.1 mL 50 mM sodium acetate pH 4.4. Polygalacturonase activity was determined according to the 2-cyanoacetamide method (Gross, 1982). One unit of PGA activity was determined to be the amount of enzyme that released 1 umol of reducing sugar (galacturonic acid) per minute.

Pectin methylesterase was extracted from 15 g of fresh tissue homogenized with 60 mL of ice-cold extraction buffer (0.1 M NaCl, 0.25 M Tris-Base, pH 8.0) for 1 min using a Waring blender at high speed. The homogenate was extracted for 3 h at 4 ^{o}C after which the mixture was filtered through two layers of Miracloth and centrifuged for 10 min at 4 ^{o}C and 10,000 *g*. The supernatant was collected and 30% ammonium sulfate added. The solution was allowed to precipitate overnight. The dispersion was centrifuged for 15 min at 4 ^{o}C and 10,000 *g* and the supernatant collected. Pectin

methylesterase activity was determined by titration using a pH stat titrator (Brinkmann, Westbury, NY) (Banjongsinsiri et al., 2004). The assay was carried out with 0.4 mL of extract added to 20 mL of a solution of 1.0% high methoxy citrus pectin with 0.1 M NaCl at pH 7.5 and 37 ^{o}C. The assay was conducted for 20 min. One unit of PME activity was determined to be the amount of enzyme that released 1 μmol of carboxylic acid group per minute.

Fructan exohydrolase was extracted and assayed using a modification of Benkeblia et al., (2005). Approximately 15 g of fresh tissue was homogenized in 45 mL of ice-cold 50 mM citrate-phosphate pH 5.0 buffer (Sigma) for 1 min using a Waring blender at high speed. The homogenate was allowed to extract overnight at 4 ^{o}C. After extraction the homogenate was filtered through two layers of Miracloth and filtrate was centrifuged for 15 min at 10,000 *g*. The supernatant was collected and a 0.5 mL aliquot was concentrated using centrifugation at 12,000 g and 4 ^{o}C for 1 h, as described in the PGA assay (Microcon YM-10, Millipore). The retentate was resuspended in 0.1 mL of 50 mM citrate-phosphate buffer and reconcentrated with centrifugation at 12,000 *g* for 20 min. The retentate was redissolved in 0.1 mL of citrate-phosphate buffer. Activity of FEH was determined in a total volume of 200 μL containing 50 μL of extract, 50 μL of citrate-phosphate buffer pH 5.0 and 100 μL of a 3% chicory inulin solution (Sigma). The mixture was incubated at 30 ^{o}C for 1 h and the reaction was stopped by heating in a boiling water bath for 5 min. The amount of fructose present in samples was determined using oxime-TMS derivitization and GC as described previously. One unit of FEH activity is defined as the amount of enzyme that release 1 μmol of fructose per minute from the inulin substrate.

Invertase was extracted from 15 g of fresh tissue homogenized for 1 min in 45 mL of cold 50mM sodium acetate buffer (pH 5) containing 10 mM sodium bisulfite, 1 mM phenylmethanesulfonylflouride, and 5 mM 2-mercaptoethanol using a Waring Blender on high speed (Benkeblia et al., 2004). The homogenate was extracted overnight at 4 ^{o}C. The extractant was filtered through two layers of Miracloth and filtrate was centrifuged at 4 ^{o}C and12,000 *g* for 15 min. The supernatant was collected and 0.5 mL was concentrated with centrifugation at 12,000 *g* for 1 h as described previously (Microcon YM-10, Millipore). This also removed residual 2-mercaptoethanol prior to conducting the total protein assay. Then 0.1 mL of 50 mM sodium acetate buffer (pH 5.0) containing 0.02% w/v sodium azide was added to the retentate solution and reconcentrated at 12,000 *g* for 30 min. The retentate was dissolved in 0.1 mL of 50 mM sodium acetate buffer. The enzyme assay was carried out in a total volume of 200 µL containing 50 µL of concentrated enzyme extract, 50 µL sodium acetate buffer, and 100 µL of substrate solution containing 200 mM sucrose. The assay was incubated at 30 ^{o}C for 1 h. The reaction was stopped by heating in a boiling water bath for 5 min. The fructose formed was determined using GC as described previously. One unit of invertase activity was defined as the amount of enzyme that could produce 1 µmol of fructose per minute from the sucrose substrate.

Protein analysis. Total protein concentrations used in each enzyme assay were determined using the bicinchoninic acid method (Smith et al., 1985). A commercially available kit was used and manufacturer's instructions followed using bovine serum albumin protein as a standard (Pierce BCA Protein Assay Kit, Pierce, Rockford, IL, USA).

Transmission electron microscopy. Samples were analyzed using TEM to determine if any structural changes could be observed in the cell wall/middle lamella between onion cultivars over storage time. All samples were prepared at 4 ^{o}C. At harvest and after 12 weeks of storage, bulbs were sampled for TEM. Five, 1-2 mm^3 samples were cut from each of 15 bulbs and immediately placed into Sorensen's phosphate buffer pH 7.2 with 4% glutaraldehyde fixative for 8 h (EMS, Hatfield, PA, USA) (Dawson et al., 1989). Then samples were washed twice for 30 min and once overnight in Sorensen's phosphate buffer. Samples were post-fixed in Sorensen's phosphate buffer containing 1% osmium tetraoxide for 2 h, and washed in Sorensen's buffer three times for 20 min each. Samples were dehydrated in a graded ethanol series (20, 30, 50, 70, 95,100% ethanol) for 20 min at each step. Propylene oxide was used as a transitional solvent prior to embedding. Samples were embedded in Spurr's low viscocity embedding resin (EMS) (Spurr, 1969).

Ultra-thin (90 nm) sections were cut using a Reichert Jung ultracut E ultramicrotome (Reichert Microscope Services, Depew, NY, USA). Sections were placed on 200 mesh copper grids and stained with uranyl acetate and lead citrate. Sections were visualized on a Zeiss 902A TEM (Carl Zeiss Microimaging, Thornwood, NY, USA). Five subsamples for each onion line x sampling time were tested.

Statistical Analysis. All data was subjected to the GLM procedure for significance of main effects, interactions between cultivar and storage time and SAS statistical software (SAS v. 9.1.3, SAS institute, Cary, N.C.). Mean separation of main effects means was performed using Duncan's means separation test. Percentage data was transformed using an arc-sin transformation prior to analysis. Significance was determined using $P< 0.05$.

Results

Weight loss, dry matter and SSC. Weight loss in bulbs during storage, measured as a percentage of fresh weight (FW) at harvest, differed among cultivar and increased during storage (Fig. 1A). There was also a significant interaction between cultivar and storage time for weight loss. Much of this interaction is the result of the large increase in weight loss experienced by MBL87-WOPL between 4 and 8 weeks of storage, compared to the smaller weight losses incurred by the other cultivars during that time. Overall, MSU4535B experienced the smallest weight loss in storage, losing 6% of its harvest weight, while MBL87-WOPL experienced the largest weight loss, eventually losing 16% of its harvest weight after 12 weeks in storage. The percentage dry matter in bulbs differed significantly by cultivar and storage time. As expected the, MBL87-WOPL, had the highest bulb dry matter concentration ranging from 15-18%, with the fresh market sweet onion, Pegasus, having the lowest (Fig.1B). There was a significant interaction between cultivar and storage time regarding dry matter concentration. This resulted from the slight increase in dry matter content in MBL87-WOPL during storage, while the other cultivars saw little change. Total SSC was affected by variety, being highest in MBL87-WOPL and lowest in Pegasus (Fig. 1C). The values for SSC obtained in this study fall within the ranges previously reported for similar lines (Randle, 1992). Total SSC was also affected by storage time. Cultivar and storage time significantly interacted to affect bulb SSC. While SSC declined slightly during storage in Pegasus and MSU4535, SSC increased slightly in MBL87-WOPL resulting in a small, though significant interaction.

Rooting/Sprouting and Respiration. Breaking of dormancy as measured by the ability of the harvested bulbs to resume growth by sprouting new shoots or roots was affected by

cultivar and storage time (Fig 2A). There was also a significant interaction between cultivar and storage regarding sprouting and rooting. At harvest the short-day line Pegasus exhibited rooting or sprouting in roughly 60% of bulbs, while the other varieties required eight weeks of storage to reach similar levels. Analogous sprouting patterns among different cultivars have been previously reported (Kopsell and Randle, 1997).

Firmness. Onion scale firmness was measured during storage. Firmness was affected by cultivar and storage time (Fig. 3). There was also a significant interaction among cultivars and storage time for bulb firmness. Firmness decreased for all lines over the 12 week storage period. The high dry matter line MBL87-WOPL had the highest average firmness at harvest (4.17 N) and saw the smallest overall decrease in firmness with an average decrease of 0.34 N after 12 weeks. In contrast the low dry matter cultivar Pegasus had the lowest average firmness readings at harvest (2.96N) and a 0.44 N average decrease in firmness after 12 weeks. The long-day line, MSU4535B, had an average firmness of 3.75 N at harvest and experienced an average decrease of 0.52 N over the 12 week storage period.

Non-structural carbohydrates. Cultivar and storage time significantly interacted to affect fructose concentrations (Fig. 4A). At harvest MBL87-WOPL and MSU4535B had significantly lower fructose levels than Pegasus. However, after 8 weeks in storage the concentration of fructose in MBL87-WOPL and MSU4535B rose slightly, while Pegasus had a slight decrease in fructose, resulting in no difference between the cultivars at 8 or 12 weeks of storage. Glucose concentrations were also different among varieties tested (Fig. 4B), but did not change over time. The glucose concentration in the Pegasus and MSU4535B lines were greater than MBL87-WOPL, which had approximately 50% as

much glucose of the other varieties. Cultivar and storage time interacted to affect sucrose concentrations. This was the result of a small decrease in sucrose concentration in MBL87-WOPL during storage and an increase in the concentration of sucrose in MSU4535B. Sucrose concentrations differed among cultivars and storage time (Fig 4C). Although significant, differences were minimal.

Fructans constitute the majority of non-structural carbohydrates in many onion cultivars (Darbyshire and Steer, 1990). There were large differences in total fructan concentration among cultivars and smaller differences over time. There was a significant interaction between cultivar and storage time, with the concentration of fructans in MBL87-WOPL increasing during storage while decreasing in MSU4535B and Pegasus. The high dry matter line MBL87-WOPL had the highest fructan concentration at all sampling times.

Total carbon differed among cultivars. The high dry matter line MBL87-WOPL had the highest concentration of carbon followed by MSU4535B and Pegasus. The concentration of carbon increased slightly in MBL87-WOPL, during the first four weeks of storage, however, carbon levels remained unchanged in the other varieties resulting in a significant, though minor interaction.

Structural Carbohydrates. Uronic acid analysis of the AIS indicated that the MBL87-WOPL line had the greatest concentration of pectin (Fig. 5A). There were slight changes in total pectin over time, but no trends were apparent. There was a significant interaction between cultivar and storage time for total pectin concentration. This was the result of the small increase in total pectin in MSU4535B coupled with the decrease in total pectin in MBL87-WOPL between harvest and 4 weeks of storage. Water soluble pectin had

large differences among cultivars, with MBL87-WOPL having 1.5 and 3 times more WSP than MSU4535B and Pegasus, respectively (Fig 5B). The concentration of WSP also changed over time. A large increase in WSP in MSU4535B between harvest and 4 weeks in storage, while the WSP in the other cultivars changed little resulted in a significant cultivar by storage interaction. The CSP fraction, which is primarily composed of calcium linked polyuronides, differed among cultivars and over time (Fig 5C). A large decline in the CSP in MBL87-WOPL during storage coupled with minimal changes in the other cultivars tested resulted in a significant cultivar by storage time interaction for CSP concentration. The ACSP fraction differenced among onion lines, with the MBL-WOPL line again having the highest concentration of ACSPs. The ACSP concentration also changed over time, decreasing in all the onion lines (Fig 5D). The ASP fraction, composed primarily of hemicellulose, differed among cultivars, but did not change over time (Fig. 5E). As was the case some of the pectin fractions, cultivar and storage time interacted to affect cellulose concentration. MBL87-WOPL which generally had the highest concentration of cellulose, experienced a small decrease in cellulose during storage, while the other cultivars changed little.

Transmission electron microscopy. Changes in cell wall and middle lamella regions were observed at during storage using TEM (Fig 6A-F). At harvest all three lines displayed similar cell morphology. After 12 weeks in storage all lines displayed some disruption of the cell wall/middle lamella region. Some samples showed a disruption in the middle lamella at the junction where three cells meet (6B). In other ther samples adjacent cell walls were pulled apart with residual carbohydrate chains remaining after adjacent cell walls separated (Fig. 6D).

Enzyme analysis. Cultivar and storage time interacted to affect INV activity. Invertase activity was several times higher in MBL87-WOPL at harvest than the other lines. Though large changes in activity during storage resulted in MSU4535 having the highest rate of activity after 8 weeks in storage. With the exception of MBL87-WOPL at harvest, INV activity was highest at eight weeks of storage. As was the case with INV, cultivar and storage time interacted to affect FEH activity. Though there were no differences among cultivars at 4 weeks of storage, activity changed over time, resulting in large differences in FEH activity after 8 weeks of storage. MBL87-WOPL, which had the highest concentration of fructans also had the highest level of FEH activity. The trends for FEH activity were similar to those seen for INV, with a significant increase occurring at eight weeks in storage.

Pectin methylesterase activity was affected by onion variety and storage time (Fig 7C). The large increase in activity of PME in Pegasus after four weeks of storage resulted in a significant cultivar by storage time interaction. Although similar to Pegasus at harvest, MBL87-WOPL consistently had the lowest level of PME activity during storage averaging between 0.1 to 0.2 units·mg protein^{-1}. Cultivar and storage time interacted to affect PGA activity. An increase in PGA activity in Pegasus, coupled with a decrease in activity in MSU4535B between 4 and 8 weeks of storage appeared largely responsible for the interaction. There were significant differences in PGA activity among cultivars throughout the study. Generally, MBL87-WOPL had the lowest level of PGA activity while activity in Pegasus and MSU4535B was higher and more variable.

Discussion

Although onions are often stored for long periods of time little is known regarding changes in quality as related to scale firmness during storage. Changes in cell walls, pectin, PGAs and PMEs have a profound affect on firmness and storability in many fruits and vegetables (Brummel, 2006). It was our intent to investigate their role in onion firmness during storage. Because previous onion storage studies have investigated changes in non-structural carbohydrates and related enzymes (FEH, INV) we chose to measure these parameters as well (Rutherford and Whittle, 1984).

It has been reported that storage potential and bulb dry matter content are positively correlated (Rutherford and Whittle, 1984). The percent of bulb dry matter and SSC were highest in the good storing cultivar, MBL87-WOPL, and lowest in the poor storing cultivar Pegasus. As expected, SSC decreased during storage for Pegasus and the MSU4535B lines. Typically, SSC decreases in bulbs during long term storage (Rutherford and Whittle, 1982). However, there was an unexpected increase in SSC in the MBL87-WOPL line occurring between harvest and eight weeks in storage. It has been previously reported that most weight loss in onion during storage was due not to loss of carbon, but to water loss (Komochi, 1990). The increase in SSC and dry matter content coupled with the relatively large weight loss between harvest and eight weeks (Fig. 1A) in MBL87-WOPL suggests that differences in water loss may responsible for the increases in dry matter and SSC.

Concentrations of non-structural carbohydrates were as expected. Fructose, glucose and sucrose concentrations were similar to those previously reported (O'Donoghue et al., 2004). The largest differences occurred with glucose, where

MBL87-WOPL had roughly 50% of the glucose as Pegasus and MSU4535B. Fructose was highest in Pegasus at harvest, underscoring the higher perceived sweetness in this cultivar (Corrigan et al., 2000).

Total fructans were highest in the high dry matter cultivar MBL87-WOPL and lowest in Pegasus. The accumulation of fructans in MBL87-WOPL is likely the primary factor for the increased dry matter and SSC in MBL87-WOPL. Fructans have a number of roles in plants, many of which are still being elucidated (Ritsema and Smeekens, 2003). It has been suggested that fructans can enhance cell water potential (Van Laere and Van Den Ende, 2002). An increase in water potential can lead to an increase in cell turgor which could result in firmer plant tissue (Gomez-Galindo et al., 2004). This suggests that the high fructan content of the MBL87-WOPL line might be responsible for it having the firmest scales (Fig. 3). However, if this were the case we would likely see large decreases in firmness in this line after eight weeks in storage due to the large weight loss (Fig. 1A), most of which is presumed to be water (Komochi, 1990). Because changes in firmness could not be correlated to changes in weight loss it may be unlikely that increases in fructans or water potential were primarily responsible for the increased firmness in MBL87-WOPL line.

Because onion bulbs are dormant when stored, bulb metabolism is expected to remain low until dormancy is broken and growth resumes. Breaks in dormancy coincide with a resumption of root and shoot growth and an increase in respiration (Kays and Paull, 2004). Previously it was reported that increases in FEH activity and INV activity as well as changes in carbohydrate composition occurred after several weeks in cold storage (Benkeblia et al., 2004; Benkeblia et al., 2005). Presumably this was in response to a

break in dormancy and an increased demand for sugars to resume growth. In this study we wanted to confirm previous results and also determine if changes in firmness were related to changes in dormancy.

Cultivars varied widely in their ability to resume growth at a given time and correlations between rooting/sprouting and changes in firmness were not significant. It has been reported that roots initiating from the outside of the basal plate of onion bulbs have little or no dormancy requirement (Komochi, 1990). Therefore some bulbs can produce roots initiating from the base plate of bulbs, but may still have greatly lowered metabolic activity resulting in a poor correlation between the two parameters measured.

As expected, increases in FEH and INV activity during storage generally coincided with an increase in respiration that occurred after eight weeks (Figs. 2A, 7A,B). Soluble INV, which catalyzes the cleavage of sucrose to glucose and fructose is closely linked to tissue growth (Roitsch and Gonzalez, 2004). Similar trends in activities of both enzymes have been previously observed (Benkeblia et al., 2004, Benkeblia et al., 2005). In addition, FEH activity was highest in the high fructan cultivar MBL87-WOPL and lowest in the low fructan cultivar, Pegasus. Other parameters measured such as firmness, pectins, PME and PGA activities did not correspond with changes in root/shoot growing, suggesting that these attributes may not be closely linked to changes in dormancy.

Pectins account for about 30% of the polysaccharides in the primary cell wall and middle lamella (Willats et al., 2001). Changes in pectin brought about by PGA and PME have been linked to softening of fruits during the ripening process, though it is clear that other enzymes such as expansin are important in softening (Brummell, 2006; Brummell

and Harpster 2004). The composition of onion pectin has been previously investigated, though little is known about how pectin changes during storage or if this would affect onion firmness (Matsuura et al., 2000; Ng et al., 1998; Redgewell and Selvendran, 1986). In this study the firmest at harvest was linked to pectin concentration. MBL87-WOPL had nearly twice the concentration of pectin as Pegasus, the softest line measured. The changes in pectin fractions during storage though do not necessarily correspond to changes in firmness. The WSP fraction increases significantly in the MBL87-WOPL between 8-12 weeks of storage, and the ASP fraction decreases accordingly. Additionally there is a large decrease in firmness that occurs in MBL87-WOPL at this time. The other cultivars however, do not behave in the same manner. Changes in firmness of Pegasus and MSU4535B do not correlate well with changes in pectin composition during storage. Thus while changes in pectin composition may result in changes in texture of MBL87-WOPL, clearly other factors must be involved in the decrease in firmness of Pegasus and MSU4535B.

Cellulose, the 1,4-β-D-glucan that gives rise to microfibrils, is also generally thought to give the cell wall much of its strength (Carptia and Gibeaut, 1993). Cellulose concentrations were highest in the MBL87-WOPL line and lowest in Pegasus. Similarly, O'Donoghue et al., (2004) found that Pukekohe Longkeeper, a firm long-storing onion, had higher concentrations of cellulose than a softer poor-storing Houston Grano bulb. This suggests that cellulose may also be linked to firmness in onion.

The activity of two common pectinases, PME and PGA were measured. Pectin methylesterases catalyses the de-methylesterification of polyalacturonan chains in the cell wall. Once de-esterified, the polygalactuonan chains may become are susceptible to

hydrolysis by PGAs, resulting in loosening of the cell wall (Micheli, 2001). The activity of PME and PGA was consistently higher in Pegasus and MSU4535B lines during storage and lowest in the MBL87-WOPL line. Furthermore, while all lines experienced softening in storage, Pegasus and MSU4535B experienced more rapid declines in firmness than the MBL87-WOPL line. Changes in the middle lamella region observed using TEM support these findings. This suggests that PME and PGA activity in onion during storage may be related to the rate of bulb softening. Previously, PGA activity has been linked to disruption in cellular adhesion in roots of *A. porrum* (Peretto et al., 1992).

Our results indicate that firmness in onion scales may be the result of several factors. One may be the concentration of pectin and cellulose in bulbs. Pegasus had the lowest concentration of pectin and cellulose and had the softest bulbs at harvest, while MBL87-WOPL had the highest levels of structural carbohydrates and were the firmest bulbs at harvest. The MSU4535 bulbs had intermediate levels of pectin and cellulose as well as firmness at harvest. A second factor related to bulb firmness in these lines may be the activity of PME and PGA. Both Pegasus and MSU4535B had higher PME and PGA activities and experienced their largest decrease in firmness relatively early during the storage period. MBL87-WOPL had lower levels of PME and PGA activity during storage and softening was delayed until 8-12 weeks in storage. In addition, PME, which acts prior to PGA in cell wall disassembly peaked in activity in Pegasus and MSU4535B four weeks prior to the maximum observed PGA activity indicating that both enzymes may act together in the Pegasus and MSU4535B cultivars.

Conclusion

Our results indicate that firmness in onion at harvest may be linked to the concentration of cellulose and pectin in the bulb. Unlike the activities of FEH and INV, PME and PGA appear to be independent of changes in bulb dormancy (respiration). Interestingly PME and PGA activity were highest in those cultivars that experienced the earliest softening during storage. However, all cultivars eventually did soften during storage. Because MSU4535B had relatively high levels of pectin and cellulose, but still experienced rapid softening during storage it seems unlikely that the abundance of pectin and cellulose in theMBL87-WOPL were entirely responsible for the delayed the expression of softening until late in storage. Other factors, such as expansin activity may also be responsible for softening during onion storage, but the results obtained here will prove useful for further investigation regarding onion softening during storage.

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Figure 1(A-C). A: Percentage of harvest weight lost, B: percentage of dry matter content, and C: percentage of soluble solids content (SSC). Each data point represents the mean (± s.e.) of four replications for Pegasus and MSU4535B and three replications of MBL87-WOPL onion (*Allium cepa* L.) cultivars measured at harvest, 4, 8 and 12 weeks of storage. Each replication consisted of 15 bulbs.

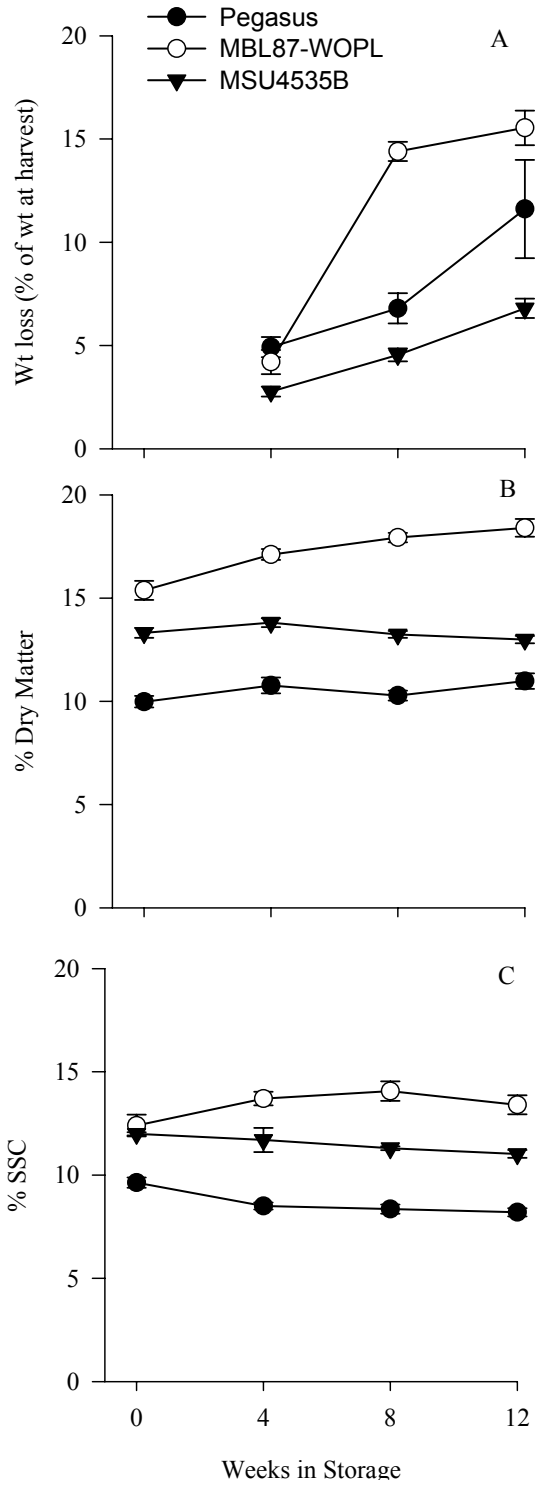
Pegasus
MBL87-WOPL
MSU4535B
A
B
C
Wt loss (% of wt at harvest)
% Dry Matter
% SSC
0
5
10
15
20
0
4
8
12
Weeks in Storage

Figure 2. Percentage of bulb slices exhibiting sprouting or rooting after ten days and B: respiration rate measured at harvest, 4, 8 and 12 weeks of storage. Each data point represents the mean (± s.e.) of four replications for Pegasus and MSU4535B and three replications of MBL87-WOPL onion (*Allium cepa* L.) cultivars. Each replication consisted of 15 bulbs.

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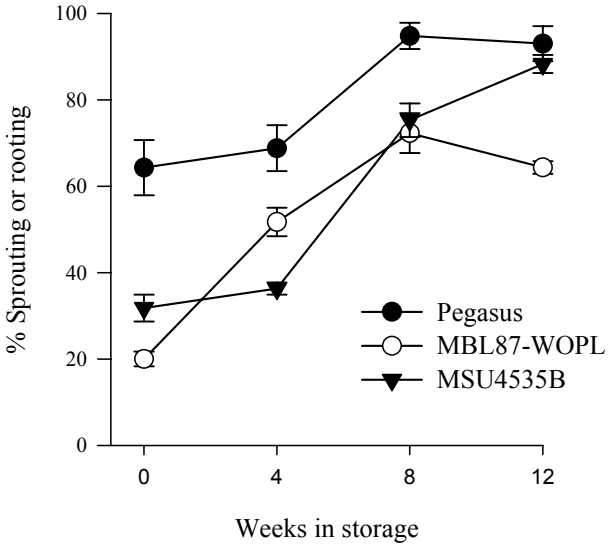

100
80
60
40
20
0
% Sprouting or rooting
0
4
8
12
Weeks in storage
Pegasus
MBL87-WOPL
MSU4535B

Figure 3. Firmness of onion (*Allium cepa L*) scales in measured in Newtons (N) at harvest, 4, 8, and 12 weeks of storage. Each data point represents the mean (± s.e.) of four replications for Pegasus and MSU4535B and three replications of MBL87-WOPL cultivars. Each replication consisted of 15 bulbs.

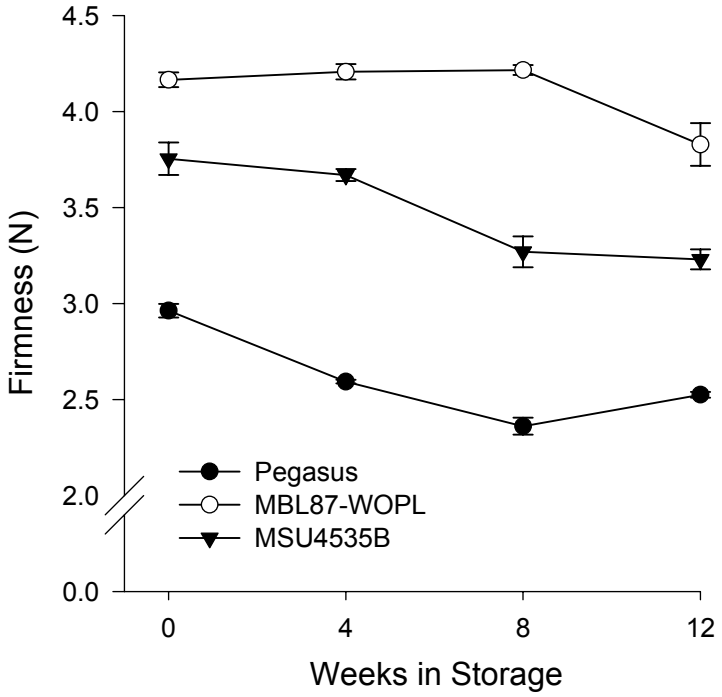

4.5
4.0
3.5
3.0
2.5
2.0
0.0
Firmness (N)
0
4
8
12
Weeks in Storage
Pegasus
MBL87-WOPL
MSU4535B

Figure 4 (A-E). A: Fructose, B: Glucose, C:Sucrose, D:Total Fructan, and E: Total carbon, measured at harvest, 4, 8, and 12 weeks of storage. Each data point represents the mean (± s.e.) in $mg \cdot g^{-1}$ fresh weight (FW) of four replications for Pegasus and MSU4535B and three replications of MBL87-WOPL onion (*Allium cepa* L.) cultivars. Each replication consisted of 15 bulbs.

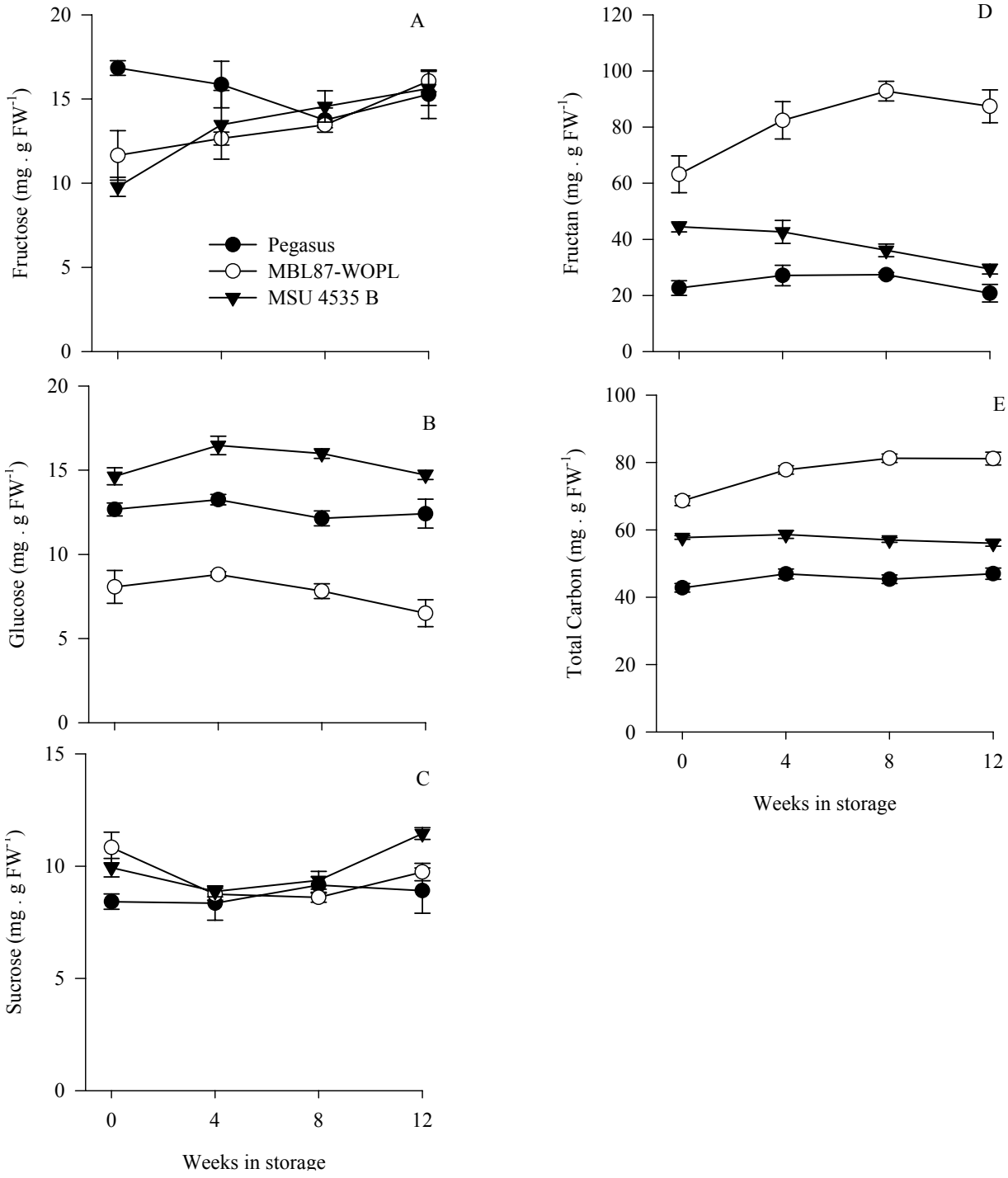

A
B
C
D
E
Fructose (mg . g FW-1)
Glucose (mg . g FW-1)
Sucrose (mg . g FW-1)
Fructan (mg . g FW-1)
Total Carbon (mg . g FW-1)
Pegasus
MBL87-WOPL
MSU 4535 B
Weeks in storage
Weeks in storage

Figure 5(A-F). A: Total uronic acid (UA) (pectin), B: Water soluble pectin (WSP), C: Chelator soluble pectin (CSP), D: Acid soluble pectin (ACSP), E: Alkaline soluble pectin (ASP), and F: Cellulose, measured at harvest, 4, 8, and 12 weeks of storage. Data points represent the mean (± s.e.) in $mg \cdot g^{-1}$ fresh weight (FW) of four replications for Pegasus and MSU4535B and three replications of MBL87-WOPL onion (*Allium cepa* L.) cultivars. Each replication consisted of 15 bulbs.

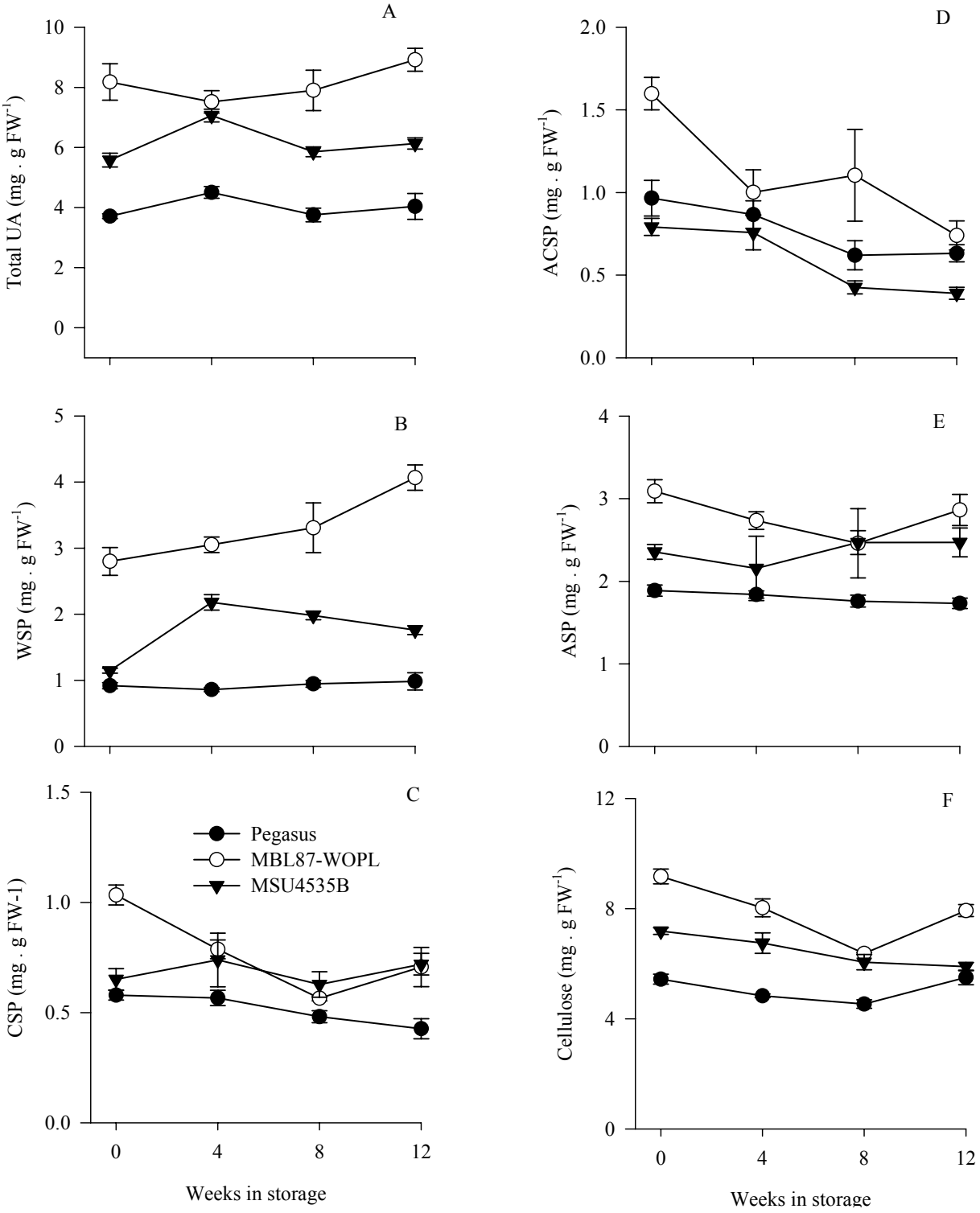

A
Total UA (mg . g FW^{-1})
B
WSP (mg . g FW^{-1})
C
CSP (mg . g FW-1)
Pegasus
MBL87-WOPL
MSU4535B
Weeks in storage
D
ACSP (mg . g FW^{-1})
E
ASP (mg . g FW^{-1})
F
Cellulose (mg . g FW^{-1})
Weeks in storage

Figure 6 (A-F). Transmission electron micrographs showing cell walls (CW), middle lamella (ML) and carbohydrate chains (CC) in three cultivars of onion (*Allium cepa L.*) at harvest and after 12 weeks of storage. A: Pegasus at harvest, B: Pegasus at 12 weeks of storage, C: MBL87-WOPL at harvest, D: MBL87-WOPL at 12 weeks of storage, E: MSU4535B at harvest, D:MSU4535B at 12 weeks of storage.

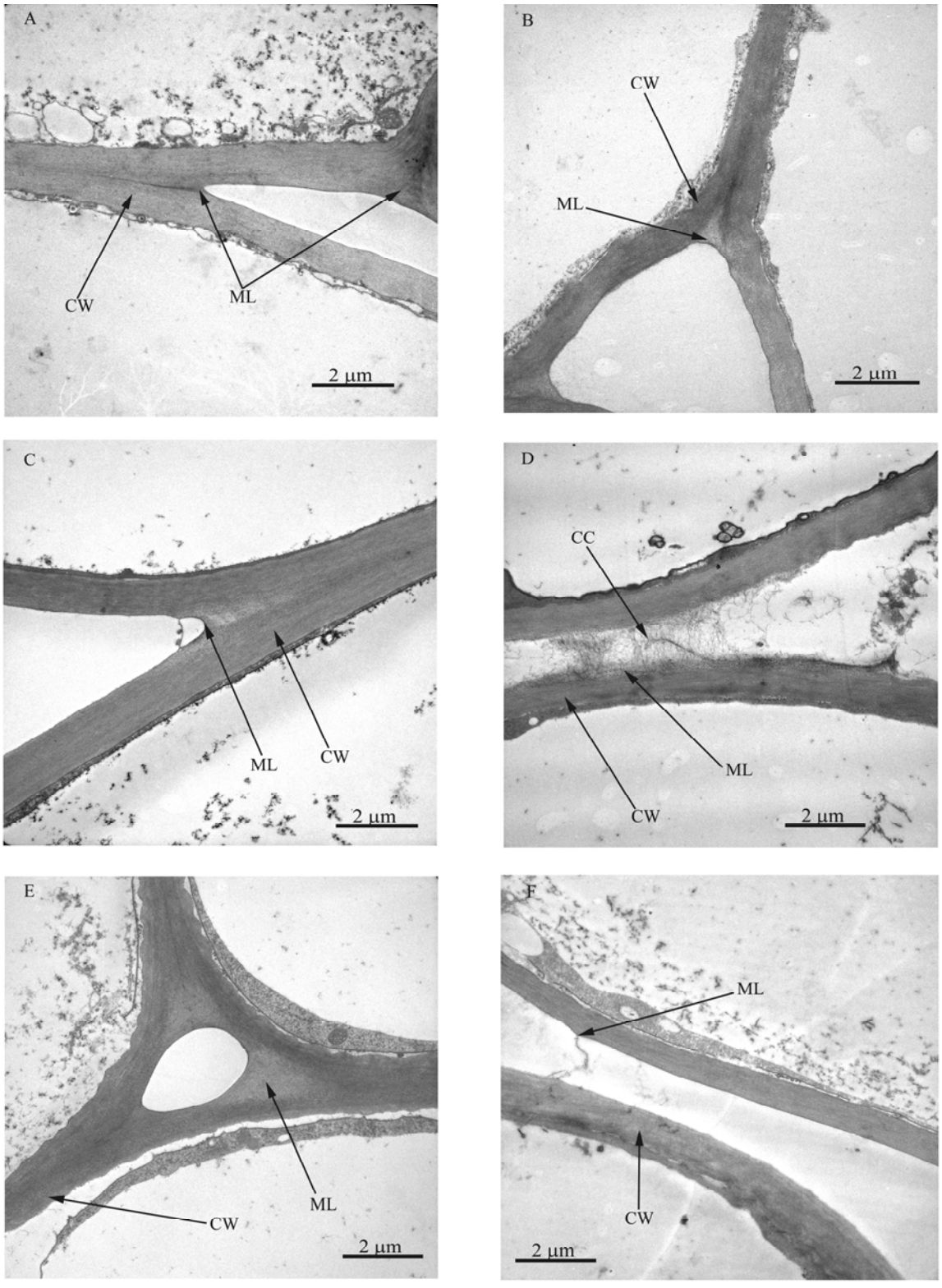
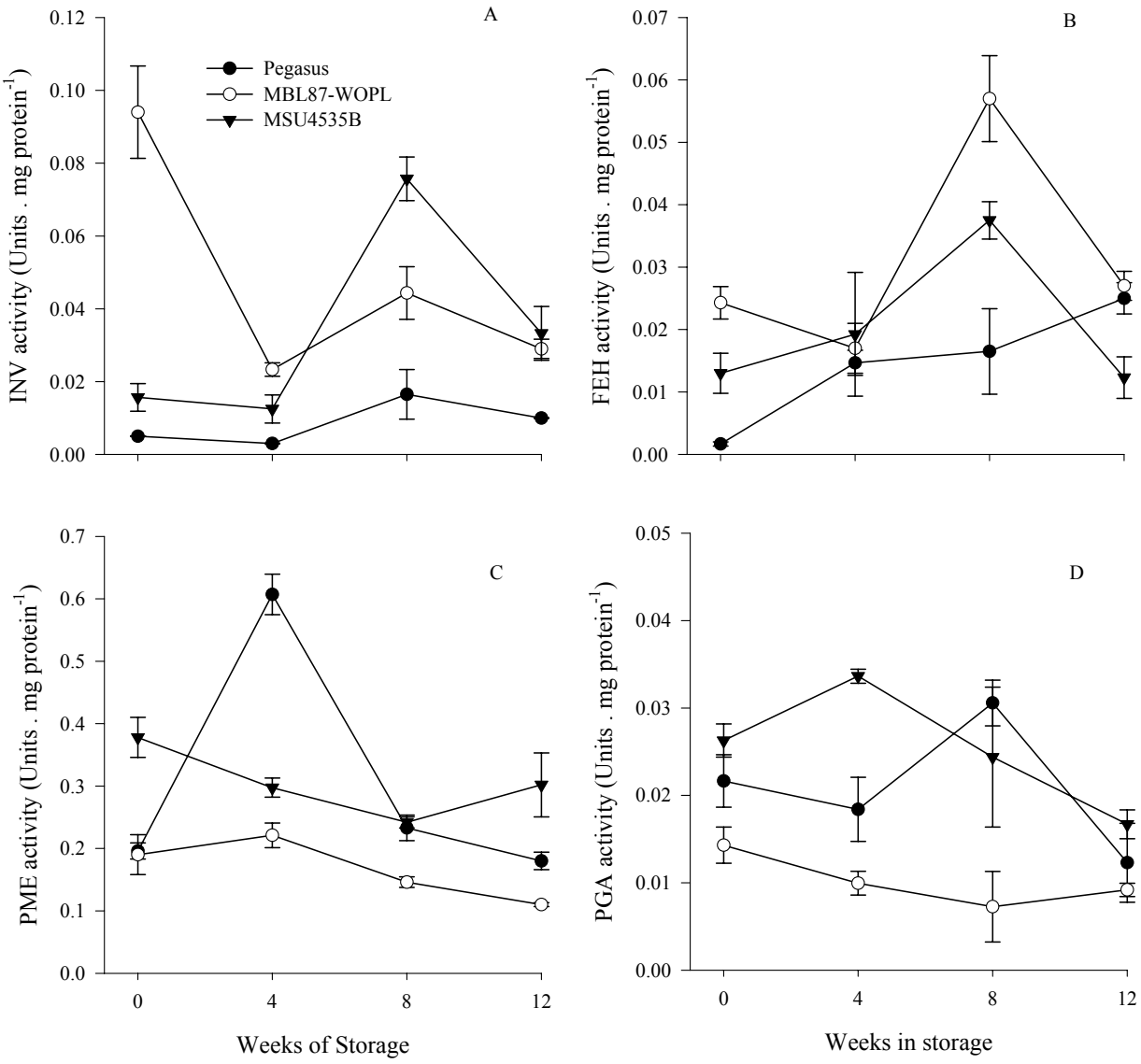

A
CW
ML
2 µm
B
CW
ML
2 µm
C
ML
CW
2 µm
D
CC
ML
CW
2 µm
E
ML
CW
2 µm
F
ML
CW
2 µm

Figure 7 (A-D). Enzyme activities measured for three cultivars of onion (*Allium cepa L.*) at harvest, 4, 8, and 12 weeks of storage. Activities of A:Invertase (INV), B:1-Fructan exohydrolase (FEH), C: Pectin methylesterase (PME), and D: Polygalacturonase (PGA). Each data point represents mean (± s.e.) in activity units·mg protein $^{-1}$ for four replications of Pegasus and MSU4535B and three replications of MBL87-WOPL. One activity unit is equivalent to 1 umol of product produced per minute from a given substrate.


A
B
C
D
Pegasus
MBL87-WOPL
MSU4535B
INV activity (Units . mg protein-1)
FEH activity (Units . mg protein-1)
PME activity (Units . mg protein-1)
PGA activity (Units . mg protein-1)
Weeks of Storage
Weeks in storage

CHAPTER 5

QUANTITATIVE ANALYSIS OF *BOTRYTIS ALLII* IN ONION[3]

[3] Coolong, T.W., R.R. Walcott, and W.M. Randle. Submitted to HortScience.

Additional index words: *Allium cepa*, real-time PCR, neck-rot

Abstract

A real-time PCR assay was developed for the detection of *Botrytis allii*, the causal agent of neck rot in onion tissue. The assay utilized the TaqMan® probe-based reaction to detect an amplicon from the *L45-550* region of *B. allii*, while utilizing the onion serine acetyl transferase gene (*SAR1*) as a control gene to guard against false negatives. The detection limits for both onion and *B. allii* DNA were 10 pg. The detection limit for lyophilized *B. allii* mycelia was 1 µg. The presence of onion tissue in the samples did not affect the performance of the assay. The assay was able to distinguish among onion disks that were artificially inoculated with different levels of *B. allii* conidia. Furthermore, assay results corresponded well with visual observations of fungal growth on inoculated onion disks. This assay will be used in upcoming studies to determine the level of *B. allii* mycelia in bulbs at harvest that is necessary to cause significant storage losses. The assay should allow for the prediction of neck rot in stored onion bulbs.

Introduction

Onion (*Allium cepa L.*) is an economically important vegetable, accounting for nearly $1 billion in farm gate income annually in the U.S. (National Onion Association, 2005). Because a large portion of the crop is marketed from storage, postharvest rots can contribute to significant losses for growers. The primary storage disease reported in onions is neck rot, caused by the fungus *Botrytis allii* (syn. *B. aclada*) (Maude, 1990; Yohalem et al., 2003). Losses to neck rot during storage can be as high as 35% in some years (Tietjen and Ceponis, 1981; Williams-Woodward, 2001).

Botrytis allii can infect onions at any stage during the growing season. Potential sources of *B. allii* inoculum include infected seeds, onion debris from previous crops, and alternate crops (Maude, 1976; Walcott et al., 2004). Culling infected bulbs at harvest is challenging; however, as onion plants can become infected without displaying visual symptoms of neck rot (Kritzman, 1983; Maude, 1990). With a latent period of 8-10 weeks, infected bulbs may appear asymptomatic at harvest, only to develop symptoms of the disease during storage, resulting in significant economic loss. (Maude 1990). Because visual inspection of intact bulbs is ineffective, alternative methods have been developed to detect *B. allii* in onion bulbs. These include culturing samples on semi-selective media, enzyme linked immunosorbent assay (ELISA), and conventional PCR (Linfield et al., 1995; Kritzman and Netzer, 1978; Nielsen, 2002). While all of these methods are useful for identifying the and detecting *B. allii* in onion, none of them can be used to easily quantify the level of *B. allii* inoculum in infected tissues. The ability to quantify the inoculum in bulb tissues is of great potential importance because it is likely that the amount of inoculum in a bulb at harvest is related to bulb rot after storage. Higher levels of initial inoculum have been shown to lead to earlier and more severe neck rot symptoms in onion tissue (Bertolini and Tian, 1997). Although other factors such as plant resistance, storage temperature, and relative humidity interact to determine severity of neck rot infection, it is clear that the levels of *B. allii* in bulbs at harvest is related to neck rot severity (Alderman and Lacy, 1984, Bertolini and Tian, 1997; Kritzman, 1983). Therefore developing a method that could precisely quantify *B. allii* inoculum in onion bulbs could be useful for predicting levels of infection during storage. Conventional diagnostic assays do not have the capacity to reliably quantify mycelial mass; however,

quantitative real-time PCR represents one technique by which fungal inoculum can be estimated.

Unlike traditional PCR, quantitative real-time PCR can simultaneously amplify and estimate the concentration of specific template DNA sequences. The ability to estimate the amount of sequence specific template DNA or RNA in a sample can allow for the simultaneous identification and quantification of pathogens in crops of interest. Current methods used to quantify template DNA utilize fluorescent dyes, which emit light of a specific wavelength during amplification (Shena et al., 2004). Fluorescence can occur either through non-specific methods, such as the fluorescence emitted by SYBR green as it is intercalated into double stranded DNA, or via sequence specific methods such as the light emitting probes used in the TaqMan® or Scorpian® PCR systems (Wilhelm and Pingoud, 2003). In any system, the amount of fluorescence increases as product is amplified. The user can set a threshold value for fluorescence, below which samples are considered negative, and above which samples are considered positive. The number of cycles required for a sample to reach the threshold is called the cycle threshold (Ct) value. The higher the concentration of template DNA, the shorter the number of amplification cycles required to reach the threshold and the lower the Ct value (Wilhelm and Pingoud, 2003). Because the amount of pathogen template DNA is proportional to the level of pathogen inoculum present, real-time PCR can be used to estimate fungal mass, viral load or bacterial cells in a given sample.

Recently Suarez et al., (2005) developed a TaqMan® based real-time PCR assay for the quantification of *B. cinerea* in *Pelargonium* species (Suarez et al, 2005). This assay facilitated the quantification of *B. cinerea* inoculum over four orders of magnitude.

Furthermore, there was a positive relationship between estimates of inoculum concentration and the visual expression of symptoms on leaf discs. The utility of quantitative real-time PCR as a tool for plant pathologists has been demonstrated numerous times as assays have been developed to identify and quantify a variety of pathogens in agronomic and horticultural crops including: *Fusarium* species in wheat, *Rhizoctonia* species in tomato and *Phytopthora ramorum* in *Quercus* species (Lievens, et al., 2006; Schnerr, et al., 2001; Tooley et al., 2006).

Quantitative real-time PCR represents a tool to quickly and reliably detect the presence and amount of *B. allii* in asymptomatic (with regard to neck rot infection) onion bulbs at harvest. The overall goal of this project is to develop and evaluate a system to predict the likelihood of neck rot development in stored onion bulbs based on the amount of initial inoculum at harvest. This assay could allow growers to make more informed decisions about whether to sell onions to fresh markets or assess the risks of neck rot development in storage. The specific objective of this study was to develop and evaluate a quantitative real-time PCR assay for the quantification of *B. allii*, mycelia in onion tissue.

Materials and Methods

Fungal isolates. Fungal isolates *B. allii 703-308*, and *B. cinerea 870-404A*, *870-404B*, *870-404C* were routinely maintained on potato dextrose agar (PDA) (Beckton-Dickinson, Sparks MD). The mycelia used for DNA extraction was produced by culturing *B. allii 703-308* in potato dextrose broth with agitation for seven days at 20 ^{o}C. Mycelia were harvested by passing the liquid culture through a 0.45 μm filter disk (Corning, Corning, NY, USA) and freezing the mycelial fragments in liquid nitrogen. The mycelia was then

lyophilized and ground into a fine powder using a mortar and pestle. Cultures from two common onion bacterial pathogens, *Pantoea ananatis 99-8* and *Burkholderia cepacia 92-1,* were maintained at -80 ^{o}C and routinely grown on LB broth. Specificity of the *B. allii* primers and probes was later tested on DNA extracted from these cultures. Disease free onion bulbs cv. 'Pegasus' were grown under greenhouse conditions using standard protocols. Approximately 100 g fresh tissue from mature bulbs was frozen in liquid nitrogen, lyophilized, and ground into a fine powder using a mortar and pestle.

DNA extraction. Genomic DNA was extracted using a modified phenol extraction method (Ellington and Pollard, 1999). To a 100 µl suspension of fungal or onion tissue, 0.4 ml of extraction buffer containing 200 mM Tris HCl, 250 mM NaCl, 25 mM EDTA, and 0.5 w/v SDS was added and heated at 65 ^{o}C for 10 min. Then 0.5 ml of 25:24:1 phenol/chloroform/isoamyl alcohol was added, and the mixture was centrifuged at 10,000 g for 1 min. The aqueous phase was removed and re-extracted in 0.4 ml of 25:24:1 phenol/chloroform/isoamyl alcohol. The DNA was precipitated with one-tenth volume of 3 M sodium acetate (pH 5.2) followed by the addition of two volumes of 70% ethanol and incubation at -20 ^{o}C for 15 minutes. The DNA solution was then centrifuged for 5 min at 10,000 g and the pellet containing DNA was washed twice with 1 ml of 100% ethanol and centrifuged for 5 min. at 10,000 g. The pellet was then dried at 25 ^{o}C for 10 min. and dissolved in 50 µl sterile water.

Primer and probe design for *B. allii.* Conventional PCR primers for detection of *B. allii* in onion were previously designed by Nielsen et al. (2002). For this study, the previously reported oligonucleotide BA2F (Table 8) was used as the forward primer. A new reverse primer (BA3R) and probe were designed using PrimerQuest™ software

(Integrated DNA Technologies (IDT), Coralville, IA, USA) based on the *L45-550* DNA sequence from *B. allii* (Nielsen et al., 2001). For real-time PCR the TaqMan® system was employed and the Taqman® probe, BaProbe, (Table 8) was dual labeled with Texas Red-X® (IDT) at the 5' end and the Black Hole Quencher 2® (BHQ2) (IDT) at the 3' end.

Primer and probe design for internal control using onion *SAT1* gene. To normalize the results obtained from quantitative real-time PCR for *B. allii*, a second PCR assay was developed as a control targeting the onion *SAT1* gene. TaqMan® primers and probes were designed based on the *SAT1* onion gene sequence recently characterized by McManus et al. (2005). This quantitative PCR assay was necessary as a control to ensure that DNA extraction and amplification efficiency was similar among samples and to guard against false negative signals due to poor DNA quality. *SAT1* was chosen because it is one of the few well characterized onion genes that is thought to be conserved within *Allium* species. Hence this would allow use with number of onion cultivars. Primers and probe were designed as previously described based on the *SAT1* gene sequence (McManus et al., 2005) (Table 8). The TaqMan® probe, SatProbe, was dual labeled with 6-carboxyfluorescein (6-FAM™) (IDT) at the 5' end and Black Hole Quencher 1® (BHQ1) (IDT) at the 3' end (Table 8).

PCR Conditions. After purification, DNA from *B. allii* mycelia and onion tissue was subjected to real-time PCR analysis. Real-time PCR of *B. allii* and onion DNA was performed in separate 25 ul reactions using the Cepheid Smart Cycler (Cepheid, Sunnyvale, CA, USA). Routinely, real-time PCR was performed using the commercially available Eppendorf RealMasterMix Probe containing HotMaster Taq DNA polymerase and self adjusting magnesium buffer (Eppendorf, Hamburg, Germany). Final primer and

probe concentrations for *B. allii* and onion control assays were 300 nM and 200 nM, respectively. The thermal profile used for DNA amplification included an initial denaturation at 95 ^{o}C for 105 sec, followed by 40 cycles of denaturation at 95 ^{o}C for 15 sec, annealing at 55 ^{o}C for 15 sec, and extension at 68 ^{o}C for 30 sec. Fluorescence was measured at the extension step in each cycle and typically samples were considered to be positive when fluorescence exceeded a set background threshold of 30 fluorescence units. For quantitative PCR, the partial cycle number at which fluorescence for a reaction surpassed the background fluorescence (Ct) value was recorded.

To confirm identities of the *L45-550* SCAR and SAT1 sequences amplified, PCR amplicon length was confirmed using gel electrophoresis (Figure 8). For electrophoresis, PCR products were separated in a 1% agarose gel containing 0.5 µg/ml ethidium bromide in a 0.04 mM Tris acetate buffer with 2 mM EDTA (Ellington and Pollard, 1999) buffer at 90 volts for 60 min. In order to obtain valid sequence information, PCR products were cloned, using the TOPO-TA cloning kit (Invitrogen Corp., Carlsbad, CA, USA) following the manufacturer's directions. Cloned DNA from *B. allii* and onion was purified using the Quiagen Plasmid Mini Kit (Quiagen Inc. Valencia, CA, USA) and sequenced on an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA) at The University of Georgia Integrated Biotech Sequence and Synthesis Facility (University of Georgia, Athens, GA, USA).

Specificity of the B. allii and onion TaqMan PCR assay. The specificity of the real-time PCR assays for the *B. allii* and onion control genes were tested using DNA from the closely related pathogen *B. cinerea* and common onion pathogens *Pantoea ananatis* (center rot) and Burkholderia cepacia (sour skin). For this DNA was extracted from

isolates of *B. cinerea 870-404A, 870-404B, 870-404C* as well as isolates of the bacterial pathogens *P. ananatis 99-8* and *B. cepacia 92-1.* Five ng of DNA was used as a template and reactions were set up as described previously. Each reaction was carried out in triplicate, and samples that yielded a Ct value greater than 39 were considered be negative.

Relationship between DNA concentration Ct value and B. allii mycelial mass. Real-time PCR was performed on *B. allii* and onion samples of known DNA concentrations in order to confirm that Ct values correlated well with DNA concentration, and to determine the detection limits for the real-time PCR assay. To do this, serial dilutions of *B. allii* and onion genomic DNA were made ranging from 100 ng/µl to 1 pg/µl. Dilutions were replicated four times and real-time PCR reactions were carried out as described above. To determine if the presence of onion DNA would interfere with the amplification of *B. allii* DNA and vice-versa, 50 ng of onion DNA and 5 ng of B. allii DNA were added to the serial dilutions of *B. allii* and onion DNA, respectively, and analyzed. Regression analysis was performed on data from *B. allii* DNA with and without onion DNA present and vice-versa to determine the precision of the test and if the presence of non-target DNA altered the results. This experiment was replicated four times.

The lower detection limit of this assay for lyophilized *B. allii* mycelia mass was also determined. To test this, serial dilutions of *B. allii* mycelial suspensions in water were made ranging from 10 mg/ml to 0.001 mg/ml lyophilized mycelia. Dilutions were replicated four times and DNA extractions and real-time PCR analysis were carried out as previously described. To determine the effects of the addition of onion tissue to the assay, five mg of lyophilized onion tissue was added each dilution of *B. allii* mycelia

ranging from 10 mg/ml to 0.001 mg/ml mycelia. This was also replicated four times and the DNA was extracted and tested using the real-time PCR assay as previously described.

In addition to testing the relationship between mycelial weight and Ct value, the accuracy and precision of the method was also determined. One mg of lyophilized *B. allii* mycelia and 5 mg of lyophilized onion tissue were added to the same vial, suspended in 1 ml of HPLC water and the DNA was extracted as described previously. This was replicated seven times. Real-time PCR assays for *B. allii* and onion were performed on DNA from each replication. Mean recovery of the 1.0 mg of lyophilized mycelia was determined using the standard curves relating mycelial mass to Ct value obtained earlier. The results of the *B. allii* assay were normalized using the values obtained for the assay of the onion DNA in each replicate.

Detecting *B. allii* in artificially inoculated onion tissue. To determine if real-time PCR quantification corresponded to visual observations of fungal growth an assay was conducted with inoculated onion disks artificially inoculated with *B. allii* conidia. Asymptomatic greenhouse grown onion bulbs, 'Pegasus,' were peeled and the first fleshy scale was removed. Single 7 mm diameter disks were taken from each bulb and washed three times for 10 sec. each in 70% ethanol and once for 30 sec. in sterile water. The average weight of a disk was 193.7 mg (±2.26 mg). Three randomly chosen disks were then plated onto individual petri dishes containing PDA. Each disk on a dish was stab inoculated with 10 µl of sterile deionized water, or suspensions of 10^2 or 10^4 B. allii conidia. Conidial suspensions were obtained from 7 – 10 day old PDA cultures of *B. allii 703-308* by flooding the colony with sterile water and gently rubbing the colony surface with a glass rod. Conidial suspensions were collected in a 50 ml tube and conidial

concentrations were enumerated using a hemacytometer. All inoculation levels were found on a single dish. One dish acted as a single replication for a particular incubation time, with each time having three replicates (dishes) each for a total of nine dishes. After inoculation, dishes were sealed with parafilm (Pechiney Plastic Packaging, Chicago, IL, USA) and incubated in the dark at 20 ^{o}C and 80% relative humidity for 0, 72, and 120 hours. After incubation disks were visually examined photographed and subjected to DNA extraction and the real-time PCR assay for *B. allii* and onion DNA.

Statistical Analysis Results were subjected to linear regression and/or the Proc Mixed procedure of SAS statistical software (SAS v. 9.1, SAS Institute, Cary, NC, USA)

Results

Specificty of the *B. allii* and onion TaqMan® assay. The identity of the 200 bp PCR products from the *L45-550* and *SAT1* regions of the *B. allii* and onion genomes, respectively, was confirmed by gel electrophoresis and sequencing (Figure 8). Blast searches of the NCBI database for the *B. allii* amplicon resulted in a 99.5% match (199/200 bp) to the 546 bp L45-550 RAPD fragment from *B. aclada* strain BA8 (GenBank accession #AJ291477). Blast searches of the onion amplicon showed a 99% match (198/200 bp) to the 1094 bp complete cDNA sequence for the onion *SAT1* gene (GenBank accession # AF212156). Specificity of primer and probe combinations was confirmed for both *B. allii* and onion primer and probe sets using template DNA from a variety of sources (Table 9). No false positives were observed.

Relationship between DNA concentration and Ct values. The real-time PCR assay showed a negative linear relationship between *B. allii* DNA concentration and Ct value (R^2=0.97) and was able to successfully detect *B. allii* DNA across five orders of

magnitude at concentrations ranging from 100 ng/µl to10 pg/µl genomic DNA (Figure 9a). The addition 50 ng of onion DNA did not change the detection limit or the overall performance of the TaqMan® assay for *B. allii* DNA (Figure 9a). When purified onion genomic DNA was tested using the TaqMan® assay developed for *SAT1* a negative linear relationship was also observed between DNA concentration and Ct value (Figure 9b). As in the *B. allii* assay, the onion assay successfully amplified and quantified onion genomic DNA across five orders of magnitude ranging from 100 ng/µl to 10 pg/µl (Figure 9b). The addition of 5 ng of B. allii DNA to each dilution of onion DNA did not affect the sensitivity or trend of response (Figure 9b).

Relationship between mycelial weight and Ct value. A standard curve was developed for detection of *B. allii* mycelia using real-time PCR. The *B. allii* assay was able to amplify DNA from *B. allii* mycelia across five orders of magnitude of mycelial weight ranging from 10 mg to 1 µg lyophilized mycelia. The response of Ct value to mycelial weight was negative and linear with Ct values decreasing as mycelia content of the sample increased (Figure 10). When 5 mg of lyophilized onion tissue was added to each sample the slope of the standard curve changed slightly but detection limits were not significantly different (P=0.288) (Figure 10). The standard curve obtained for mycelial weight plotted against Ct value in the presence of onion tissue was: Ct value=-1.47Ln(mycelia wt) + 23.42. This standard curve was used to calculate recovery of lyophilized mycelia and infection levels in subsequent experiments.

Using the curve developed for the relationship between mycelial weight and Ct value the percentage recovery for *B. allii* was determined. After normalization, samples containing 1mg of lyophilized mycelia and 5 mg lyophilized onion tissue displayed a

mean recovery of 0.992 mg ± 0.202 mg mycelia. This equates to an average recovery of 99.2% with a coefficient of variation of 20.4%.

Quantitative real-time PCR assay of artificially inoculated onion tissue. The real-time PCR assay was also tested to determine the ability to detect and quantify artificially inoculated onion tissue. As expected, *B. allii* was not detected in control onion disks. Estimated *B. allii* mycelial mass was significantly higher in the 10,000 conidia treatment than the 100 conidia treatment ($P<0.001$). Furthermore, mycelial mass increased significantly with incubation time ($P<0.001$) (Figure 11a). Immediately after inoculation *B. allii* was only detected in disks inoculated with 10,000 conidia (Figure 11a). However, at 72 h of incubation *B. allii* was detected in disks inoculated with 100 and 10,000 conidia giving average mycelial masses of 0.022 and 0.486 mg of mycelia/disk, respectively. After 120 h of incubation mean fungal growth was 1.5 and 2.4 mg mycelia for disks inoculated with 100 and 10,000 conidia, respectively (Figure 11a). The response to both conidial number and time was similar with Ct values decreasing as both conidia and incubation time increased. The results obtained from the real-time PCR assay correspond well to visual observations of fungal growth on the onion disks at the time of sampling. Fungal mycelia appeared on onion disks after 72 hours and were easily seen on disks inoculated with 10,000 conidia. At 120 hours profuse mycelial growth was observed (Figure 12).

Onion template DNA concentration was estimated to be significantly different for each incubation time ($P<0.001$), decreasing by more than 50% over the 120 h incubation, but did not differ according to inoculation treatment ($P=.133$). Because the onion template DNA concentration significantly decreased in the onion disks over time

average onion DNA concentrations at each sampling time were used for normalization of *B. allii* data (Fig 11b).

Discussion

Neck rot is one of the most economically important diseases of stored onion bulbs. Although the open wound resulting from the removal of foliage at harvest is an important site for bulb infection, many plants are infected early in the growing season (Maude 1990). Conidia produced during the season can infect plants at the seedling stage (du Toit, et al., 2004; Maude et al., 1982; Maude and Presly, 1977), resulting in asymptomatic plants, which can develop symptoms of neck rot during storage (Maude, 1990). Because *B. allii* is ubiquitous in many onion growing regions (du Toit et al., 2004), simply testing for the presence of *B. allii* at harvest would yield a high percentage of positives. This information alone would be useless as it would result in the excessive culling of bulbs that would not necessarily rot in storage. It is likely that there is a level of fungal infection of onion bulbs at harvest that could be tolerated without noticeable bulb rot in storage. Based on knowledge of this infection threshold, an assay measuring bulb inoculum at harvest might be useful in predicting the storability of bulbs.

In this study a quantitative real-time PCR assay was developed to predict losses to onion neck rot during storage. Although quantitative PCR assays that utilize SYBR Green technology (Wilhmelm and Pingoud, 2003) are more common, they have several pitfalls that make them less appropriate for disease quantification. First, a probe-based system adds another level of specificity to the assay, as both primers and a probe must hybridize to the template DNA sequence prior to amplification and signal generation. Second SYBR Green-based assays require post-amplification dissociation curve analysis

(melting point analysis) to ensure that non-specific amplicons did not contribute to the SYBR green fluorescence observed (Wilhelm and Pingoud, 2003). Additionally, although not employed in this study, a probe-based system could allow for the detection and quantification of multiple target sequences. This could allow simultaneous analysis of an internal control, such as the *SAT1* gene of onion, and the *B. allii* target sequence, thereby increasing the efficiency of the assay. While multiplex real-time PCR assays have been successfully developed for pathogen quantification, (Schena et al., 2006; James et al, 2006) attempts to develop such a system in this study demonstrated significant competition between the amplification reactions of *B. allii* and the *Sat1* gene. Hence PCR reactions for the two targets were conducted in separate tubes

The assay developed in this study facilitated the quantification of *B. allii* DNA over at least five orders of magnitude ranging from 10 pg to 100 ng ul^{-1} DNA. As expected, this assay was as sensitive as the conventional PCR assay for *B. allii* (Nielsen et al., 2002). Additionally, the detection sensitivity of the assay was not affected by the presence of onion DNA. The assay detected DNA extracted from lyophilized *B. allii* mycelia over five orders of magnitude with a detection limit of 1 ug of mycelia. This is important, as one of the goals of this study was to employ real-time PCR to accurately measure mycelial mass. The high percentage recovery observed further indicates that this method is accurate. However, the relatively high CV value of 20.2% shows that but precision could be improved.

One possible explanation for the high CV observed is that very small differences in Ct value are magnified when amounts of starting template are calculated using standard curves. For example, in this test the average Ct value for one mg of *B. allii*

mycelia was 22.1 cycles with a standard deviation of 0.52 cycles. This equates to a CV 2.3% when calculated based on Ct value alone. This should be considered when comparing the precision of all real-time PCR assays. Running samples in duplicate or triplicate could further reduce this variability.

The use of the *SAT1* gene as internal control in *B. allii* quantification should prevent misleading false negatives. During the onion tissue inoculation experiment onion template DNA concentrations decreased significantly. Therefore we had to use the average DNA concentration obtained at each sampling time to normalize the mycelial weights obtained for each time. Failure to do so would have resulted in overestimating mycelial growth at later incubation times. Other studies have reported similar decreases in the recovery of DNA from degraded plant tissues that were incubated at room temperature for extended periods of time (Ceccherini et al., 2003). The senescence of detached leaf tissue, especially at room temperature and high humidity can result in significant reductions of recoverable DNA (Rostage, 1992; Thomas and Stoddart, 1980). This explanation might apply to the observations in this study.

Suarez et al., (2005) reported a positive relationship between visual symptoms of disease in *Pelargonium* leaf disks infected with *B. cinerea* spores and real-time PCR results. Disks infected with a 1000 *B. cinerea* spores showed more severe symptoms earlier than those infected with 10 spores. The more severely infected leaves also showed a concomitant rise in Ct value when assayed using a TaqMan® assay for *B. cinerea*. Similarly, in this study there was good correlation between the visual observation of fungal growth and real-time PCR data.

In future studies, the real-time PCR assay developed in this study will be used to determine the threshold of *B. allii* inoculum in onion bulbs at harvest that will lead to neck rot in storage. If such a threshold can be determined and if it is within the range of detection for the real-time PCR assay, growers and extension agents would have a tool to make informed decisions to improve the management of post harvest neck rot of onion. Information generated by real-time PCR could help growers and brokers to decide whether to sell infected bulbs (with high risk for rot development) to fresh markets or store them with reduced risk of yield reduction. This study represents the first phase of this project.

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Table 8 Nucleotide acid sequences of TaqMan primers and probes used to detect *Botrytis allii* and onion (*Allium cepa L.*) DNA used in this study

Name	Sequence 5'-3'	Function	Source
SatF1	GAT CGG TGA AAC TGC AGT CAT AGG	Onion *SAT1* forward primer	This study
SatR1	CAT CGA TCA GAA CAA CAG ACC CTG	Onion *SAT1* reverse primer	This study
SatProbe	6-FAM-AGG GAA TAT CAG AAT CGG TGC TGG G-BHQ-1	Onion *SAT1* probe for TaqMan® assay	This study
BA2F	GTG GGG GTA GGA TGA GAT GAT G	*B. allii L45-550* forward primer	Nielsen et al., 2002
BA3R	TTG AAT TGG GAG AGC GTT CCT TCG	*B. allii L45-550* reverse primer	This study
BaProbe	TexRed-TCC GCC CTT GTT GAT GAA GTC GAG AA-BHQ2	*B. allii L45-550* probe for TaqMan® assay	This study

Table 9 Specificity of the *Botrytis allii* and onion (*Allium cepa* L) assays when performed with DNA from common fungal and bacterial pathogens of onion bulbs. Each reaction was performed in triplicate with 5 ng/ul DNA template. Samples with cycle threshold values of 39 or higher with a background fluorescence of 30 units or less were considered to be negative.

Pathogen	PCR result with *B. allii* PCR assay	PCR results with *SAT1* PCR assay
Botrytis cinerea 870-404A	-	-
Botrytis cinerea 870-404B	-	-
Botrytis cinerea 870-404C	-	-
Pantoea ananatis	-	-
Burkholderia cepacia	-	-
B. allii	+	-
Onion	-	+
Water	-	-

Figure 8 PCR amplification of the *Botrytis allii* L45-550 fragment and onion (*Allium cepa L.) SAT1* gene using primers and probes described in Table 1. Lanes 1 & 8: the 100 bp ladder ranging from100-1000 bp, lanes 3 & 4 contain duplicate 200 bp amplicons for the onion *Sat1* gene, lanes 6 & 7 contain duplicate 200 bp amplicons from the *L45-550* fragment from *B. allii*, lanes 2 & 5 contain negative controls of onion and *B. allii* template DNA, respectively with no Taq polymerase.

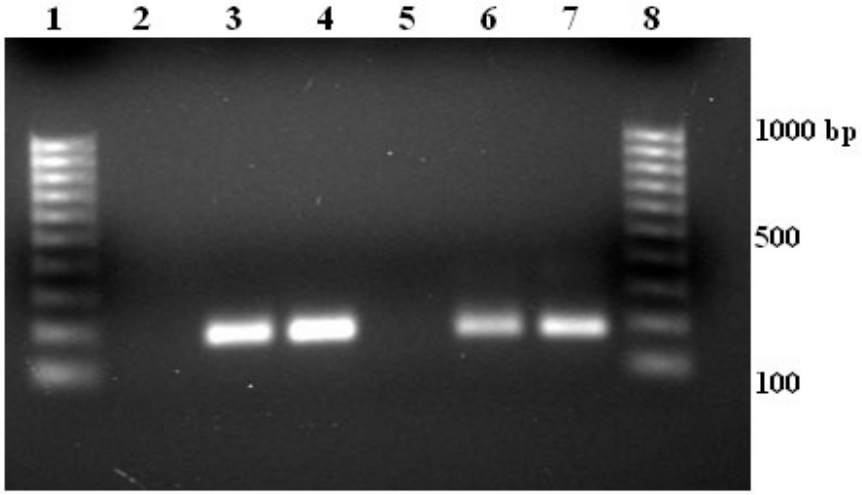

1
2
3
4
5
6
7
8
1000 bp
500
100

Figure 9 (A&B) A: Detection of *Botrytis allii* DNA using a real-time PCR assay based on the *L45-550* DNA sequence. ─●─ *B allii* DNA, ··· ○··· *B allii* DNA with 50 ng onion genomic DNA in each sample. B:.Detection of onion DNA using the real-time PCR assay for the *SATI* genomic region. ─●─ Onion DNA, ··· ○··· onion DNA with 5 ng *B. allii* DNA in each sample. Analyses at each concentration (A and B) were replicated four times. In each example the threshold for positive response was a fluorescence value of 30.

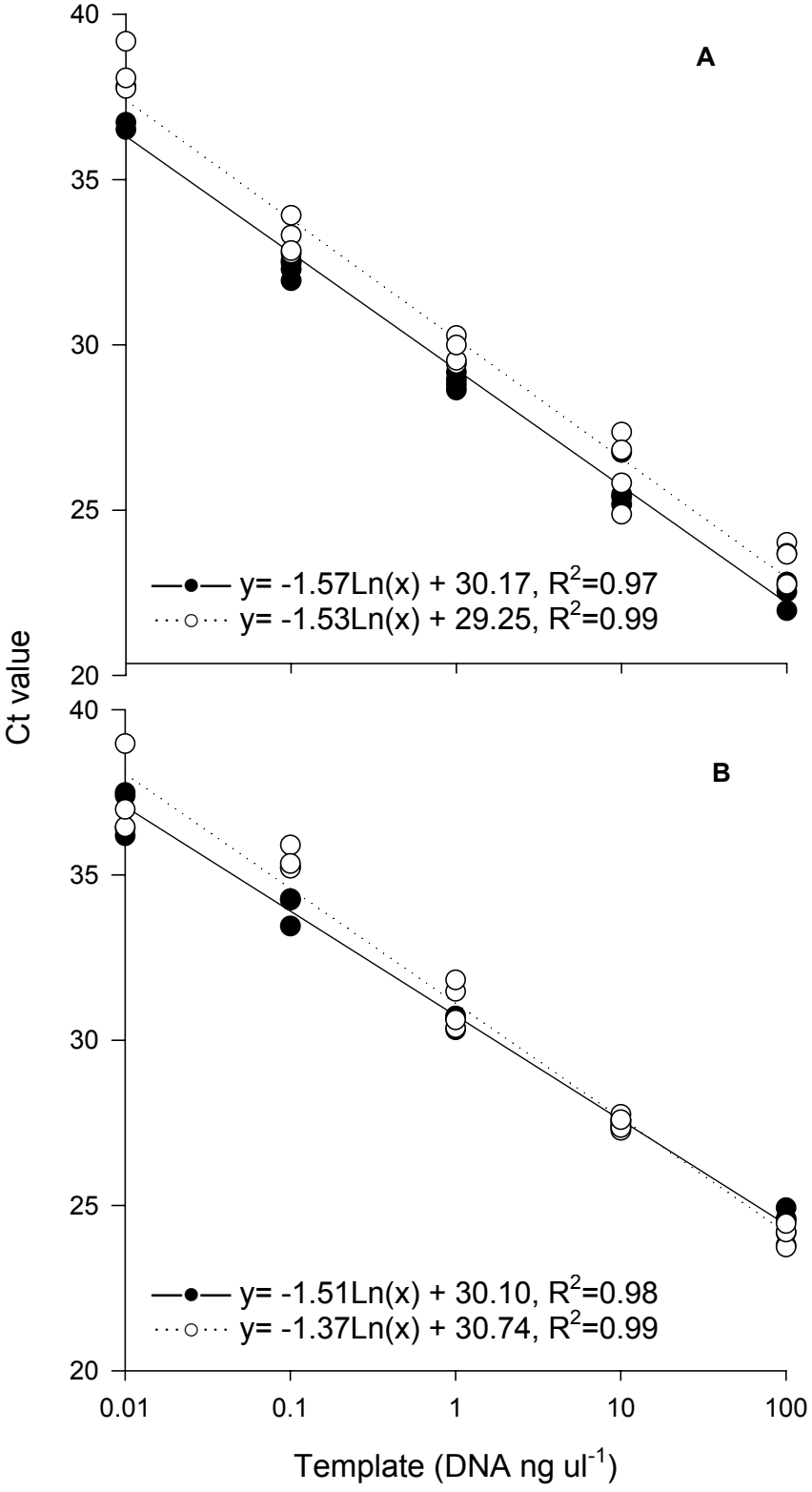

A
B
Ct value
Template (DNA ng ul-1)
y= -1.57Ln(x) + 30.17, R2=0.97
y= -1.53Ln(x) + 29.25, R2=0.99
y= -1.51Ln(x) + 30.10, R2=0.98
y= -1.37Ln(x) + 30.74, R2=0.99
40
35
30
25
20
0.01
0.1
1
10
100

Figure 10 Standard curves for detection of *Botrytis allii* mycelia using the real-time PCR assay for the *L45-550* region of the *B. allii* genome. —●— *B allii* mycelia, ··· ○··· *B allii* mycelia with with 5 mg lyophilized onion tissue in each sample. Each mycelial weight was replicated four times with and without onion tissue. In addition, a positive detection threshold of 30 fluorescence units was used for both curves.

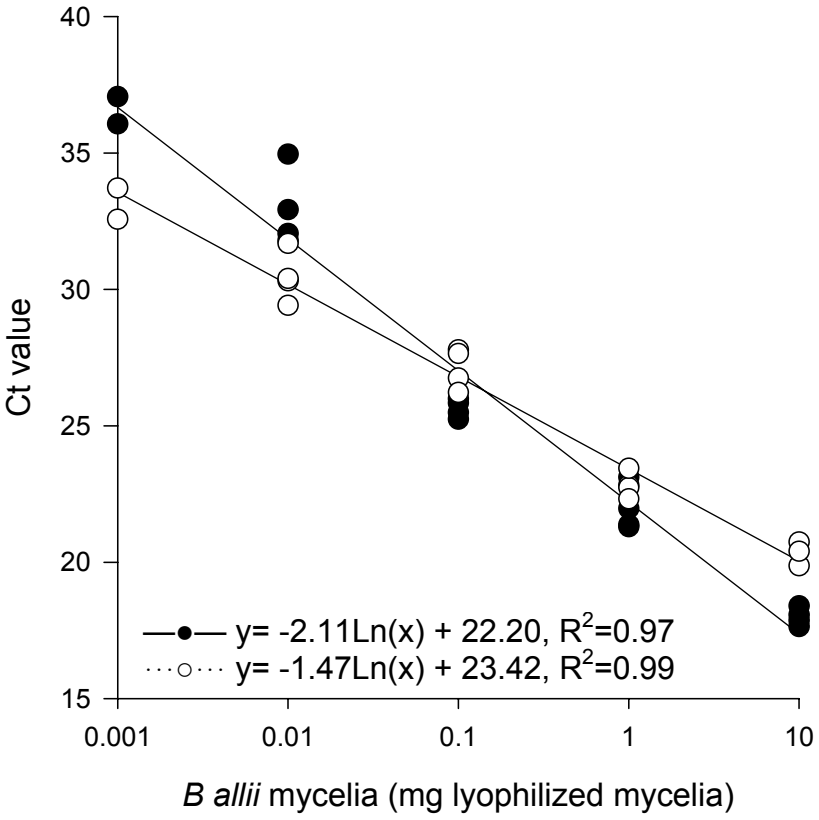

40
35
30
25
20
15
Ct value
0.001
0.01
0.1
1
10
y= -2.11Ln(x) + 22.20, R2=0.97
y= -1.47Ln(x) + 23.42, R2=0.99
B allii mycelia (mg lyophilized mycelia)

Figure 11 (A&B) A**:** Average level of *Botrytis allii* mycelial weight with standard error bars in onion disks stab inoculated with *B. allii* conidia. Each bar represents three replicates. B The average amount of onion DNA present in onion disks inoculated with conidia.

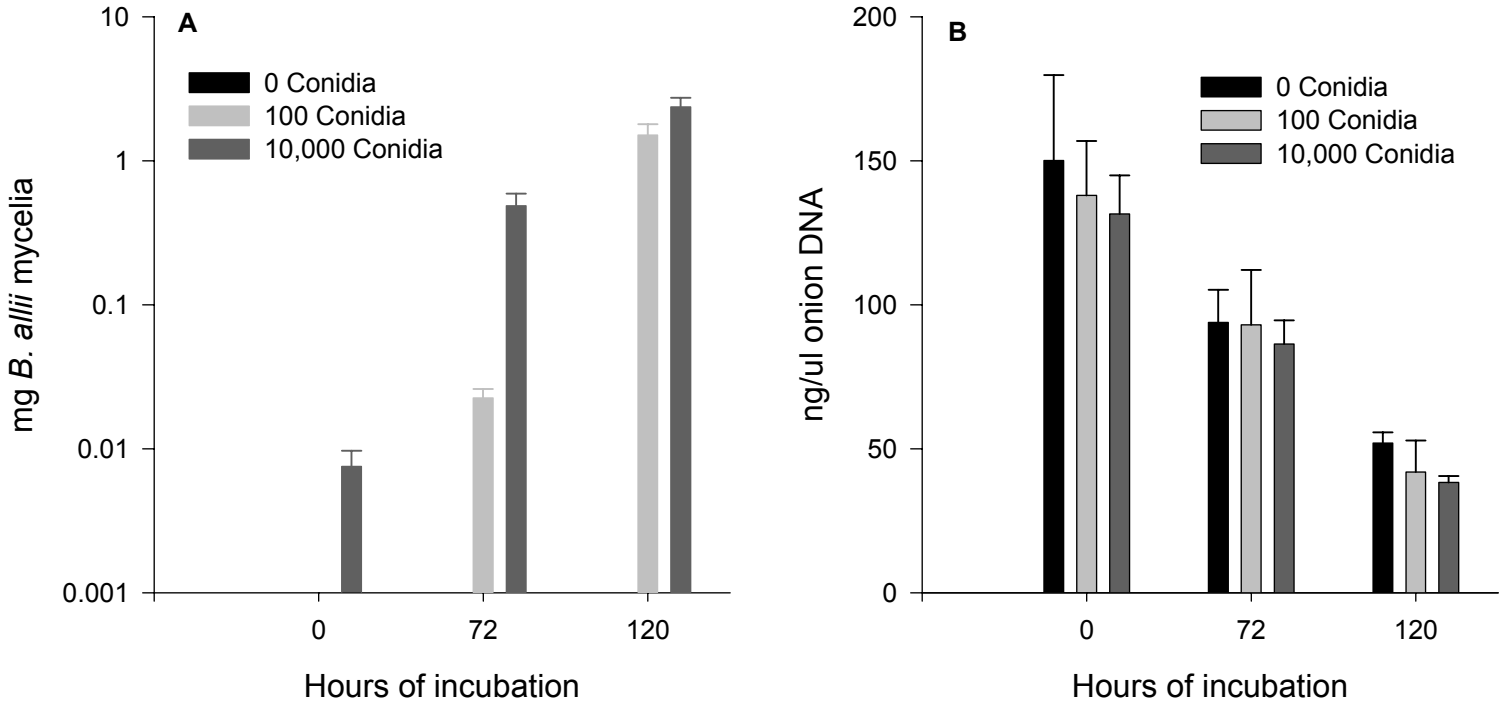
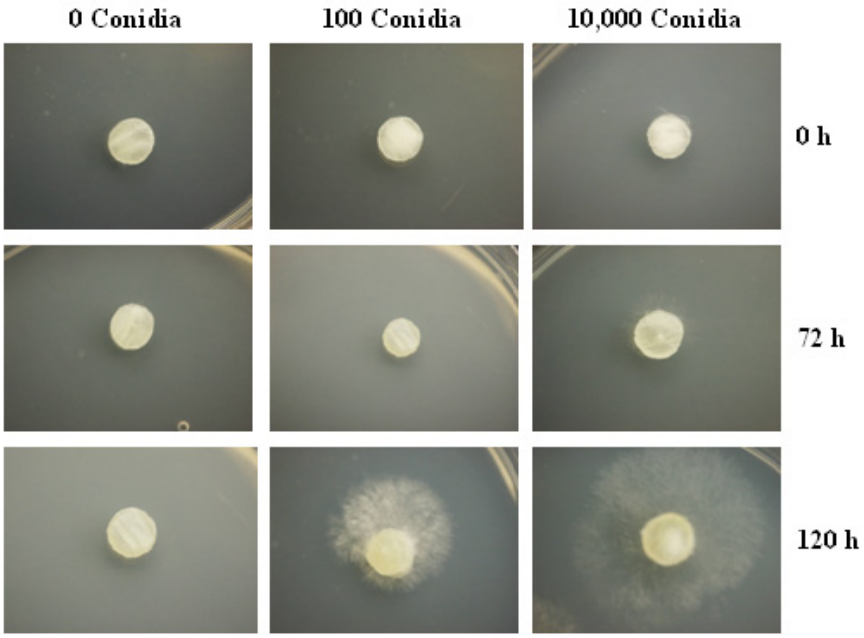

A
0 Conidia
100 Conidia
10,000 Conidia
mg B. allii mycelia
10
1
0.1
0.01
0.001
0
72
120
Hours of incubation
B
0 Conidia
100 Conidia
10,000 Conidia
ng/ul onion DNA
200
150
100
50
0
0
72
120
Hours of incubation

Figure 12 Photos of 7 mm onion (*Allium cepa* L) disks stab inoculated with 0, 100, and 10,000 conidia from *Botrytis allii* and incubated on plates of potato dextrose agar for 0, 72, and 120 hours.


0 Conidia
100 Conidia
10,000 Conidia
0 h
72 h
120 h

CHAPTER 6

CONCLUSIONS

The study discussed in Chapter 3 of this document investigated the effects of supplemental calcium chloride combined with different levels of ammonium sulfate on storage parameters in onion. Preliminary data collected had suggested that calcium chloride applications would both increase firmness and reduce storage rot in onion. Because sulfur nutrition is an important issue for sweet onion growers we felt that it would be appropriate to not only determine the effects of calcium chloride, but to investigate whether there was an interaction between calcium chloride and sulfur fertility, supplied as ammonium sulfate. There was no interaction, and as expected, increases levels of ammonium sulfate fertility lead to more intensely flavored bulbs. Additionally, our results provided insight into improving onion storability. Calcium chloride improved firmness in bulbs at harvest. However, we did not expect to see the large decrease in firmness that occurred in bulbs during storage. Furthermore, in an attempt to determine the mechanism by which calcium chloride affects firmness, we measured the pectin composition of bulbs. Interestingly we found that there were significant changes in pectins during onion storage. This had not previously been reported in onion. Although unexpected, these results lead us to investigate the role of pectin, cellulose, polygalacturonase (PGA) and pectin methylesterase (PME) in onion storability.

Due to results obtained after the first year of the field study I chose to investigate the role of pectin and pectinases in onion storage. By growing three onion varieties with different firmness attributes I was able to determine what factors appear to influence firmness in bulbs at harvest and during storage. Because this was the first attempt to investigate changes in pectin, cellulose, PGA and PME in onion at storage there was very little data available to give insight into what type of results to expect. Therefore I chose to measure non-structural carbohydrates, dormancy, respiration, soluble invertase and 1-fructan exohydrolase to guage the current study against previous work as well as to investigate carbon status in the bulbs. Previously, total pectin content of bulbs was found to decrease during storage and I wanted to determine if this was due to a demand for carbon in the bulb or was caused by another factor.

The results obtained in the second study have allowed us to draw some conclusions regarding bulb firmness. The most important result from this study seems to be that bulb firmness is related to the amount of pectin and cellulose present in bulbs. Although only three varieties were tested the concentration of pectin and cellulose on a fresh weight basis was tightly correlated to firmness readings. Because there were large differences in the percentage dry matter in bulbs, this large difference would not have been observed if data was presented on a dry weight basis. However, firmness is measured while onion tissue is fresh and therefore we felt that this was the most appropriate measure to use. In addition, transmission electron micrographs (TEM) confirmed our results.

In addition to being able to relate pectin and cellulose concentrations to firmness in bulbs we were also able to observe changes that occur during storage using the TEM.

The most obvious changes were that the middle lamella regions of cell appeared to weaken causing adjacent cells to separate. This could explain the mechanism by which firmness in bulbs decreases in storage. Other measurements such as respiration and non-structural carbohydrate concentrations were as expected and served to validate our results. Although we associated firmness with storability in onion in the previous studies,onion storage poten storability is much more than maintaining firm bulbs. In fact, the most important determinant of storability in many crops, including onions, is disease resistance. In all of our studies we observed significant disease losses in storage. In most cases the causal agent was neck rot caused by *Botrytis allii*. Neck rot is ubiquitous in the onion industry and there are very few treatments available to growers. Although high temperature curing at harvest is effective many growers choose not to cure their bulbs. In addition, onion bacterial diseases such as center rot (*Pantoea ananatis*) and sour skin (*Burkholderia cepacia*) thrive at high temperatures and can cause problems in cured bulbs. Therefore we felt that a new way to manage neck rot in storage was necessary.

In Chapter 5 a method for the detection and quantification of *B. allii* in onion tissue is described. In successfully developing a reliable method by which *B. allii* inoculum can be estimated in bulbs at harvest we may be able to determine which bulbs have the highest risk of rotting in storage and sell those first, while storing the lowest risk bulbs. This method would be an excellent management tool that is environmentally sound and could be used by organic growers. The first stage of developing a neck rot management system for onion storage using real time PCR is completed. The method is also the first to relate real time PCR signal to mycelial weight of a pathogen. Although the latter stages of the study can only be completed if funds are obtained, a strong

foundation has been built. As I mentioned previously, the ability to store any fruit or vegetable for long periods of time is affected by a number of factors. Disease resistance, changes in texture, weight and water loss, flavor alterations and color changes all contribute to the suitability of a particular crop to store for long periods. Onions are naturally suited for storage because they themselves are a storage organ that is in a state of dormancy. However, onions grown for the sweet onion market, store poorly. Although the use of controlled atmosphere storage extends the season for many growers, it is expensive and reduces the profit margin for bulb sold from storage. In addition, controlled atmosphere storage is not available for most of the farmers in the world. The purpose of these studies was to find ways to improve the postharvest life of onion and to determine what factors would be most important in onion decay during storage. Ultimately breeding will lead to the largest improvements in the storage ability of onions. In the future some of the data collected from these studies may help breeders in determining what traits are important for improving onion postharvest quality. In the immediate future, some of the cultural and management strategies investigated here, such as calcium chloride and assaying for neck rot may help improve the postharvest quality of short-day onion.