TEMPERATURE, NITROGEN AND SULFUR FERTILITY INFLUENCE THE FLAVOR PATHWAY IN ONION (ALLIUM CEPA L.)

by

TIMOTHY WAYNE COOLONG

(Under the Direction of William M. Randle)

ABSTRACT

The effects of temperature and developmental age, nitrogen fertility, and nitrogen and sulfur fertility interactions on onion flavor were tested. Temperature and developmental age effects on onion flavor were tested by growing onions between 16.5 and 32.2 ^{o}C for 50 days and until maturity. Bulbs responded to increasing in temperature with increases in total bulb sulfur, pungency, and flavor precursors.

The effects of nitrogen fertility were also studied. Total bulb sulfur, pungency and flavor precursors responded quadratically to nitrogen level, increasing and then decreasing as nitrogen levels increased.

In a third study, nitrogen and sulfur nutrition were shown to interact, affecting the flavor pathway. At low levels nitrogen and sulfur interacted to affect most flavor parameters. Additionally, nitrogen and sulfur fertility were shown to influence the accumulation of different precursors, suggesting that the two nutrients regulate flavor precursor accumulation at different points within the flavor pathway.

INDEX WORDS: alkenyl cysteine sulfoxide, methyl cysteine sulfoxide, 1-propenyl cysteine sulfoxide, propyl cysteine sulfoxide, sulfate, pyruvic acid

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DEDICATION

I would like to dedicate this work to my fiancé YunYong. She has always taken the time, even when she had little to spare. For the constant support and advice she has given me, and her "willingness" to listen to my discussions about my research I would like to thank her.

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Soon after starting my graduate program I realized how little I actually knew, and how much I would need to know in order to succeed. I would like to sincerely thank my major professor Dr. William Randle, for the constant advice and attention he has given me, and for his wealth of knowledge that he has freely shared on a daily basis. I would also like to thank my committee members, Dr. Stanley Kays and Dr. David Kissel for taking the time to make contributions to my work.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Onions (*Allium cepa* L.) are valued primarily for their ability to infuse flavor into the diet. Flavor development in onion occurs when intact tissue is crushed and vacuoles are lysed, releasing the enzyme alliinase. Alliinase hydrolyzes the *S*-alk(en)yl cysteine sulfoxides (ACSO) that are found in the cytoplasm of the bulb (Lancaster and Boland, 1990). The three naturally occurring ACSOs that have been isolated in bulb onions are *S*-methyl-L-cysteine sulfoxide (MCSO), *trans-(+)-S*-(1-propenyl)-L-cysteine sulfoxide (1-PRENCSO), and (+)-*S*-propyl-L-cysteine sulfoxide (PCSO). The hydrolysis reaction of the ACSOs yields pyruvic acid, ammonia, and a number of sulfenic acids (Block, 1992). The sulfenic acids immediately condense to produce a number of thiosulfinates, the true flavor compounds of onion.

Because flavor is essential in determining the value of an onion, researchers and growers have been interested in determining those factors, which influence flavor development in onion. Several environmental factors have been identified, which can alter onion flavor. Sulfur (S) nutrition has been reported to strongly influence flavor in onion (Freeman and Mossadeghi, 1970; Randle et al., 1995). High levels of nitrogen (N) availability, growing temperature and irrigation status have also been reported to alter onion flavor (Freeman and Mossadeghi, 1973, Platenius and Knott, 1936; Randle, 2000; Yamaguchi et al., 1975).

The scope of some of the above-mentioned investigations is limited, however. Randle (2000) demonstrated that flavor quality and intensity were affected by N level in solution culture; however, the N levels imposed on the plants were very high, and thus nothing is known concerning the effects of lower N levels flavor. Furthermore, there have been several reports of N nutrition influencing S metabolism in plants, thus presenting questions of how N availability would affect the biosynthesis of the S-containing flavor compounds in onion (Brunold and Suter, 1984; Koprivova et al., 2000; Takahashi et al., 1996). Platenius and Knott (1936) demonstrated that growing temperature influenced the production of S volatiles in onion, but the results were confounded by developmental stage. Additionally, newer, more representative, analytical methods may yield different results (Platenius, 1935). Yamaguchi et al., (1975) reported that soil temperature influenced gross flavor intensity, but was not concerned with flavor quality parameters or S metabolism in the plant. Thus, there is still much work that can be done to gain a better understanding as to the influence of environment on flavor in onion.

The purpose of my investigations was to determine how growing temperature, N availability, and N x S fertility interacted to influence flavor development in onion. We hoped to gain a better understanding of how these environmental factors would affect several parts of the flavor biosynthetic pathway so that in the future we can make better decisions with respect to onion production, particularly the sweet onion industry in Georgia.

CHAPTER 2

LITERATURE REVIEW

Allium History

Onions have been used as flavoring agents, religious artifacts and medicinal cures for nearly 5000 years. Though no records exist describing the domestication of onion, carvings found in Egyptian tombs dating to 3200 B.C. depict the consumption of onions (Hanelt, 1990). Several varieties of onion were described by Theophrastas, (322 B.C.), with each variety being named for the region where they were grown (Fenwick and Hanley, 1985). Carbonized remains of onions and garlic dating to 79 A.D. have been found in Pompeii, and by the Middle Ages onion had become a widespread crop over much of Western Europe and England (Hanelt, 1990). In the 16th century onion had become so widespread that red, white, bluish, long, flat, round, sweet and pungent types had all been recorded (Fenwick and Hanley, 1985). Onions were also one of the first cultivated plants brought to the New World. Planted in the gardens of The Isabella Islands by Columbus in 1494, onions were being cultivated in Massachusetts by the early 17th century (Fenwick and Hanley, 1985).

The impact of the onion on some cultures is evident in architecture as well. Onion domes first appeared on Russian churches during the 11th century and are still used today in church architecture. Built in the 17th century, the Taj Mahal in India is also adorned with onion domes.

Onion and other *Alliums* have held religious significance in many cultures for nearly as long as they have been cultivated. Onions were used as religious offerings in Ancient Egypt, and often accompanied mummies in their tombs. Conversly, for those living in India in the first century A.D., onions were thought to be evil, and were not to be eaten by anyone who was pursuing a spiritual life (Fenwick and Hanley, 1985). The use of *Alliums* is also noted in several places in the Bible.

Alliums, particularly onion and garlic have also been used medicinally since antiquity. In 79 A.D. Pliny listed 61 garlic and 28 onion based remedies that were said to be effective against maladies ranging from hemorrhoids to rheumatism. Onion juice was reported to be effective against dog bites, and when applied to the eye was thought to improve vision (Fenwick and Hanley, 1985). A concoction of crushed garlic and wine, called Four Thieves' Vinegar, was apparently used by four condemned thieves who had been ordered to bury the dead during a terrible plague in Marseilles, France in 1721. Despite their constant exposure to disease, none of the thieves ever fell ill. Their immunity was attributed to their consumption of the "garlic wine." In fact one can still purchase the brew today in Marseilles, France under the name *vinaigre des quatre voleurs* (Block, 1986).

During the American Civil War, General Grant would not move his army until he received a supply of onions, believing they were necessary to avoid widespread epidemics of dysentery among his troops (Peirce, 1987). Louis Pasteur reported antibacterial effects of garlic in 1858, and garlic based antibiotics are still used today in many parts of Russia and Japan (Block, 1986). Recent research has focused on the use of

onion and garlic as antithrombic agents, as well as the use of *Alliums* to combat colon cancer (Dorsch, 1997; Ernst, 1998).

The Origin 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, and onion appears to have originated from a region of the world that is considered present day Turkey and Afghanistan. This geographical area marks the primary center of diversity among the genus, responsible 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 generally 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 one chromosome nucleus, making it roughly 107 times the size of *Arabidopsis thaliana* (Arumuganathan and Earle, 1991; Havey, 2002).

The Botany of Onion

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 is adaxial surface (DeMason, 1990). Each succeeding leaf increases in size, until bulbing is initiated. During bulbing, newly formed leaves become smaller until no blade is 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 between five and ten ^{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 if favorable conditions present themselves (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" (SD), "intermediate-day" (ID), or "long-day" (LD) 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 LD 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 which 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).

Onion Storage

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. Onions are typically cured at high temperatures immediately prior to storage in order to suppress disease, and to dry the outer scales of the bulb, preventing rapid dessication during storage (Rubatzky and Yamaguchi, 1997). Recommended curing times and temperatures for commercial Granex onions are 48-96 hours at 32.2-35^{o}C.

Storage temperature is critical in maintaining bulb dormancy. Bulbs stored at the incorrect temperature will quickly vernalize and sprout. Recommended storage conditions for onion bulbs are near 0^{o}C and 65-75% relative humidity (Peirce, 1987).

Nutritional Composition of Onion

Onions are considered very poor nutritionally. 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).

Most 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. Sugar content can vary with cultivar, location within the bulb and growing conditions (Breu 1996; Darbyshire, 1978).

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, querciting-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 outside and top of the bulb.

Sulfur Metabolism

Onions and other *Alliums* have been valued for their flavoring attributes for more than 5000 years. Therefore, it should be of no surprise that the chemistry of *Allium* flavor has been the subject of investigation since the 19th century, when several sulfur (S) containing compounds were isolated from garlic (Block, 1992). However, it was not until the mid to late 20th century when the true complexity of *Allium* chemistry was fully realized. The characteristic flavor of onion and other *Alliums* is due to the presence of a large number of organosulfur compounds, which can contribute up to five percent of the dry-weight in some *Allium* species (Block, 1992). Because of the integration of large amounts of S into *Allium* flavor compounds, an understanding of S assimilation is useful for those invesigating *Allium* flavor chemistry. Therefore I have chosen to review S metabolism in plants in conjunction with my discussion of *Allium* flavor chemistry. Methionine, an S containing amino acid, is not thought to be directly involved in S metabolism leading up to the production of the flavor compounds in *Allium*, and will not be discussed.

The primary source of S for plant utilization is sulfate (SO_4^{2-}) in the soil. Although plants may take up S via other means, such as sulfur dioxide and hydrogen sulfide absorption through leaves, the overwhelming majority of S taken up by the plant is as SO_4^{2-} at the roots (Marschner, 1995). Sulfate appears to be actively transported into roots by a membrane localized SO_4^{2-} transporter. The transporter is driven by an electrochemical gradient that is established by a plasma membrane protein adenosine tri-phosphatase (ATPase) (Leustek et al., 2000). Sulfate uptake at the roots has been reported to be regulated by internal sulfate concentrations (Anderson, 1990), as well as by cysteine (Cys) concentrations (Datko and Mudd, 1984). Sulfate transport into the roots displays complex uptake kinetics, however; and much has yet to be resolved.

Upon uptake by the roots, SO_4^{2-} is "loaded" into the xylem and transported to the leaves. Once in the leaves, SO_4^{2-} is "unloaded" from the xylem and transported into photosynthesizing cells. Transport across the plasma membrane was once considered to occur via a single, low affinity SO_4^{2-} transporter that could be inhibited by group IV anions such as selenate, arsenate and chromate because they compete with SO_4^{2-} for binding (Leustak, 1996). The current view of SO_4^{2-} transport across the plasma membrane into cells, is that there are at least three sulfate permeases that act as H^+/ SO_4^{2-} cotransporters to move SO_4^{2-} across the cell membrane (Takahashi et al., 1997; Takahashi et al., 2000). Seven genes have been identified in *A. thaliana* that code for these permeases (Saito, 2000). It has been reported that the activity of these permeases can be regulated by the S levels in the cell. Sac1, an integral membrane protein, can sense the S status of a cell, and in response to a deficiency, initiate a signal cascade system that up-regulates SO_4^{2-} transport into the cell (Leustek et al., 2000). In addition to increasing S

uptake, signals are sent to the chloroplast causing a reduction in photosynthesis, until S concentrations in the cell return to sufficient levels.

Once in the cell, SO_4^{2-} must be transported into chloroplasts, as virtually all of the reduction of SO_4^{2-} to sulfide (S^{2-}) occurs here (Saito, 2000). There are currently two hypotheses concerning the nature of the plastid transporter. The first theory is that there is a SO_4^{2-} transporter in the plastid membrane that is analogous to those found in the plasma membrane of the plant cells (Leustek et al., 2000). The second theory is that there is an integral membrane ATP dependent sulfate/thiosulfinate transporter complex that is entirely different in structure from the plasma membrane SO_4^{2-} transporters (Leustek et al., 2000). This transporter has not yet been identified in vascular plants. Additionally, SO_4^{2-} does not have to enter the chloroplast immediately; instead it can be stored in the vacuole until needed.

Little is known about the transport of SO_4^{2-} through the tonoplast (Mornet et al., 1997). The partitioning of SO_4^{2-} in the vacuole and its relation to reduced S, however, has been the subject of some investigations. Randle et al., (1999) demonstrated that the relative percentage of total S in onion that resided as SO_4^{2-} increased as S fertility increased. It has also been reported that withdrawing S nutrition from oilseed rape (*Brassica rapa*) results in a rapid decline in the amount of S in the leaves that is partitioned as SO_4^{2-}. This indicates that SO_4^{2-} may be remobilized in times of S starvation (Blake-Kalff et al., 1998).

Once in the chloroplast, SO_4^{2-} is reduced to S^{2-} in a series of steps that require eight electrons and roughly twice the energy that is needed for nitrate reduction (Fig 1.) (Hell, 1997). Sulfate enters the plastid where it is activated by ATP sulfurylase to form

adenosine 5'-phosphosulfate (APS) from an ATP molecule. Because APS is a potent competitive inhibitor of ATP sulfurylase, ATP sulfurylase could be a pathway regulating rate-limiting enzyme (Leustek, 1996).

The SO_4^{2-} reduction pathway splits with the formation of APS. Upon synthesis, APS can be acted on by APS kinase, which adds a phosphate group from ATP to form 3'-phospho-5'-phosphadenylylsulfate (PAPS) (Leustek et al., 2000). There are a number of sulfotransferases, which use PAPS as a sulfuryl donor (Varin et al., 1997). The PAPS pathway appears to be a branch point, at which, S can be used to sulfonate a variety of S-containing compounds, including the glucosinolates and brassinosteroids (Leustek et al., 2000).

If APS continues down the reduction pathway and does not form PAPS, then it will be reduced in a reaction catalyzed by APS reductase. This enzyme uses two electrons from glutathione (GSH) to reduce APS to a sulfite (SO_3^{2-}) molecule (Bick and Leustek, 1998). Sulfite reductase then takes six electrons from a ferredoxin molecule to reduce SO_3^{2-} further to S^{2-} (Saito, 2000). Now that SO_4^{2-} has been reduced to S^{2-}, it can be utilized to synthesize Cys. Interestingly, while the reduction of SO_4^{2-} to S^{2-} takes place almost entirely in the chloroplast, the incorporation of S^{2-} into Cys can occur in the chloroplast, cytoplasm or mitochondria (Leustek, 1996).

Cysteine is formed in a reaction in which S^{2-} reacts with *O*-acetylserine (OAS) to form Cys and acetate. The reaction is catylized by OAS thiol-lyase. The formation of OAS is catylized by serine acetyltransferase (SAT) and is the result of a reaction between serine and acetyl CoA (Leustek et al., 2000). There is also an OAS thiol-lyase-SAT complex involved in Cys synthesis. However, the OAS thiol-lyase-SAT complex is

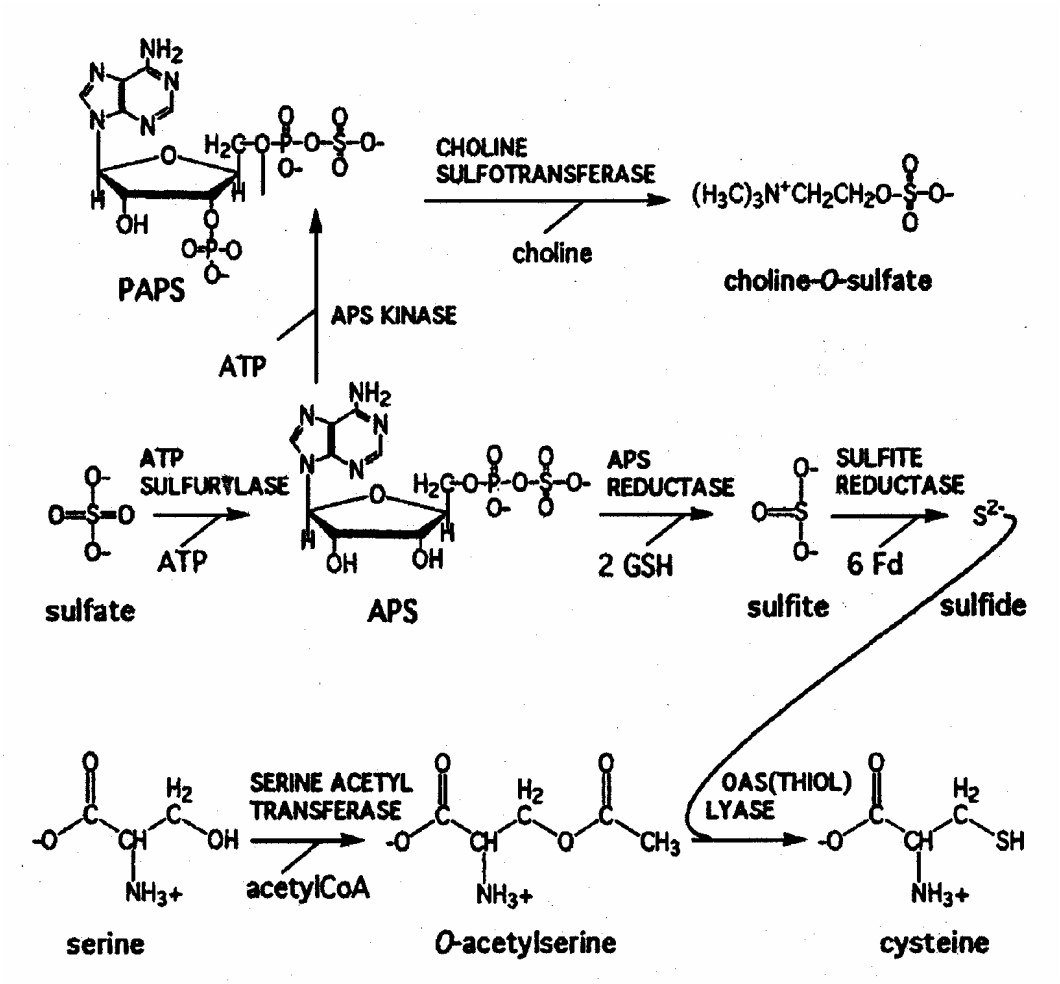

Figure 1. An overview of the proposed pathway of SO_4^{2-} reduction and S^{2-} incorporation into cysteine (Leustek et al., 2000).

much less efficient in synthesizing Cys than free OAS thiol-lyase (Droux et al., 1998). Furthermore, the ratio of OAS thiol-lyase to SAT in the chloroplast is roughly 300:1. Therefore, the OAS thiol-lyase in the free form is thought to be responsible for most Cys synthesis (Leustek et al., 2000).

Regulation of Cys formation as well as SO_4^{2-} transport is thought to be partially controlled by the OAS thiol-lyase-SAT complex, which has been found to be up-regulated by the presence of S^{2-}, and down-regulated by Cys and glutathione (GSH) (Leustek et al., 2000). Recently Noji et al. (1998) demonstrated that there were three distinct SATs, for the cytosol, mitochondria and chloroplast. Interestingly, only the cytosol SAT was affected by feedback inhibition of Cys. This indicates that cytosolic Cys concentrations are especially important in regulating Cys synthesis. Recently calcium (Ca^{2+}) ions have also been shown to be involved in the inhibition of Cys synthesis. When plants were subjected to S starvation, Ca^{2+} levels rose, and this increase in Ca^{2+} seemed to densensitize SAT to the effects of cytoplasmic Cys, thus decreasing the level of inhibition by Cys when the plant was stressed for S (Saito, 2000).

Nitrate (NO_3^-) assimilation has also been reported to influence SO_4^{2-} reduction. Because all amino acids contain N as an amine group, N levels in the plant will affect amino acid production. When N levels fall, the pool of available serine, which is used in the synthesis of Cys, will decrease. A decrease in serine will ultimately influence Cys formation and the prerequisite reduction of SO_4^{2-} in the plant. It has been reported that high NO_3^- to SO_4^{2-} ratios led to increased levels of OAS in transgenic *A. thaliana*, but low NO_3^- to SO_4^{2-} ratios decreased OAS levels, indicating that N and S fertility are important in regulating Cys production (Kim et al., 1999). Additionally, activity of sulfite

reductase, the enzyme catalyzing the reduction of sulfite to S^{2-}, has been reported to be depressed by high levels of NO_3^- availability in leek (*Allium tuberosum*) seedlings, especially when SO_4^{2-} was limited (Takahashi et al., 1996). When *A. thaliana* plants were N starved for 72 hours, the activity of APS reductase was reduced up to 70% of the control group (Koprivova et al, 2000). APS reductase messenger RNA levels were also reduced by N starvation, suggesting that N fertility influences S assimilation by down-regulating transcription of genes coding for APS reductase. Brunold and Suter (1984) reported that omitting NO_3^- for 24 hours from nutrient solutions of the duckweed (*Lemna minor*) resulted in a subsequent decrease in ATP sulfurylase and APS reductase, as well as a decrease in extractable protein. Migge et al., (2000) reported that NO_3^- reductase genes could be down-regulated in S starved tobacco (*Nicotiana tabacum* L.) further suggesting that N and S metabolism in the plant are intimately related.

Although Cys is an intermediate in the formation of a number of essential S compounds in the plant, levels of cellular Cys are quite low, often less than 10 μM. This is because and the turnover rate (flux) is very high (Giovanelli et al., 1980). Free Cys is quickly employed in the synthesis of methionine, proteins, and GSH. Glutathione, a tripeptide consisting of Cys, glycine (Gly), and glutamine (Glu) residues, is incredibly important in that it seems to be the primary sink for reduced S in the plant. Sekiya et al., (1982) reported that up to 74% of the reduced S in the cells of cucumber leaves was as GSH. Glutathione is also a key intermediate in the production of the organosulfur flavor compounds in *Allium* species.

Synthesis of GSH from Cys is a two-step process that can occur in the cytosol or the chloroplast (Leustek et al., 2000). When Cys is produced, it can quickly combine

with Glu in an ATP dependent reaction catalyzed by gamma (γ) glutamylcysteine synthetase to form the dipeptide γ-glutamyl-L-cysteine (Rennenberg, 1982). A Gly residue is then added to the C-terminal end of γ-glutamyl-L-cysteine to form GSH in another ATP dependent reaction. This reaction is catylized by glutathione synthetase (Bergmann and Rennenberg, 1993). Glutathione is produced in the leaves and is then translocated throughout the entire plant, and appears to be the primary transport form of reduced S in the plant (Rennenberg, 1982). As a stable form of reduced S in the plant, GSH can be used in the production of a number of organosulfur compounds including, phytochelatins and the flavor compounds in *Allium* (Block, 1992; Rauser, 1995).

Flavor Chemistry in Onion

Twenty-four γglutamyl peptides have been isolated in *Allium* species, with 18 of them containing S (Lancaster and Boland, 1990). The link of the peptides to the generation of flavor compounds in *Allium* is still unclear. Because the γ-glutamyl peptides are not hydrolyzed by the flavor generating enzyme, alliinase, they do not directly contribute to the flavor of crushed onions. It has been proposed that the γ-glutamyl peptides function as a storage reservoir for N and S in the bulb (Randle and Lancaster, 2002).

As was discussed previously, Cys can go through a two-step reaction to form GSH in plants. The first step in the reaction yields γ-glutamyl-L-cysteine, which can either bind a Gly residue to form GSH, or in *Allium* spp. combine with methacrylic acid to form γ-glutamyl-S-2-carboxypropylcysteine. Gamma-glutamyl-S-2-carboxypropylcysteine can then undergo decarboxylation to form γ-glutamyl-*S*-1-propenylcysteine, which is then oxidized by γ-glutamyl-*S*-1propenylcysteine S-oxide to

form γ-glutamyl-*trans*-(+)-*S*-(1-propenyl)-cysteine sulfoxide (GPRENCSO) (Block 1992). As the penultimate peptide in the production of the flavor precursor, *trans*-(+)-*S*-(1-propenyl)-L-cysteine sulfoxide (1-PRENCSO), GPRENCSO is generally regarded as the most abundant γ-glutamyl peptide in onion (Carson, 1987). A γ-glutamyl transpeptidase acts on GPRENCSO to produce 1-PRENCSO (Fig 2.). Interestingly, Kopsell (1999) reported that the loss in GPRENCSO was proportional to an increase in 1-PRENCSO that occurred during long-term storage of onion bulbs. This has relevance because γ-glutamyl transpeptidase has not been found in dormant bulbs, suggesting that another enzyme may be involved in the cleavage of GPRENCSO *in vivo* (Lancaster and Boland, 1990). In onion, and several other *Allium* spp, γ-glutamyl-S-1-propenylcysteine can gain a hydrogen atom to become γ-glutamyl-S-1-propylcysteine, and then undergo an oxidation and cleavage by a transpeptidase to form the flavor precursor (+)-*S*-propyl-L-cysteine sulfoxide (PCSO) (Block, 1992).

Alternatively, a γ-glutamyl peptide pathway, in which GSH involved, has also been proposed. In *Alliums*, GSH may receive a methacrylic acid residue and form *S*-2-carboxypropylglutathione (2-Carb) (Block, 1992). 2-Carb can then lose a Gly residue to form γ-glutamyl-S-2-carboxypropylcysteine, which can then undergo a series of reactions to form 1-PRENCSO and PCSO, as was discussed above. Most interesting however, is the methylation of GSH to form *S*-methylglutathione. Upon formation, a Gly residue is cleaved from *S*-methylglutathione forming γ-glutamyl-*S*-methylcysteine, which then undergoes an oxidation and cleavage by a transpeptidase to form the flavor precursor (+)-*S*-methyl-L-cysteine sulfoxide (MCSO) (Block, 1992). The pathway leading to the formation of MCSO is unique in that it has been found to exist in all *Allium* species

analyzed thus far (Lancaster and Boland, 1990). Furthermore, it shares no γ-glutamyl peptides with the pathways leading to the production of the other precursors.

There are still questions outstanding regarding the synthesis and destruction of the γ-glutamyl peptides in *Allium*. Many peptides, including several in the MCSO pathway are found in such small quantities that they have yet to be isolated *in vivo*. Additionally, quantitative analysis of onion bulb and leaf tissue at various times of development has shown that the two major peptides, GPRENCSO and 2-Carb, were absent prior to bulbing (Lancaster and Shaw, 1991). However, the flavor precursors, which are the end products of the peptide pathways, are present prior to bulbing (Lancaster et al., 1986). This suggests that the peptides are in a state of high flux prior to bulb development, and accumulate only during bulb maturation. Furthermore, it has been reported that the activity of γ-glutamyl transpeptidase in sprouted *Allium sativum* bulbs increased when compared to dormant bulbs (Ceci et al., 1992). This strengthens the argument that the γ-glutamyl peptides are a reserve for N and S, to be used when the bulb sprouts in the second year of growth.

When onion tissue is macerated, the enzyme alliinase is released from the vacuole. This causes the hydrolysis of the *S*-alk(en)yl cysteine sulfoxide (ACSO) flavor precursors, which gives rise to the characterisic flavor and aroma of onion. Three ACSOs have been reported to exist in bulb onion (Block, 1992; Lancaster and Boland 1990; Randle 1997). They are (+)-*S*-methyl-L- cysteine sulfoxide (MCSO), *trans*-(+)-*S*-(1-propenyl)-L-cysteine sulfoxide (1-PRENCSO) and (+)-*S*-propyl-L-cysteine sulfoxide (PCSO), and as was discussed in the preceding section, they are synthesized from the γ-glutamyl peptides (Randle and Lancaster, 2002).

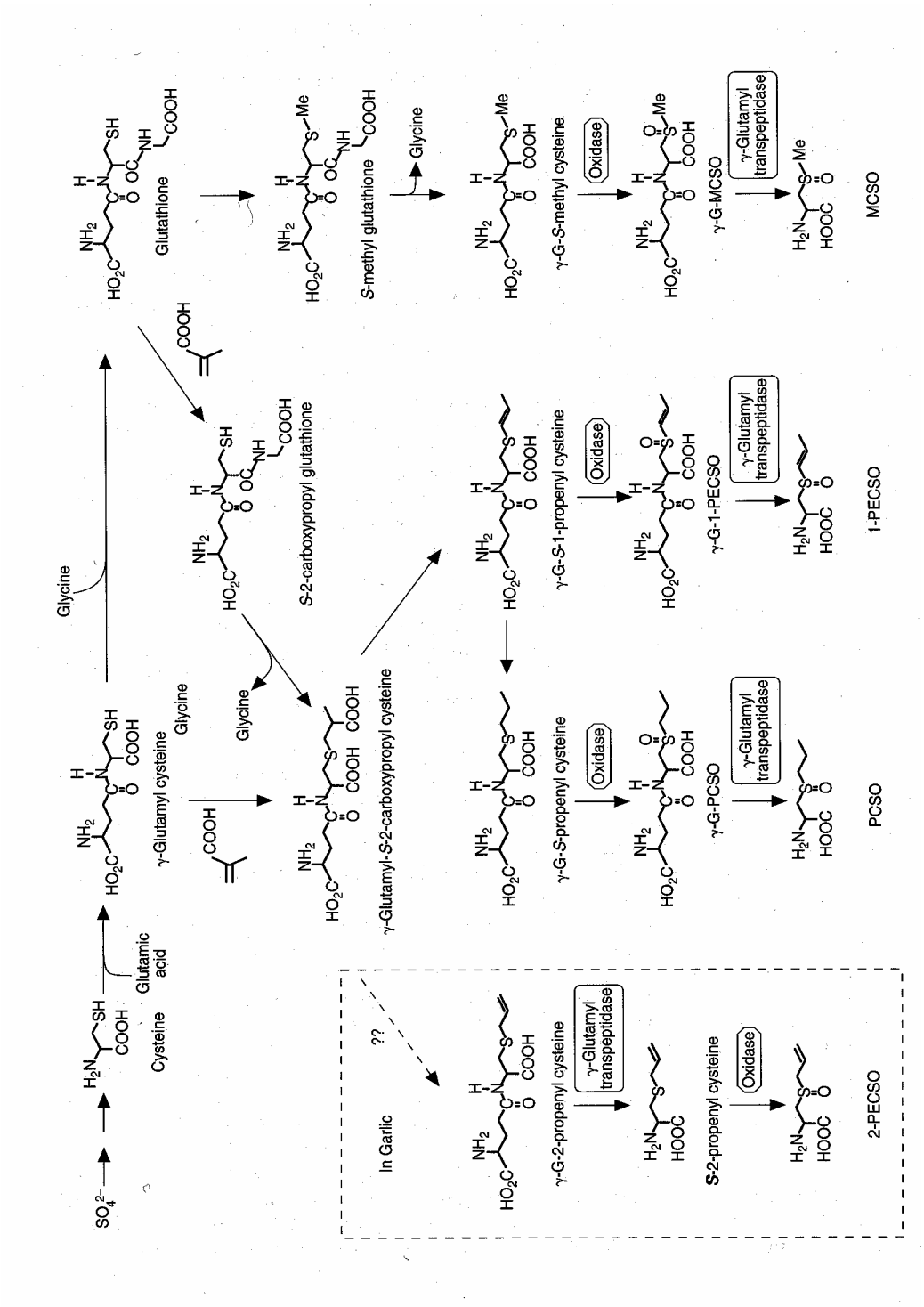

Figure 2. A schematic of the biosynthetic flavor pathway in *Allium* species (Randle and Lancaster, 2002)

Upon hydrolysis, each ACSO contributes to the formation of various thiosulfinates, each of which imparts a different flavor sensation. MCSO tends to lend a cabbage-like and fresh onion sensation, while 1-PRENCSO is responsible for the tearing and mouthburn affiliated with pungent onions. Chive and fresh green onion flavors have been associated with the hydrolysis of PCSO (Randle et al., 1994). ACSOs are produced in onion leaves throughout plant development, and are translocated to the bulb during maturation and bulb development (Lancaster et al., 1986). It has been reported that the 1-PRENCSO is located in the endoplasmic reticulum of onion cells, where it is physically separated from the enzyme alliinase, which resides in the vacuole (Lancaster and Collin, 1981). The actual flavor reaction in onion occurs when vacuoles are lysed and the ACSOs interact with alliinase.

Allinase

The officially accepted name for the flavor enzyme, alliinase, is alliin alkyl-sulphate-lyase. It is also known as *S*-alk(en)yl-L-cysteine lyase, alliin lyase and cysteine sulfoxide lyase (Randle and Lancaster, 2002). A major protein in *Allium* species, it can account for up to 6% of the total soluble protein in onion bulbs (Nock and Mazelis, 1987). Alliinase has been reported to exist as a trimer or tetramer, with molecular masses of 53.3 and 51.6 kDa for the two isoform subunits (Clark et al., 1998). Alliinase-like enzymes have been isolated in nearly all members of *Allium*, as well as *Albizia, Acacia,* and several *Brassica* spp. (Kopsell, 1999). Alliinase is found in leaf, root, bulb and shoot tissue of *Allium* species and is localized within vacuoles (Lancaster and Boland, 1990; Lancaster et al., 2000). When released from the vacuole, it 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 (Randle and Lancaster, 2002). Thus, the products of the hydrolysis reaction are pyruvate, ammonia, and a sulfenic acid. The sulfenic acids immediately condense with each other to form a number of thiosulfinates, which are the flavor compounds that one perceives when eating an onion (Block, 1992).

Historically, those investigating onion flavor have used enzymatically produced pyruvate (EPY) as a measure of onion pungency. Because EPY is produced by the hydrolysis of the ACSOs, the overall flavor intensity (pungency) of the onion can be measured. Schwimmer and Weston (1961) initially developed a method for using EPY to measure pungency, which was later refined for batch processing by Randle and Bussard (1993a). Wall and Corgan (1992) reported a high (0.92) correlation between EPY and flavor perception for onion bulbs. Because determining EPY is quick and inexpensive it is often used as a gross indicator for determining flavor intensity in onions.

The three ACSOs found in onion undergo varying levels of hydrolysis *in vivo*. In fact, it is this difference in activity that gives various *Alliums* their unique flavors (Block, 1992). Alliinase tends to hydrolyze 1-PRENCSO more completely than MCSO and PCSO in onion. Lancaster et al., (1998) demonstrated that upon maceration, nearly all of the intact 1-PRENCSO measured in bulbs had been hydrolyzed, whereas a maximum of 30% and 40% of MCSO and PCSO respectively had been hydrolyzed. This suggests that 1-PRENCSO in particular has a greater impact than the other ACSOs on the final flavor of onion. Because 1-PRENCSO is hydrolyzed to a much greater extent than MCSO and PCSO, propenyl-sulfenic acids and corresponding thiosulfinates tend to dominate the

flavor profile. Among the thiosulfinates formed, is propanethiol *S*-oxide, the lachrymatory factor (LF) of onion (Block, 1992). The LF is highly volatile, and most will be lost from solution within 20 seconds of formation. Once volatilized, the LF will bind to nerve cells, triggering lachrymation in humans (Randle and Lancaster, 2002). Measuring the LF is very difficult due to the unstable nature of the compound. Kopsell et al., (2002) demonstrated that the LF must be extracted from onion juice within ten seconds, otherwise, much will be lost to the atmosphere.

In addition to the thiosulfinates, a number of other S containing compounds are formed from the hydrolysis of the ACSOs. These include capaenes, which are a relatively new class of organosulfur compounds, the zwiebalanes and a number of mono, di, tri-sulfides (Bayer et al., 1989; Breu 1996)

Environment and Onion Flavor

Although onion flavor potential is ultimately determined by gentics, growing environment has been shown to have a pronounced effect on both flavor intensity and quality in onion. Investigations into onion flavor demonstrated that the same varieties of onion grown in different geographical regions can have much different flavors (Lancaster et al., 1988; McCallum et al., 2001 Platenius and Knott 1936). Furthermore, yearly fluctuations in onion pungency have also been observed (Vavrina and Smittle, 1993). Because environmental factors such as soil type, climate, and fertility vary between locations and over time, researchers have attempted to isolate those environmental factors, which influence onion flavor.

Because S is an integral part of the flavor compounds in onion, S fertility and its influence on flavor has been the subject of numerous investigations. Several groups have

reported increases in bulb pungency in response to different levels of S fertility (Freeman and Mossadeghi, 1970, Randle and Bussard 1993b). It appears, however, that onions become "saturated" when S is supplied in sufficiently high amounts. At high levels, pungency no longer responds to increases in S fertility (Hamilton et al., 1998). Randle et al., (1995) also reported changes ACSO composition within the bulb in response to different levels S fertility. This has implications for total flavor quality, as well as flavor intensity. Selenium, which is thought to compete with S for uptake by the plant, has been reported to influence bulb pungency. Kopsell and Randle (1999) demonstrated that increasing sodium selenate availability led to a decrease in onion pungency in some cultivars.

The effect of N availability on flavor quality also been investigated. Nitrogen form in a hydroponic solution was reported to influence pungency in onion (Gamiely et al., 1991). Randle (2000) reported that growing 'Granex 33' onions hydroponically with very high levels of N availability led to bulbs with MCSO as the predominate ACSO. Typically, 1-PRENCSO has been reported to be the dominant ACSO in field-grown onion bulbs, with MCSO being found in the second largest concentrations (Block, 1992; Edwards et al., 1994; Yoo and Pike, 1998). Previously, MCSO has been shown to be the principal precursor only when bulbs are grown with minimal S fertility and high levels of sodium selenate (Kopsell and Randle, 1999; Randle et al., 1995). The mechanism by which low S and high N availability act to cause an increase in the partitioning of organically bound S into the MCSO pathway has yet to be determined.

Because growers must use lime (Ca^{2+} carbonate) to increase the pH of their soil and gypsum (Ca^{2+} sulfate) as a source of S nutrition, Ca^{2+} fertility has also been

investigated for its effects on flavor. Randle (1995) demonstrated that Ca^{2+} did not influence onion pungency, except at extremely high levels of Ca^{2+}.

Flavor intensity, as measured by EPY, has been reported to increase as growing temperature increased (Randle et al., 1993). Platenius and Knott (1941) reported an increase in the production of S volatiles when plants were grown under increasing temperatures; however, bulbs were harvested and tested at different stages of development, thus confounding the results. Additionally, Yamaguchi et al., (1975) reported and increase in pungency in response to increasing soil temperatures for onions. Unfortunately little is known about the effect of growing temperature on the individual ACSOs and flavor quality.

Through careful greenhouse studies, researchers have been able to isolate those factors, which will most likely influence onion flavor, thus providing a strong information base for those involved with growing onions, as well as those interested in the unique organosulfur chemistry of the *genus Allium*.

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CHAPTER 3

TEMPERATURE INFLUENCES FLAVOR INTENSITY AND QUALITY IN 'GRANEX 33' ONION[1]

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Abstract

The effects of temperature and developmental age on flavor intensity and quality were tested by growing 'Granex 33' onions (*Allium cepa* L.) at 16.5, 22.1, 26.7, and 32.2 (±0.4) ^{o}C for 50 days and to maturity. Plants were harvested and evaluated for growth characteristics. Bulbs were then analyzed for sulfur (S) assimilation and flavor development parameters. Total bulb S increased linearly with temperature regardless of bulb age. Bulb sulfate changed little over temperatures, indicating that organically bound S increased with temperature. Total pyruvic acid content (pungency), total S-alkenyl cysteine sulfoxide (ACSO) content and individual ACSOs increased linearly in response to temperature when measured at the two developmental stages. Though *trans*-(+)-S-(1-propenyl)-L-cysteine sulfoxide was the predominant ACSO at most temperatures, (+)-S-methyl-L-cysteine sulfoxide accumulation was greatest among the individual ACSOs in mature bulbs grown at 32.2 ^{o}C. Additionally, (+)-S-propyl cysteine sulfoxide was present in the least amount at all treatment levels and developmental stages. Gamma glutamyl propenyl cysteine sulfoxide and 2-carboxy propyl glutathione peptides in the flavor biosynthetic pathway also increased linearly with temperature. When ACSOs were assessed in onion macerate as a measure of alliinase activity, levels of degraded ACSOs increased linearly with growing temperature. The relative percentage of most ACSOs hydrolyzed, however, did not change in response to growing temperature. This suggested that the activity of alliinase was proportional to the amounts of flavor

precursors synthesized. Growing temperature, therefore, should be considered when evaluating and interpreting yearly and regional variability in onion flavor.

Introduction

Onion (*Allium cepa* L.) is prized throughout the world for its ability to infuse flavor into our diet. Because of the emphasis on obtaining quality flavor from onion, investigations into flavor development have been frequent. Of particular interest has been sulfur (S) uptake and utilization within the flavor pathway. Once in the plant, sulfate (SO_4^{2-}) is successively reduced to sulfide, incorporated into cysteine and then into methionine or glutathione. In many plants, most of the organic S is found in these three compounds, whether unbound or incorporated into various proteins (Anderson, 1990). Onions and other S-accumulating species of *Allium* are unique in that they accumulate large quantities of organic S into secondary S compounds, particularly the γ-glutamyl peptides (γGP) and S-alkenyl cysteine sulfoxides (ACSO) associated with flavor. When an onion is eaten, the ACSOs are hydrolyzed by the enzyme alliinase, and form a number of volatile S-compounds, which give rise to the flavor attributes associated with onion.

Yearly and regional variation in the flavor potential of onion can be a problem for growers wishing to produce a consistent product and for the consuming public. Though the limits of flavor potential are ultimately determined by genetics, multiple environmental factors can act to influence flavor within physiological limits. Flavor intensity of a given onion cultivar can vary depending on the region where it is grown (Lancaster et al., 1988; McCallum et al., 2001; Platenius and Knott, 1936). Yearly differences in flavor intensity have also been observed (Bedford, 1984; Vavrina and Smittle, 1993). Environmental factors that vary between locations and years include

fertility and irrigation practices, soil type and growing temperature, and have therefore been the subject of numerous investigations. Through controlled experiments, SO_4^{2-} and nitrogen (N) availability, and irrigation regime have all influenced onion flavor (Freeman and Mossadeghi, 1970, 1973; Platenius, 1941; Randle, 2000; Randle et al., 1995).

Observations made on commercial sweet onion production suggest that yearly temperature variations were possibly linked to changes in onion flavor quality (personal observation). Growing temperature was reported to affect total-S accumulation, pungency and the production of total S-volatiles (Platenius, 1941; Randle et al., 1993; Yamaguchi et al., 1975). Pungency and total-S volatiles, though useful in describing flavor intensity, give limited insight into the overall flavor quality, determined by changes in individual ACSOs, or in how S is metabolized in onion. To gain knowledge of the flavor pathway and flavor quality, the ACSOs and their intermediates should be investigated. Furthermore, results obtained by Platenius (1941) concerning onion flavor were confounded with the developmental age of the bulb. In those studies, plants were grown for a set period of time at different temperatures. Plant development is influenced by growing temperature and developmental age can influence onion flavor intensity (Hamilton et al., 1998; Platenius and Knott, 1936).

The purpose of this research was to determine the effect of growing temperature on flavor intensity and quality in onion by measuring the composition of the ACSOs and their related peptides in onion. To determine if alliinase activity was affected by growing temperature, decomposed ACSOs were assessed in onion bulb macerates. And finally, to determine if the effect of temperature on flavor was independent of the developmental age, two experiments were conducted. First, plants were grown for a specific period of

time. As such, plant development varied with temperature, but the time of exposure to the different temperatures was the same. In the second experiment, plants were grown to maturity where plant development was similar, but the exposure time to the different temperatures varied.

Materials and Methods

PLANT CULTURE. Experiment 1 [Plant development varied, exposure time to temperature was similar]. On 18 Nov. 2000 seeds of 'Granex 33' onion (Asgrow Seeds, Kalamazoo, Mich.) were seeded into greenhouse flats containing Fafard 3b Custom Mix artificial medium (Fafard Corp., Anderson, S.C.) and watered as needed. Each flat was fertilized twice-weekly with 4 L of Peter's 20N-8.8P-16.6K (The Scotts Company, Marysville, Ohio) at a concentration of 100 $mg \cdot L^{-1}$ N. Seedlings were greenhouse grown under natural photoperiods and light intensities (~ 34 ^{o} N latitude) at day/night 26/22 ^{o} C set points for 6 weeks. Plants were then transplanted into boxes (0.74 m x 0.36 m x 0.2 m) containing Fafard 3b Custom Mix artificial medium (Fafard Corp.). Ten plants were evenly spaced in each box. Boxes were placed into growth chambers and grown for six weeks at 22 ^{o} C under 10-h photoperiods. Growth chambers were tested and adjusted to provide uniform conditions. Average radiant flux measured at the plant base was 420 $\mu mol \cdot m^{2} \cdot s^{-1}$ (Basic Quantum Meter, Spectrum Tech. Plainfield, Ill.). Plants were fertilized weekly with a full strength Hoagland's #2 solution (Hoagland and Arnon, 1950), at the rate of 100 mL of solution per plant. Additionally, plants were watered with deionized water as needed. After six weeks at 22 ^{o}C, 14-h photoperiods (to promote bulbing) and temperature treatments were imposed. Treatments consisted of growing plants at constant temperatures of 15.6, 22.1, 26.7, or 32.2 (±0.4) ^{o}C for 50 d. These

treatments were chosen because they represent the range of temperature over which onions will bulb (Brewster, 1990). Each treatment contained four boxes, with 10 plants/box. A broad-spectrum insecticide (*O,O*-dimethyl S-(1,2 dicarboxyethyl) phosphorodithioate; Malthion; Cheminova, Inc., Wayne, N.J.) was applied as needed for thrips (*Thrips tabaci*) control.

Plants were harvested on 4 Apr. 2001. Bulb and leaf fresh weights (FW) were recorded at this time and bulbs were processed for chemical analysis immediately thereafter.

Experiment 2 [Bulb development similar, exposure time to temperature varied]. On 2 Apr. 2001 plants of 'Granex 33' onion (Asgrow Seeds) were established and grown under the conditions outlined in Experiment 1, with the exception that plants were grown to maturity at each temperature. Plants were classified as mature when the pseudostems became soft and treatments were harvested when 50% of the plants displayed soft pseudostems. Treatments were harvested from 22 Aug. 2001 to 13 Sept. 2001. Upon harvest, bulb and leaf FW were obtained, after which bulbs were cured for one week at ambient greenhouse temperatures before chemical analyses. Bulbs from both experiments were analyzed in the same manner. Analyses were performed on the combined bulb tissue of each 10-bulb group. Three 2-5 mm-thick wedges were cut longitudinally from bulbs. One wedge was used to measure total bulb S and SO_4^{2-} concentrations, a second to measure intact ACSOs and γGPs, and a third to measure total pyruvic acid (TPY), soluble solids content (SSC), and ACSOs remaining in onion macerates.

MINERAL ANALYSIS**.** Bulb tissue was dried at 65 ^{o}C in a forced air oven (Linberg Blue, Asheville, N.C.) for 5 d. Dried tissue was then ground through a 0.5 mm screen with a Cyclotec Mill (model 1093, Tector, Hoganas, Sweden). Total bulb S was determined using 0.25 g of tissue on a Leco 232 S determinator (Leco Corp., St. Joseph, Minn.). Samples received 0.1 g of vanadium pentoxide accelerant before analysis (Leco Corp.). Calibration and S quantification were done using certified coal standard reference materials (Leco Corp.).

Sulfate concentrations were determined using anion analysis and high performance liquid chromatography (HPLC). Sulfate was extracted from 0.25 g of ground tissue in 50 mL of HPLC grade water. Flasks were shaken on an orbital shaker for 30 min at 150 rpm. Solution sub-samples were filtered through 0.22 μm nylon syringe filters (Fisher Scientific, Pittsburg, Pa.) into 1 mL plastic vials. Analysis was done on a Waters 2690 Separations Module with an attached autosampler and a Waters 432 Conductivity Detector (Waters Corp., Milford, Mass.). Fifty microliters of extract were injected into a 4.6 x 75 mm IC-PAK Anion HR column coupled to an IC-Pak Anion Guard Pak (Waters Corp.) Column temperature was maintained at 30 ^{o}C, and an isocratic sodium borate-gluconate eluent was used at a flow rate of 1.0 mL$\cdot$min^{-1}. The eluent was composed of 40 mL of a sodium borate-gluconate concentrate, with 40 mL of butanol and 120 mL of acetonitrile brought to 1 L with HPLC grade water. The sodium borate-gluconate concentrate was composed of 16 g of sodium gluconate, 18 g of boric acid, 25 g sodium tetraborate, and 250 ml of glycerol brought to 1 L with HPLC grade water. Peaks were integrated against a standard curve developed using sodium sulfate standards on Millenium Chromatography Software (Version 3.05, Waters Corp.).

SOLUBLE SOLIDS CONTENT AND TOTAL PYRUVIC ACID. The bulb tissue from each treatment/replication was juiced in a pneumatic press. Several drops of the fresh juice were placed on a hand-held refractometer to determine soluble solids content (SSC) (Kernco, Tokyo, Japan). Gross flavor intensity of the onion was determined by measuring the total pyruvic acid (TPY) in 0.5 mL of the juice as described in the method of Randle and Bussard (1993a). Pyruvic acid is a decomposition product of ACSO degradation and is often used as an indicator of gross onion flavor intensity (Schwimmer and Weston, 1961). Historically, pyruvic acid content has been reported as enzymatically produced pyruvic acid where background pyruvic acid is subtracted from the analysis. Because background pyruvic acid is difficult to accurately measure, and Yoo and Pike (2001) reported that these concentrations in onion are uniform and low, we choose to report only TPY.

ACSOs AND THEIR PEPTIDE INTERMEDIATES. The ACSO and γGP contents from intact bulb tissue were determined according to Randle (2000). The tissue wedges from each ten bulb group were weighed and the ACSOs and γGPs were twice extracted (using 5mL$\cdot$g^{-1} FW) in 12 methanol (MEOH) :3 water and once in 12 ethanol (ETOH) : 3 water over a 3 d period. Extracts were then combined into one solution from which 15 mL was analyzed. S-Methyl glutathione, γ-L-glutamyl-L-glutamic acid, and (±)-S-1-butyl-L-cysteine sulfoxide were used as internal standards in concentrations of 0.5, 0.2 and 1.0 mg$\cdot$g^{-1} FW respectively, and added to the 15 mL of extract solutions which were then dried using forced air (Evap-o-Rac; Cole Parmer, Vernon Hills, Ill.). The ACSOs measured were, (+)-S-methyl-L-cysteine sulfoxide (MCSO), *trans*-(+)-S-(1-propenyl)-L-cysteine sulfoxide (1-PRENCSO) and (+)-S-propyl-L-cysteine sulfoxide (PCSO). The

two γGPs measured were 2-carboxypropyl glutathione (2-Carb) and γ-glutamyl propenyl cysteine sulfoxide (γGPECSO).

ACSOs remaining in the onion macerates (MACSO) were extracted using the method of Lancaster et al. (1998). After an incubation period of 10 min at room temperature (25 ^{o}C), a 0.5 mL aliquot of onion juice macerate was placed in a 20 mL scintillation vial to which 10 mL of 12 MEOH: 3 water was added. Internal standards were added as described above and the entire solution was dried using forced air.

The dried ACSO and MACSO samples were redissolved in 1 mL of HPLC grade water. A 0.75 mL aliquot of each solution was then subjected to ion exchange chromatography using a 10 x 40 mm column (Bio-Rad, Hercules, Calif.) with 3 mL of Dowex 1 x 8 resin (200 to 400 mesh; Bio-Rad). The ACSOs were separated from the γGPs using four successive concentrations of glacial acetic acid (0.1, 0.2, 2 and 5 M). The ACSOs and γGPs were contained in the 0.1 and 2 M fractions while the 0.2 and 5 M fractions were discarded. The collected fractions were dried under forced air.

The levels of ACSOs, MACSOs, and γGPs were determined using HPLC analysis according to Randle (2000). Dried sample fractions were redissolved in 1.0 mL of HPLC water, from which 100 μL was pipetted into a 1.5 mL microcentrifuge vial and then dried in vacuo using a Labconco Centrivap Concentrator (Labconco, Kansas City, Mo.). Upon dryness, 250 μL of 1 ETOH: 1 Triethylamine (TEA): 1 HPLC water was added to each vial and dried again. Samples were then derivitized by adding 7 ETOH: 1 TEA: 1 phenylisothiocyante : 1 HPLC water. Vials were immediately flushed with nitrogen gas, capped, and stored at room temperature for 18 min. Vials were then uncapped and dried.

Dry samples were redissolved in 1 mL of 7 HPLC water: 2 acetonitrile and transferred to 2.0 mL borosilicate vials for HPLC analysis.

Samples were analyzed on a Waters 2690 Separations Module (Waters Corp.) equipped with an autosamper and coupled to a Waters 996 photodiode array (PDA) detector (Waters Corp.). Fifty microliters of sample was injected into a 250 x 4.6 mm, 5 μm column (Spheri- 5 RP-18; Applied Biosystems, Foster City, Calif.) mated to a 15 x 3.2 mm, 7 μm guard column (RP-18 Newgard; Applied Biosystems) for separation. Column temperature was maintained at 30 ^{o}C. Eluents were: A) aqueous acetonitrile (60%), B) 0.14 M sodium acetate with 0.05% TEA buffered to a pH of 6.35 using glacial acetic acid. All eluents were filtered through 0.45 μm nylon filters (Millipore, Molsheim, France). The flow rate was set at 1.0 mL·min^{-1} for the duration of the run. A gradient run was programmed as follows: 15% A for 1.1 min, 15% - 45% over the next 21.1 min, then 45% -100% A over the next 1.0 min, and then hold at 100% A for the next 14 min. The gradient was returned to the initial 15% A : 85% B over the next 1.0 minute, and the column was conditioned at that setting for the next 12.9 min until the next sample was injected.

Compounds were detected on a photodiode array (PDA) detector set at 254 nm. Data were collected and peaks integrated using Millenium Chromatography Software (Version 3.05; Waters corp.). Peaks were assigned by comparing retention times of authentic standards prepared according to the method of Armstrong and Lewis (1951) as described in Lancaster and Kelly (1983) and Randle et al. (1995)

Statistical Analysis. Data were subjected to linear and polynomial regression analyses when appropriate, using SAS statistical software. (Version 8.2, SAS institute,

Cary, N.C.). Percentage data were tested for normality, and arcsin transformations were done if necessary.

Results and Discussion

BULB AND LEAF WEIGHT. The response of bulb FW to increasing temperature from 50 d and mature plants was quadratic (Table 1). Fresh weight was lowest in plants grown at 15.6 and 32.2 ^{o}C, and highest for plants grown at 22.1 and 26.7 ^{o}C for both 50 d and mature plants. Others have reported similar responses of bulb weight to growing temperatures (Brewster, 1979; Yamaguchi et al., 1975). This suggests that the highest bulb FW yields would be obtained when onions are grown at temperatures between 22.1 and 26.7 ^{o}C.

Leaf FW was not affected by temperature for 50 d plants, but mature plants responded quadratically to temperature. However, unlike bulb FW, leaf FW was lowest at 22.1 and 26.7 ^{o}C. Additionally, leaf FW of mature plants was less than that of 50 d plants. This would be expected because leaves translocate materials to the swelling leaf bases during bulb maturation.

Growing temperature did not influence bulb dry weight percentage (DW) in 50 d bulbs. However, the DW of the mature bulbs responded in a negative, linear fashion, with increasing temperature, suggesting that DW gains would be higher at lower temperatures. Steer (1982) reported similar effects of temperature on bulb DW.

SSC AND TPY. A meaningful significant trend could not be fitted for the response of SSC to growing temperature in 50d bulbs. The SSC of mature bulbs had a negative linear response to increasing temperature (Table 1). The SSC of mature bulbs ranged from 9.7% to 6.7% among the temperature treatments. Bulb SSC also was higher

in mature bulbs than those harvested when immature. This substantiates the observation that SSC increases with bulb maturity (unpublished data).

The TPY from 50 d bulbs and mature bulbs increased linearly in response to increasing temperature (Table 1). Pungency ranged from 3.8 to 7.2 $\mu mol \cdot g^{-1}$ FW, which is within the range expected for 'Granex 33' grown under these conditions. Others have shown a similar response when measuring the response of flavor intensity to increasing temperature (Platenius, 1941; Yamaguchi et al., 1975). Although values for TPY were greater in onion bulbs grown to maturity than for the 50 d bulbs, developmental age did not change the trend of increasing pungency as growing temperature increased.

TOTAL BULB S AND SO_4^{2-}. Though essential to all plants, S is of particular interest to those investigating onion flavor. Onions accumulate large amounts of S, much of which is utilized in the production of flavor precursors and related compounds. Increasing growing temperature resulted in a linear increase in total bulb S in 50 d and mature bulbs. Total bulb S ranged from 0.16% to 0.46% and from 0.17% to 0.69 % DW for 50 d and mature bulbs, respectively. The trends of increasing S were similar between 50 d and mature bulbs, suggesting the response was due to temperature and not developmental age. Interestingly, these data hold many similarities with studies where S fertility was manipulated. For example, total bulb-S accumulation in plants grown at 15.6 ^{o}C was similar to that found in mild onions grown at low-S fertility levels, even though S availability was similar across temperature treatments (Randle et al. 1995; Randle et al., 1999). Furthermore, the rise of total bulb S with temperature was similar to increases found when S fertility increased.

Temperature had no effect on bulb SO_4^{2-} levels measured in 50 d bulbs. Bulb

SO_4^{2-} measured at maturity, however, responded in a positive linear fashion (Table 2). Bulb SO_4^{2-} accumulates and usually remains in the vacuole where it is unlikely to immediately contribute to the S-containing compounds in the onion flavor biosynthetic pathway (Mengel and Kirby, 1982). The percentage of total bulb S that is not SO_4^{2-} is likely bound organically and can be referred to as organic S. Much of the organic S in onion can enter the flavor precursor biosynthetic pathway (Block, 1992).

Organic S as a percentage of total bulb S increased linearly with temperature for both 50 d and mature bulbs (Table 2). Organic S ranged from 5.6% to 64.4 % in 50 d bulbs and from 8.5% to 64.6% in mature bulbs. Bulb developmental age appeared to have no effect on the level of organic S as a percentage of total S when plants were exposed to increasing temperatures. Similar results were found with organic S levels in onion bulbs when grown with increasing S-fertility levels (Randle et al., 1999). When considering S-accumulation patterns in bulb onion, this study demonstrated that temperature influences both the amount of total S that accumulates in the bulb as well as the amount of total S that becomes organic S, which has flavor implications. Our data suggest that milder bulbs developing at the lower growing temperatures result from both lower total S accumulations with most of the S stored as SO_4^{2-}. Conversely, the more pungent bulbs that occur at the higher growing temperatures have up to a 4x increase in total S accumulation with the majority forming as organically bound S.

INTACT FLAVOR PRECURSORS AND INTERMEDIATES. Total flavor precursor accumulation responded linearly to increasing temperature in both 50 d and mature bulbs (Table 3). In both experiments, ACSO concentrations from bulbs grown at 15.6 ^{o}C were roughly a third of those grown at 32.2 ^{o}C. Total ACSOs responded to

temperature increases in the same manner regardless of developmental age or exposure time.

Temperature also had a significant effect on the three individual flavor precursors and their peptide intermediates. Changes in the ratios of individual precursors can ultimately result in changes in flavor quality (Randle et al., 1994). The concentration of MCSO increased linearly in response to temperature for both 50 d and mature bulbs (Table 3). Though developmental stage did not influence how MCSO responded to temperature, mature bulbs had more MCSO on average than did 50 d bulbs. At 15.6 ^{o}C, MCSO accounted for between 21% and 29% of the total ACSOs present, whereas at the highest temperature, MCSO made up between 45% and 56% of the ACSOs measured. This would indicate that as growing temperature increased, so did relative S metabolism through the pathway leading to MCSO synthesis. This was especially evident when bulbs were grown at 32.2 ^{o}C and harvested mature. Historically, 1-PRENCSO was reported to be the main ACSO in onion (Block 1992; Lancaster and Boland, 1990). However, increasing evidence suggests that MCSO can accumulate at high levels in onions grown outside of optimal conditions. These would include growing onions with very high levels of N (Randle, 2000), high sodium selenate (Kopsell and Randle, 1999), low S fertility (Randle et al., 1995), and now high growing temperatures.

Like MCSO, 1-PRENCSO concentrations responded linearly to increasing temperature in both 50 d and mature bulbs (Table 3). At 15.6 ^{o}C, 1-PRENCSO was between 64% and 73% of the ACSOs present, while at 32.2 ^{o}C 1-PRENCSO made up between 35% and 50% of the ACSOs measured, depending on the maturity of the bulb analyzed. This would indicate that as growing temperature increased, the metabolism of

available S through the 1-PRENCSO pathway declined relative to the other ACSO pathways. Additionally, the regression coefficients of MCSO were greater than 1-PRENCSO in both experiments, indicating that MCSO accumulation per ^{o}C increase was greater than 1-PRENCSO over the range of temperatures used in these studies.

For both stages of development, PCSO was found in the least concentration of any of the ACSOs at all temperatures (Table 3). Propyl cysteine sulfoxide increased linearly for both 50 d and mature bulbs in response to growing temperature.

In order to understand more about S metabolism through the flavor biosynthetic pathway in onion, it was appropriate to measure detectable γGP intermediates. Their role in the flavor pathway has not yet been fully described, though they are believed to function as a sink for organic S prior to incorporation into the flavor precursors (Lancaster and Boland, 1990; Randle et al., 1995; Randle, 2000). 2-Carboxy propyl glutathione responded linearly to increasing temperatures in both 50 d and mature bulbs (Table 3). However, levels of 2-Carb in the bulb were lower for the 50 d bulbs than for those grown to maturity. Lancaster and Shaw(1991) observed similar responses of 2-Carb in long-day onions measured at different plant maturities. Additionally, γGPECSO increased linearly in response to temperature for both 50 d and mature bulbs. If we consider the content of the peptide intermediates relative to ACSO content at the different temperatures, we find that at 15.6 ^{o}C, 2-Carb was between 9% and 13% of the ACSOs, while γGPECSO was between 3% and 9% of ACSO content. This would suggest that at lower temperatures, metabolized S accumulated slightly more at the beginning of the ACSO pathway than at the end. However, this trend changed at the higher growing temperature. 2-Carboxy propyl glutathione was between 10% and 12% of ACSO content

at 32.2 ^{o}C, while γGPECSO was between 25% and 30% of the ACSOs present, suggesting that S now pooled in the later stage of the 1-PRENCSO pathway. High γGPECSO accumulation was also reported with higher S fertility levels in onions (Randle et al., 1995).

ACSO IN ONION MACERATES. The action of alliinase on the ACSOs was originally characterized in vivo in macerated onion tissue by Lancaster et al. (1998). It was found that alliinase acted quickly, but differently on the individual ACSOs, such that some of the flavor precursors were more completely degraded than others. They reported that the amount of 1-PRENCSO degraded in vivo was nearly 100%, while the amount of MCSO and PCSO degraded after 5 min was near 40% and 45%, respectively. This indicated that 1-PRENCSO could contribute more to the overall flavor sensation than would MCSO or PCSO, even if 1-PRENCSO was not found to be the most abundant ACSO in the intact bulb tissue. In our study, the amount of ACSOs that were degraded responded linearly to increasing temperatures for both 50 d and mature bulbs (Table 4). The total amount of MCSO degraded increased linearly with temperature for 50 d and mature bulbs. Upon decomposition, MCSO can give rise to a cabbage and fresh onion flavor, and thus the ratio of MCSO to the other precursors is important in determining flavor quality.

Temperature also influenced the total amount of 1-PRENCSO that was degraded (Table 4). Mature bulbs had less 1-PRENCSO hydrolyzed than did 50 d bulbs. This coincided with the slightly higher amount of intact 1-PRENCSO seen in the immature bulbs when compared to mature bulbs. The rate of 1-PRENCSO degradation was linear in response to increasing temperature for both immature and mature bulbs. Upon

decomposition, 1-PRENCSO forms propanethial *S*-oxide, which gives rise to tearing and mouth burn associated with pungent onions (Block, 1992), and thiosulfinates which are responsible for other onion flavors (Randle et al., 1994).

The total amount of PCSO degraded demonstrated significant linear trends for both 50 d and mature bulbs (Table 4). There was a high degree of variation among groups, however, resulting in poor values for the coefficients of determination. Upon degradation, the thiosulfinates formed from PCSO impart fresh onion and chive flavors (Randle et al., 1994)

With the exception of PCSO, the relative amounts of the individual ACSOs that degraded were of the same magnitude regardless of temperature or developmental stage, suggesting that alliinase activity was proportional to ACSO accumulation (Table 4). The amount of 1-PRENCSO that was degraded averaged nearly 97% of the intact values, while MCSO was about 77%. The amount of PCSO that was degraded did vary however. Roughly 21% of the intact PCSO was degraded, on average, in 50 d bulbs, but an average of 70% was degraded in mature bulbs. The maximum amount of PCSO degraded, however, only represented 9% of the total ACSOs degraded. Similar results regarding the near-complete degradation of 1-PRENCSO were found by Lancaster et al. (1998) and Kopsell et al. (2002). However the amounts of MCSO and PCSO degraded in our study were higher than those previously reported.

Conclusion

The purpose of our experiments was to determine how growing temperature influenced flavor intensity and quality in 'Granex 33' onion bulbs and whether plant development was a confounding factor in assessing the effects of temperature. Total bulb

S and organic S increased linearly with temperature regardless of developmental age, indicating that temperature influenced both S uptake and the amount of S partitioned into organic compounds. Moreover, changes in flavor intensity and quality in response to increasing temperature occurred in two ways. First, the amount of total S absorbed and metabolized increased with increasing temperature. Second, flavor intensity was influenced by the amount of S that accumulated as SO_4^{2-}. With low temperatures, S accumulated mainly as SO_4^{2-} and did not enter the flavor biosynthetic pathway. Increases in S accumulation have been shown to increase flavor potential and quality in onion (Freeman and Mossadeghi, 1970; Randle and Bussard 1993b; Randle et al., 1995). Changes in flavor intensity as a result of increasing temperatures were similar to the responses of onion to increasing S fertility, though the affected precursors were different. With low temperature exposure, 1-PRENCSO accumulated as the major ACSO. With low S fertility, MCSO accumulated as the major ACSO. These responses reversed at high temperature and high S fertility. Most measurements related to the accumulation of S flavor compounds increased linearly with temperature, regardless of developmental stage, suggesting that age was not a confounding factor. Alliinase activity was proportional to the changes in ACSO content at the different growing temperatures. As a result, growing temperatures should be considered when assessing and interpreting yearly or regional variations in flavor intensity and quality.

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Table 1. Effects of four growing temperatures on leaf and bulb fresh weight (FW), percentage dry weight (DW), soluble solids content (SSC), and gross flavor intensity (as measured by total pyruvic acid) (TPY) of 'Granex 33' onion bulbs harvested at 50 d and at maturity.

Growing	50 day					Mature				
temp. ^{o}C	Bulb FW (g)	Leaf FW (g)	DW (%)	SSC (%)	TPY (µmol·g FW)	Bulb FW (g)	Leaf FW (g)	DW (%)	SSC (%)	TPY (µmol·g FW)
15.6	97	189	9.3	6.6	3.8	133	76	12.0	9.7	5.3
22.1	190	186	8.5	7.2	6.2	157	48	10.0	8.1	6.0
26.7	189	192	7.5	6.4	5.7	149	51	9.5	7.9	6.8
32.2	99	168	7.3	6.1	7.1	50	66	8.3	6.7	7.2
Regression[z]	Q	NS	NS	NS	L	Q	Q	L	L	L

[z]Linear (L), quadratic (Q), or nonsignificant (NS) regression equations, respectively ($P \leq 0.01$). The regression equations for each significant response are as follows: bulb FW 50d (y= -652.6 + 71.3T - 1.5T^2, R^2=0.79), mature (y=-306.1 + 43.7T - 1.0T^2, R^2=0.89), leaf FW mature (y=255.3 - 16.9T + 0.3T^2, R^2=0.70), bulb DW mature (y= 0.15 - 0.002T, R^2 = 0.92), bulb SSC mature (y=12.2 - 0.17T, R^2 = 0.90), bulb TPY 50d (y = 1.6 + 0.2T, R^2 = 0.64) mature (y=3.4 + 0.12T, R^2 = 0.64).

Table 2. Effects of four growing temperatures on percentage total bulb sulfur (Tot S), the percent of total bulb sulfate (SO_4^{2-}-S), and the percentage of total bulb S that is organically bound (Org-S) in 50 d and mature 'Granex 33' onion bulbs grown at four temperatures.

Growing	50 d (%)			Mature (%)		
temp. ^oC	Tot-S	SO_4^{2-}-S	Org-S	Tot- S	SO_4^{-2}-S	Org-S
15.6	0.16	0.15	5.6	0.17	0.15	8.5
21.2	0.22	0.16	24.3	0.26	0.17	33.5
26.7	0.36	0.15	57.9	0.33	0.18	46.2
32.2	0.46	0.16	64.4	0.69	0.24	64.6
Regression[z]	L	NS	L	L	L	L

[z]Linear (L), or nonsignificant (NS) regression equations respectively ($P \leq 0.01$) The regression equations for each significant response are as follows: total S 50 d (y= -0.2 + 0.02T, R^2 = 0.93) mature (y= -0.3 + 0.03T, R^2 = 0.83), S-SO_4^{-2} mature (y=0.06 + 0.005T, R^2 = 0.75), organic-S 50 d (y= -54.2 + 3.9T, R^2 = 0.76) mature (y= -40.1 + 3.3T, R^2 = 0.88)

Table 3. Effects of four growing temperatures on the means for total flavor precursor (ACSO) concentration, methyl cysteine sulfoxide (MCSO), 1-propenyl cysteine sulfoxide (1-PRENCSO), propyl cysteine sulfoxide (PCSO), and the biosynthetic intermediates, 2-carboxypropylglutathione (2-Carb) and γglutamyl cysteine sulfoxide (γGPECSO), in 'Granex 33' onion bulbs harvested at 50 d and at maturity.

Growing			50 day			mg·g^{-1} FW			Mature			
temp ^{o}C	ACSO	MCSO	1-PRENCSO	PCSO	2-Carb	γGPECSO	ACSO	MCSO	1-PRENCSO	PCSO	2-Carb	γGPECSO
15.6	0.76	0.16	0.56	0.04	0.07	0.02	0.99	0.29	0.63	0.06	0.13	0.09
22.1	1.57	0.46	1.07	0.03	0.08	0.31	1.30	0.50	0.74	0.06	0.17	0.25
26.7	1.72	0.61	1.05	0.06	0.16	0.61	1.46	0.63	0.76	0.08	0.22	0.44
32.2	2.32	1.05	1.17	0.09	0.28	0.81	2.97	1.66	1.04	0.27	0.32	0.76
Regression[z]	L	L	L	L	L	L	L	L	L	L	L	L

[z]Significant linear (L) regression equation ($P \leq 0.01$) The regression equations for each significant response are as follows: ACSO 50 d (y = -0.50 + 0.09T, R^2 = 0.73), mature (y = -0.96 + 0.11T, R^2 = 0.73), MCSO 50 d (y = -0.63 + 0.05T, R^2 = 0.76) mature (y = -1.06 + 0.08T, R^2 = 0.73), 1-PRENCSO 50 d (y = 0.16 + 0.03T, R^2=0.56) mature (y = 0.26 + 0.02T, R^2 = 0.73), PCSO 50 d (y = -0.02 + 0.003T, R^2=0.61) mature (y = -0.15 + 0.01T, R^2 = 0.53), 2-Carb 50 d (y = -0.15 + 0.01T, R^2 = 0.80) mature (y = -0.05 + 0.01T, R^2 = 0.83), γGPECSO 50 d (y = -0.72 + .05T, R^2 = 0.91) mature (y = -0.58 + 0.04T, R^2 = 0.88).

Table 4. Effects of four growing temperatures on the amounts of intact total S-alk(en)yl cysteine sulfoxides (ACSO), methyl cysteine sulfoxide (MCSO), 1- propenyl cysteine sulfoxide (1-PRENCSO), propyl cysteine sulfoxide (PCSO) that were degraded in onion macerates for 50 d and mature 'Granex 33' onion bulbs. Percentages of intact precursors that were degraded are shown in parentheses.

Growing			50 day	mg·g^{-1} FW			Mature	
temp. ^{o}C	ACSO	MCSO	1-PRENCSO	PCSO	ACSO	MCSO	1-PRENCSO	PCSO
15.6	0.68 (90)	0.13 (77)	0.54 (97)	0.01 (17)	0.89 (91)	0.23 (78)	0.62 (98)	0.05 (77)
22.1	1.44 (92)	0.40 (83)	1.04 (97)	0.01 (05)	1.16 (89)	0.40 (79)	0.72 (97)	0.04 (71)
26.7	1.43 (83)	0.46 (74)	0.96 (92)	0.01 (12)	1.26 (86)	0.48 (76)	0.74 (97)	0.05 (59)
32.2	2.08 (89)	0.90 (74)	1.15 (98)	0.04 (37)	2.35 (79)	1.14 (68)	1.02 (98)	0.20 (72)
Regression[z]	L	L	L	L	L	L	L	L

[z] Significant linear (L) regression, equations ($P \leq 0.05$) The regression equations for each significant response are as follows: ACSO 50 d (y = -0.49 + 0.08T, R^2 = 0.70) mature (y = - 0.52 + 0.08T, R^2 = 0.65), MCSO 50 d (y = -0.58 + 0.04T, R^2 = 0.66) mature (y = - 0.66 + 0.05T, R^2 = 0.60), 1-PRENCSO 50 d (y = 0.10 + 0.034T, R^2 = 0.60) mature (y = 0.24 + 0.02T, R^2 = 0.72), PCSO 50 d (y = - 0.019 + 0.001T, R^2= 0.29) mature (y = - 0.11 + 0.01T, R^2 = 0.46)

CHAPTER 4

NH_4NO_3 FERTILITY LEVELS INFLUENCE FLAVOR DEVELOPMENT IN HYDROPONICALLY GROWN 'GRANEX 33' ONION[2]

[2] Coolong, T.W. and W.M. Randle. Submitted to J. Sci. Food Agr.

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Abstract

High levels of nitrogen (N) fertility have been shown to influence bulb flavor characteristics in onion (*Allium cepa* L.). To test the effects of lower levels of N fertility on onion bulb flavor, 'Granex 33' onions were grown hydroponically in a greenhouse with varying solution N levels. Eleven levels were tested by increasing the concentration of NH_4NO_3 in solutions from 20 to 140 mg·L^{-1} N. Mature plants were harvested and evaluated for plant leaf and bulb fresh weights (FW), bulb soluble solids content (SSC), bulb total pyruvic acid, bulb total sulfur (S), and bulb sulfate (SO_4^{-2}). To determine the effect of N on the flavor biosynthetic pathway of onion, total and individual *S*-alk(en)yl cysteine sulfoxides and related peptide intermediates were also tested. Leaf and bulb FW responded quadratically to N concentration as did total bulb S. Bulb SO_4^{-2} and SSC, though significantly influenced by N concentration, did not respond with a meaningful trend. Bulb pyruvic acid increased linearly with N level increases, as did (+)-S-propyl-L-cysteine sulfoxide. Total precursors, (+)-S-methyl-L-cysteine sulfoxide, and *trans*-(+)-S-1-propenyl-L-cysteine sulfoxide responded quadratically to N levels. At lower N levels, *trans*-(+)-S-1-propenyl-L-cysteine sulfoxide content was highest relative to the other precursors. However, at elevated N levels, (+)-S-methyl-L-cysteine sulfoxide accumulated in the highest concentrations. Peptide intermediates 2-carboxypropyl glutathione and γglutamyl propenyl cysteine sulfoxide responded linearly and quadratically to increasing N fertility levels, respectively. Nitrogen fertility levels can influence flavor intensity and quality and may need to be considered by growers.

Intoduction

Onions (*Allium cepa* L.) are consumed throughout the world for their unique flavor. Onion flavor develops when vacuoles are broken and the enzyme alliinase hydrolyzes individual flavor precursors collectively called *S*-alk(en)yl cysteine sulfoxides (ACSO)*. The three ACSOs found in onion are, (+)-*S*-methyl-L-cysteine sulfoxide (MCSO), *trans*-(+)-*S*-1-propenyl-L-cysteine sulfoxide (1-PRENCSO), and (+)-*S*-propyl-L-cysteine sulfoxide (PCSO) (Lancaster and Boland, 1990). Upon decomposition, the precursors form a number of thiosulfinates, which give onions their characteristic aroma and flavor. Individual precursors impart varying flavor sensations upon breakdown. Because sulfur (S) is present in onion flavor compounds, S nutrition and its effects on flavor in onion have been well characterized (Freeman and Mossadeghi, 1970; Hamilton et al., 1998; Randle and Bussard, 1993a; Randle et al., 1995). The effects of other nutrients, such as nitrogen (N), on flavor development in onion have been less thoroughly examined, however.

Because sulfur metabolism is tied to nitrate assimilation in the plant, it would be expected that N nutrition would influence S uptake by onion and the subsequent formation of the S-containing ACSOs (Anderson, 1990; Brunold and Suter, 1984; Koprivova et al., 2000). However, while N has been well characterized in its effects on growth and yield in onion, (Brewster and Butler, 1989; Brown et al., 1988; Drost et al.,

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- *ACSO (*S*-alk(en)yl cysteine sulfoxide)
- MCSO ((+)-*S*-methyl-L-cysteine sulfoxide)
- 1-PRENCSO (*trans*-(+)-S-1-propenyl-L-cysteine sulfoxide)
- PCSO ((+)-*S*-propyl-L-cysteine sulfoxide)
- γGP (gammaglutamyl petptides)
- 2-Carb (2-carboxypropyl glutathione)
- γGPECSO (gammaglutamyl propenyl cysteine sulfoxide)

2002; Hussaini et al., 2000; Neeraja et al., 2001; Sypien et al., 1973) much less is known about its influence on flavor (Randle, 2000)

Increases in N fertility have been reported to influence sweet corn flavor by increasing concentrations of dimethyl sulfide (Wang et al., 1995). In onion, N-form was reported to influence overall flavor intensity (Gamiely et al., 1991). Alternatively, Randle (2000) showed that high-N levels changed the ratios of the flavor precursors such that MCSO accumulated at elevated levels that did not represent flavor quality from a typical field-grown onion (Block, 1992). In that study N was applied at rates that were much higher than those recommended for commercial onion production (Boyhan et al., 2001). The purpose of this experiment was to determine the influence of lower levels of N nutrition on flavor development and S assimilation in onion. More importantly, we were interested in determining the level(s) of N at which 1-PRENCSO accumulation exceeded that of MCSO, a relationship more representative of a field-grown onion.

Materials and Methods

PLANT CULTURE. On 18 Dec. 2000, seeds of 'Granex 33' onion (Asgrow Seeds, Kalamazoo, Mich, USA) were sown into sheets of 2.54 cm rock wool cubes (Grodan; Hedenhusene, Denmark) and watered as needed. Upon emergence, seedlings were fertilized twice weekly with a full strength Hoagland's #2 solution (Hoagland and Arnon, 1950). The solution consisted of 0.61 $g \cdot L^{-1}$ KNO_3, 0.95 $g \cdot L^{-1}$ $Ca(NO_3)_2 \cdot 4H_2O$, 0.49 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.12 $g \cdot L^{-1}$ $NH_4H_2PO_4$, 0.01 $g \cdot L^{-1}$ iron chelate (Sequestrene 330), 2.86 $mg \cdot L^{-1}$ H_3BO_3, 1.81 $mg \cdot L^{-1}$ $MnCl_2 \cdot 4H_2O$, 0.22 $mg \cdot L^{-1}$ $ZnSO_4 \cdot 7H_2O$, 0.08 $mg \cdot L^{-1}$ $CuSO_4 \cdot 5H_2O$, 0.02 $mg \cdot L^{-1}$ $Na_2MoO_4 \cdot 2H_2O$. Plants were grown under natural photoperiods (~ 34 ^{o} N latitude) at day/night set points of 26/22 ^{o}C. Illuminance as

measured in mid-afternoon on a clear, sunny day was 66420 lx. On 21 Jan. 2001 seedlings were transplanted in 37.9-L plastic tubs. (Rubbermaid, Inc., Wooster, OH, USA). Ten plants were placed in 2.54 cm holes spaced 7 X 15 cm on each lid. Welded wire mesh was attached to each lid to support the foliage.

Tubs were filled with 28 L of deionized water and nutrients added. Each tub contained a modified nutrient solution consisting of 0.22 $g \cdot L^{-1}$ KCL, 0.22 $g \cdot L^{-1}$ $CaCl_2$, 0.25 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.06 $g \cdot L^{-1}$ $NH_4H_2PO_4$, 0.01 $g \cdot L^{-1}$ iron chelate (Sequestrene 330), 1.43 $mg \cdot L^{-1}$ H_3BO_3, 0.91 $mg \cdot L^{-1}$ $MnCl_2 \cdot 4H_2O$, 0.11 $mg \cdot L^{-1}$ $ZnSO_4 \cdot 7H_2O$, 0.04 $mg \cdot L^{-1}$ $CuSO_4 \cdot 5H_2O$, 0.01 $mg \cdot L^{-1}$ $Na_2MoO_4 \cdot 2H_2O$. Treatments were constructed by varying NH_4NO_3 such that there were eleven treatments at: 20, 32, 44, 56, 68, 80, 92, 104, 116, 128, and 140 $mg \cdot L^{-1}$ N. This included the 7 $mg \cdot L^{-1}$ N contributed by $NH_4H_2PO_4$, which was present in all treatments. The experiment was a completely randomized block, with ten individual bulb replications for each N-treatment level. Each tub was aerated via two-2.54 cm aquarium airstones. Tubs were filled to volume daily with deionized water and nutrient solutions were completely changed to original concentrations every two weeks. Electrical conductivity (EC) (model 09-326; Fisher Scientific, Pittsburgh, PA, USA) of the solutions was measured at the start and end of each two-week period. The EC of the initial solutions ranged from 1325-1930 micro-siemens (μS), increasing with N concentration. Initially, EC was nearly unchanged after two weeks, but during active bulbing the EC fell to 895-1695 μS over a two-week period. Throughout the experiment pH measurements of initial solutions ranged between 4.9 and 5.9. Solution pH changed little at the start of the experiment, but fell to 3.4-3.8 in a two-week period during active bulbing. Unreplicated macro-nutrient analysis from each

solution showed that the pH change did not appear to influence macro-nutrient uptake. Plants were sprayed with a broad-spectrum insecticide as needed for *Thrips tabaci* control (Karate; Syngenta, Greensboro, NC, USA).

Mature plants were harvested 26 Apr to 7 May 2001. Plants grown in the lower N levels matured first. Maturity was determined to be when 50% of the plants in a treatment had soft psuedostems. Bulb and leaf fresh weights (FW) were taken at harvest. Bulbs were then allowed to cure for one week at ambient greenhouse temperatures in mesh bags. Analysis was then performed on eight representative bulbs from each N treatment. Three, five mm wedges were cut longitudinally from each bulb. One wedge was used to determine total bulb S and SO_4, second to measure ACSOs and related γGPs, and a third to measure total pyruvic acid (TPY) and soluble solids content (SSC).

MINERAL ANALYSIS. Bulb tissue was dried at 65 ^{o}C in a forced air oven (Linberg Blue, Asheville, NC, USA) for five days. Dried tissue was then ground through a 0.5 mm screen with a Cyclotec Mill (model 1093, Tector, Hoganas, Sweden). Total bulb S was determined by combusting 0.25-0.30 g of tissue with a Leco 232 S determinator (Leco corp., St. Joseph, Minn, USA). Samples received 0.1 gram of vanadium pentoxide accelerant (Leco corp.). Calibration and S quantification were done using certified coal standard reference materials (Leco, corp.).

Sulfate concentrations were determined using anion analysis and high performance liquid chromatography (HPLC). Sulfate was extracted from 0.25 g of ground tissue in 50 ml of HPLC grade water in 125 ml Erlenmeyer flasks. Flasks were shaken on an orbital shaker for 30 min at 150 rpm. Solution subsamples were filtered through 0.22 μm nylon syringe filters (Fisher Scientific, Pittsburg, Pa, USA) into 1 ml

plastic vials. Analysis was done on a Waters 2690 Separations Module mated to an autosampler and a Waters 432 Conductivity Detector (Waters Corp., Milford, Mass, USA). Forty µL of extract were injected into a 4.6 x 75 mm IC-PAK Anion HR column, coupled to an IC-Pak Anion Guard Pak (Waters corp.) Column temperature was maintained at 30 ^{o}C, and an isocratic sodium borate-gluconate eluent was used at a flow rate of 1.0 ml·min^{-1}. The eluent was composed of 40 ml of a sodium borate-gluconate concentrate, with 40 ml of butanol and 120 ml of acetonitrile brought to 1 L with HPLC grade water. The sodium borate-gluconate concentrate was composed of 16 g of sodium gluconate, 18 g of boric acid, 25 g sodium tetraborate, and 250 ml of glycerol brought to 1 L with HPLC grade water. Peaks were integrated against a standard curve developed using sodium sulfate standards on Millenium Chromatography Software (Version 3.05, Waters corp.).

SSC AND TPY. The bulb tissue from individual bulbs was juiced in a hand-held garlic press. A few drops of the fresh juice were placed on a hand-held refractometer to determine SSC (Kernco, Tokyo, Japan). Pungency of the onion was determined by measuring the TPY in 0.25 ml of the juice as described in the method of Randle and Bussard (1993b). Pyruvic acid, is a product of precursor hydrolysis is often used to indicate onion pungency (Schwimmer and Weston, 1961). Traditionally, pyruvic acid content has been reported as enzymatically produced pyruvic acid, (Randle and Bussard, 1993b) in which background pyruvic acid is subtracted from the total pyruvic acid measured. It has been reported that background pyruvic acid contributes little to the TPY and is likely greatly overestimated (Yoo and Pike, 2001). Therefore, TPY was reported here.

PRECURSOR AND PEPTIDE INTERMEDIATES. The ACSO and γGP content from intact bulb tissue were determined according to Randle (2000) Fresh wedges were weighed and ACSOs and γGPs were twice extracted in 12:3 methanol:water (5ml·g^{-1} fresh weight) and once in 12:3 ethanol (ETOH):water (5ml·g^{-1} fresh weight) over several days. Extracts were then combined into one solution from which 15 ml was analyzed. Extracts were stored at -20 ^{o}C until analysis. Gamma-L-glutamyl-L-glutamic acid (Sigma-Aldrich, St. Louis, MO, USA), and (±)-S-1-butyl-L-cysteine sulfoxide, synthesized according to the method of Armstrong and Lewis (1951) were added to the 15 ml of extract as internal standards in concentrations of 0.2 and 1.0 mg·g^{-1} FW, respectively. The solutions were dried using forced air (Evap-o-Rac; Cole Parmer, Vernon Hills, Ill, USA)

The dried ACSO and γGP samples were redissolved in 1 ml of HPLC grade water. A 0.50 ml aliquot of each solution was subjected to ion exchange chromatography using a 10 x 40 mm column (Bio-Rad, Hercules, Calif, USA) with 3 ml of Dowex 1 x 8 resin (200 to 400 mesh; Bio-Rad). The ACSOs were separated from the γGPs using four concentrations of glacial acetic acid (0.1, 0.2, 2 and 5 M). The ACSOs and γGPs were contained in the 0.1 and 2 M fractions while the 0.2 and 5 M fractions were discarded. The collected fractions were dried under forced air.

The dried fractions were dissolved in 1 ml of HPLC grade water, of which 100 µL was pipetted into a 1.5 ml microcentrifuge vial and then dried *in vacuo* using a Labconco Centrivap Concentrator (Labconco, Kansas City, Mo, USA). Upon dryness, 250 µL of 1:1:1 ETOH:Triethylamine (TEA):HPLC water was added to each vial and dried again. Samples were then derivitized by adding 7:1:1:1

ETOH:TEA:phenylisothiocyante:HPLC water. Vials were flushed with nitrogen gas, capped immediately after flushing, and stored at room temperature for 18 minutes. Vials were then uncapped and dried, *in vacuo*. Dry samples were dissolved in 1 ml of 7:2 HPLC water:acetonitrile and transferred to 2 ml borosilicate vials for HPLC analysis.

Samples were analyzed on a Waters 2690 Separations Module (Waters corp.) equipped with an autosamper and mated to a Waters 996 photodiode array (PDA) detector (Waters corp.). Fifty µL of sample was injected into a 250 x 4.6 mm, 5 µm column (Spheri- 5 RP-18; Applied biosystems, Foster City, Calif, USA) coupled to a 15 x 3.2 mm, 7 µm guard column (RP-18 Newgard; Applied biosystems) for separation. Column temperature was maintained at 30 ^{o} C for the duration of analysis. Eluents were: A) aqueous acetonitrile (60%), B) 0.14 M sodium acetate with 0.05% TEA buffered to a pH of 6.35 using glacial acetic acid. All eluents were filtered through 0.45 µm nylon filters (Millipore, Molsheim, France). The flow rate was set at 1.0 ml·min^{-1}. An eluent gradient run was programmed as follows: 15% A for 1.10 min, 15 - 45% over the next 21.1 min, then 45 -100% A over one min, and then held at 100% A for the next 14 min. The gradient was returned to the initial 15% A: 85% B over the next one minute, and the column was conditioned and equilibrated at that setting for the next 12.9 min until the next sample was injected.

Compounds were detected on a PDA detector set at 254 nm. Data was collected and peaks integrated using Millenium Chromatography Software (Version 3.05; Waters corp.). Peaks were assigned by comparing retention times of authentic standards as described in Randle et al., (1995).

STATISTICAL ANALYSIS. Data were subjected to analysis of variance (ANOVA), and single degree of freedom contrasts for trend analysis procedures, using SAS statistical software. (Version 8.2, SAS institute, Cary, NC, USA). Outlying data which was determined to be data points that were more than two standard deviations from the mean of remaining data points were eliminated before analysis.

Results and Discussion

BULB AND LEAF FW. N treatments affected both bulb and leaf FW ($P \leq 0.001$; $P \leq 0.001$, respectively) (Fig 3). Average bulb FW ranged from 160-503 $g \cdot bulb^{-1}$. The response of bulb FW to N concentration was quadratic ($P \leq 0.001$), with average bulb FW increasing from 20-116 $mg \cdot g^{-1}$ N, and then falling. Leaf FW also responded quadratically ($P \leq 0.001$) to N concentration. High N levels have been shown to cause excessive foliar growth, which in-turn, may decrease bulb yield (Brewster, 1990) In this study, however, bulb and leaf FW responded similarly.

SSC AND TPY. Though SSC was influenced by N levels ($P \leq 0.02$), no meaningful relationship could be ascertained. Gross flavor intensity, as measured by TPY, was also affected by N ($P \leq 0.05$) (Fig 4). TPY concentrations ranged from 3.52 to 5.55 $\mu mol \cdot g^{-1}$ FW and the response was linear with increasing N ($P \leq 0.005$). TPY also increased when onions were grown at higher N concentrations (Randle, 2000)

TOTAL BULB SULFUR AND SULFATE. Onions accumulate a large amount of S compared to most other plants. With sufficient S fertility, onion bulb S concentrations have been reported to exceed one percent on a dry weight basis (Randle 1992). Sulfur is taken up by the plant as SO_4^{-2} and can be stored in the vacuole, or incorporated into S containing organic compounds such as cysteine, methionine, and

glutathione. A large portion of the organically bound S that occurs in onions, however, is found in the S containing compounds of the flavor biosynthetic pathway (Lancaster and Boland, 1990) Because of the presence of high amounts of S in the flavor pathway in onion, factors which influence S uptake and partitioning in the bulb, are of importance to those investigating onion flavor. Our data demonstrated that total bulb S responded significantly to N concentration ($P \leq 0.001$) (Fig 5) and the response was quadratic ($P \leq 0.001$), ranging from 1.9 to 4.2 $g \cdot kg^{-1}$ on a dry weight basis. These values are similar to those reported for 'Granex 33' onions grown with high N and similar S fertility levels (Randle, 2000)

Because SO_4^{-2} is generally stored in the vacuole and unable to immediately contribute to the production of flavor compounds in onion, it is important to determine the amount of total S that is contributed by SO_4^{-2}. By knowing total S and SO_4^{-2}-S, the amount of S that is bound organically (organic-S) in the bulb can be estimated. In onion, the flavor precursors are a strong sink for available S and much of the organic-S in the bulb may be used in their synthesis (Randle et al., 1995). Though N did significantly influence SO_4^{-2}-S levels in bulbs ($P \leq 0.012$), no meaningful trend could be fitted. Levels of SO_4^{-2}-S ranged from 1.0 to 1.5 $g \cdot kg^{-1}$ dry-weight. The amount of organically bound S in the bulbs was significantly affected by N level ($P \leq 0.001$), and like total bulb S, organically bound S responded quadratically to N levels ($P \leq 0.001$) (Fig 5). Sulfate was responsible for the majority of the total S measured in those bulbs grown at the lowest levels of N. Therefore, at low N fertility, much less S accumulates in the bulb and most of that S that is left as SO_4^{-2}, thus leaving little S for incorporation into the flavor biosynthetic pathway.

PRECURSOR AND PEPTIDE INTERMEDIATES. Flavor precursors and related peptide intermediates were also measured in order to gain a better understanding of the influence of N fertility on the quality of the onion flavor and S metabolism in the bulb. Products from the three precursors of onion confer different flavor sensations. Thus, altering the ratios of individual flavor precursors can influence the overall flavor attributes of the onion (Randle et al., 1994)

Total precursor concentration was significantly affected by N level ($P \leq 0.001$) (Fig 6A). Total average ACSO concentrations ranged from 1.2 $mg \cdot g^{-1}$ FW at 20 $mg \cdot L^{-1}$ N, to 6.3 $mg \cdot g^{-1}$ FW at 116 $mg \cdot L^{-1}$ N. The response was quadratic ($P \leq 0.04$). Total ACSO concentration has previously reported to be influenced by very high N fertility levels.[15]

N fertility also influenced the concentration of MCSO in the bulb ($P \leq 0.001$) (Fig 6A). Average bulb concentrations of MCSO ranged from 0.48 to 4.32 $mg \cdot g^{-1}$ FW. A significant quadratic trend for MCSO ($P \leq 0.07$) was found in response to N level. The thiosulfinates resulting from the breakdown of MCSO by alliinase lend a fresh onion and cabbage-like taste to the bulb upon eating (Randle et al., 1994). Higher N levels, therefore, should accentuate these flavors.

N fertility also influenced concentrations of 1-PRENCSO in the bulb ($P \leq 0.001$). Average bulb concentrations ranged from 0.69 $mg \cdot g^{-1}$ FW to 1.83 $mg \cdot g^{-1}$ FW. 1-PRENCSO best fit a quadratic response to N fertility levels ($P \leq 0.03$). Upon decomposition, 1-PRENCSO contributes to the lachrymatory factor responsible for the burning and tearing sensations, and other onion flavors (Randle et al., 1994)

PCSO was affected by N fertility levels concentrations ($P \leq 0.007$) and ranged from 0.04 to 0.19 $mg \cdot g^{-1}$ FW, which was a five-fold increase. The response was linear ($P \leq 0.02$). While PCSO is a minor precursor, it can accumulate to as much as 10% of the total precursor content (Randle et al., 1994), as was the case in this study. Upon decomposition, PCSO is responsible for the formation of thiosulfinates that result in fresh onion and chive-like flavor sensations (Randle et al., 1994)

Of the three precursors, MCSO had the greatest average change in response to increasing N levels (Fig. 6A). As such, the composition of the flavor precursors varied significantly. For example, at 92 $mg \cdot L^{-1}$ N, MCSO contributed, on average, 61% of the total precursors measured, while 1-PRENCSO was responsible for about 34% of the total. PCSO constituted approximately 5% of the total precursor content. In contrast, at N levels of 80 $mg \cdot L^{-1}$, MCSO was responsible for 45% of the total precursors on average, while 1-PRENCSO accounted for 51% of the total. At 20 $mg \cdot L^{-1}$ N, MCSO made up about 40% of the total precursor content, with 1-PRENCSO accounting for nearly 57%, with PCSO contributing about 3% on average. This trend was reversed at highest N treatment where MCSO made up about 54% of the total ACSOs, and 1-PRENCSO contributed 39% of the total. Our data therefore suggests that N levels ranging between 56 and 80 $mg \cdot L^{-1}$ result in similar amounts of MCSO and 1-PRENCSO synthesis. N levels below this range cause a substantial shift in the biosynthetic pathway which favors 1-PRENCSO synthesis, while higher N levels favor MCSO. Similarly, the previous study using high solution N levels resulted in high MCSO synthesis and accumulation (Randle, 2000). Ultimately, changes in these precursor ratios will result in bulb flavor changes, as individual ACSOs impart different flavor sensations (Randle et al., 1994).

Historically, 1-PRENCSO was reported to be the main ACSO in onion (Block, 1992; Lancaster and Boland, 1990). Evidence now suggests that MCSO synthesis is enhanced and may exceed 1-PRENCSO when onions are subjected to abnormal growing conditions such as low-S fertility (Randle et al., 1995), high sodium selenate fertility (Kopsell and Randle, 1999), and now, high N fertility.

Intermediates in the flavor biosynthetic pathway were also influenced by N fertility (Fig. 6B). The concentration of 2-carboxypropyl glutahione (2-Carb) in the bulb was significantly influenced by N fertility ($P \leq 0.001$) and the response was linear ($P \leq 0.002$). Gammaglutamyl propenyl cysteine sulfoxide (γGPECSO) was the other intermediate in the flavor biosynthetic pathway that was significantly influenced by N level ($P \leq 0.002$) with a quadratic response to increasing N levels ($P \leq 0.001$). Because γGPECSO is the penultimate precursor in the synthesis of 1-PRENCSO, it could be expected that these two would respond similarly to N. Levels of 1-PRENCSO and γGPECSO were also shown to be associated when responding to varying levels of S fertility (Randle et al., 1995).

Conclusion

The purpose of this study was to determine how S metabolism and onion flavor were influenced by a broad range of N fertility levels. In particular we were interested in N levels that would cause major shifts in the synthesis and accumulation of the individual flavor precursors. The importance in assessing changes in precursor accumulation is that major shifts would affect onion flavor quality. Total bulb S responded quadratically to N fertility levels while bulb SO_4^{-2} accumulation was unresponsive, indicating that increasing N up-regulated S reduction and its subsequent assimilation into organic forms. Of

particular interest were the preference for MCSO accumulation at the higher N levels, and conversely, the preference for 1-PRENCSO accumulation at lower N levels. The redirection of metabolized S through the biosynthetic pathways leading to precursor accumulation as a response to environmental change is now a well-documented occurrence in onion (Freeman and Mossadeghi, 1970; Kopsell and Randle, 1999; Randle and Lancaster, 2002; Randle 2000; Randle et al., 1994; Randle et al., 1995). These data have significance and usefulness for growers of onions where flavor quality and intensity are of particular importance. A shift in the relative concentration of the individual bulb precursors will ultimately affect flavor quality (Block, 1992; Randle and Lancaster, 2002; Randle et al., 1994). The mechanism(s) which drive S redirection through these pathways, however, remains unknown and requires further investigation. Because onions are grown primarily for their flavor attributes, cultural practices such as N fertility levels, need to be considered for their effects on flavor as well as yield.

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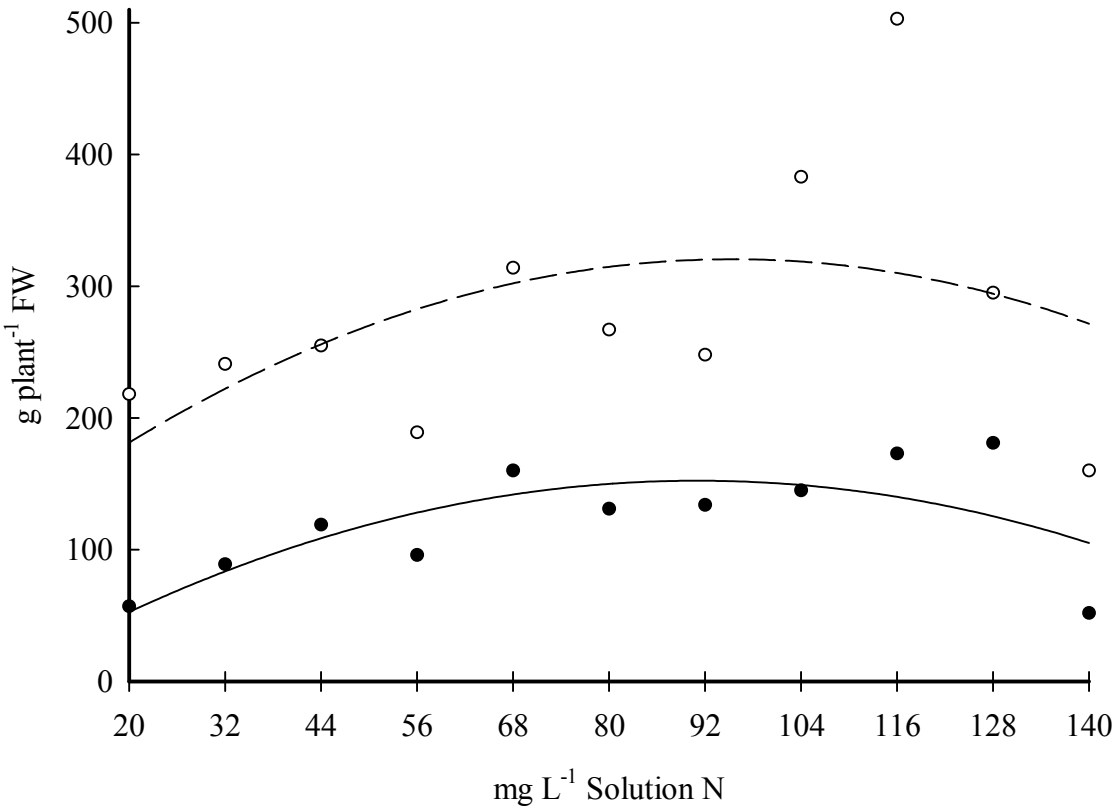
Figure 3. The response of leaf (●—, quadratic $P \leq 0.001$) and bulb (o---, quadratic $P \leq 0.001$)) fresh weight to varying nitrogen levels in hydroponically grown ‘Granex 33’ onions (*Allium cepa* L.).


500
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100
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g plant-1 FW
20
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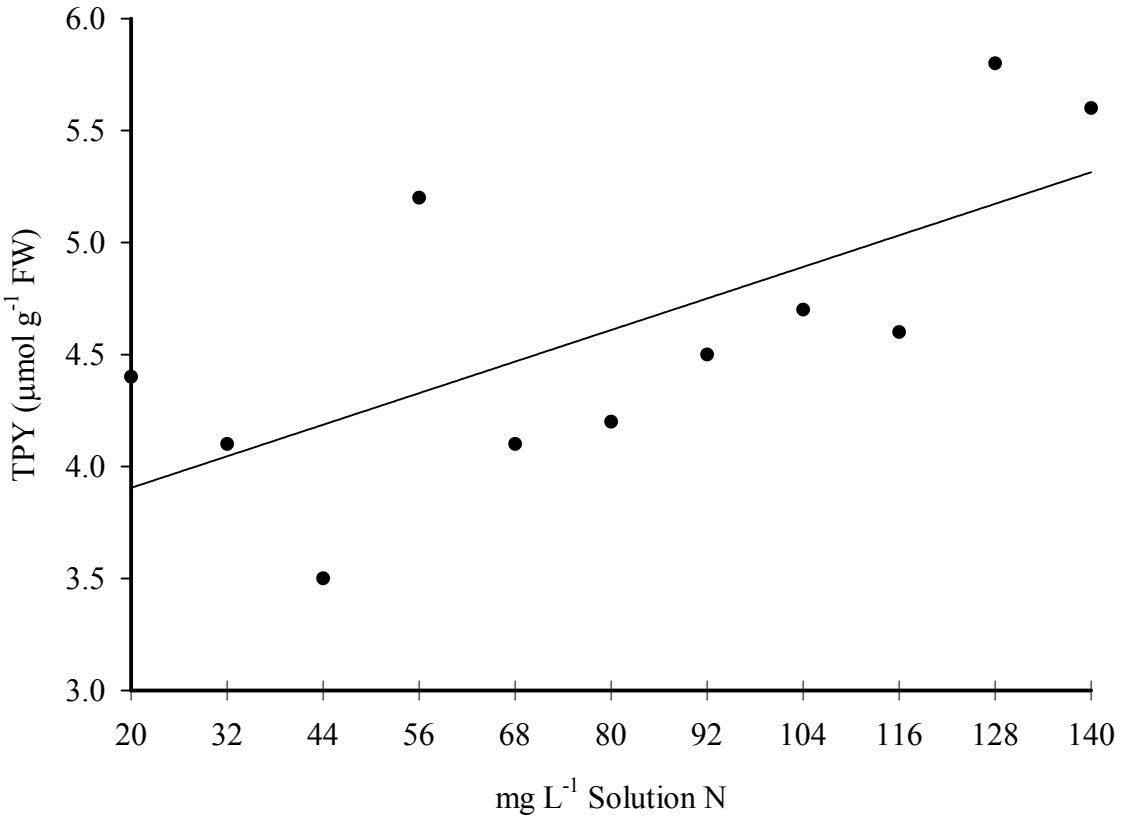
56
68
80
92
104
116
128
140
mg L-1 Solution N

Figure 4. The effect of increasing nitrogen solution levels on the concentration of total pyruvic acid (linear $P \leq 0.001$) in 'Granex 33' onion (*Allium cepa* L.).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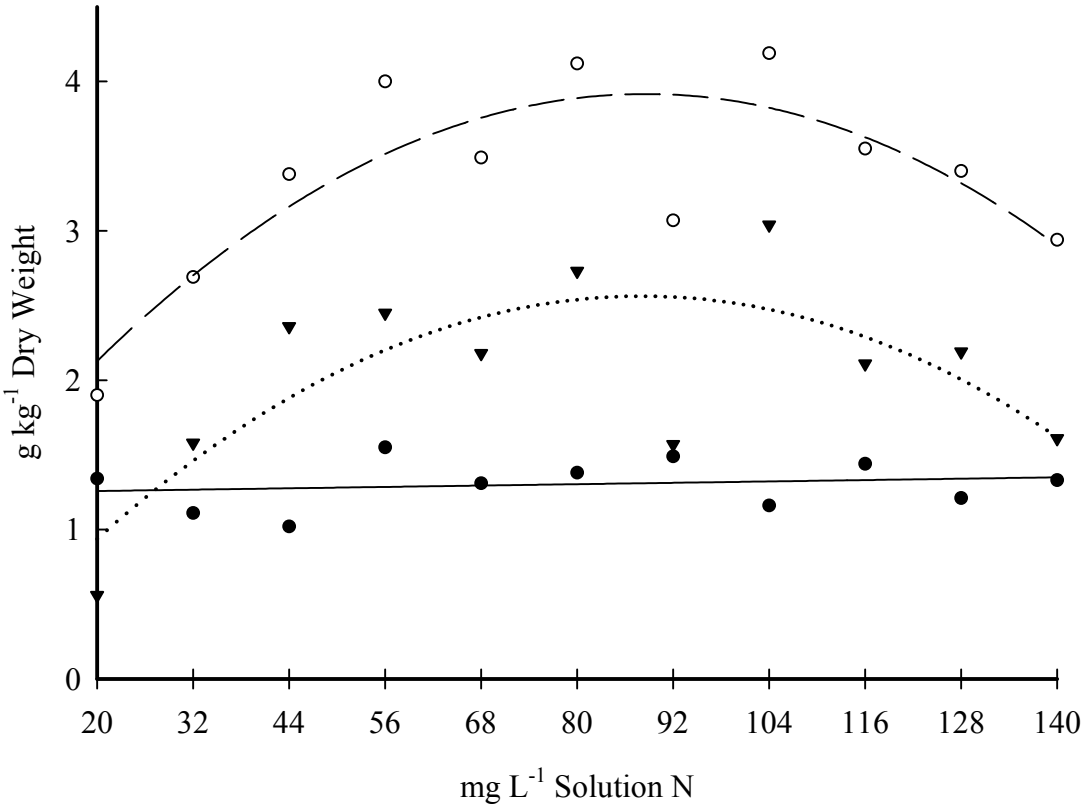
mg L-1 Solution N

Figure 5. The effect of nitrogen solution levels on the response of the concentrations of total bulb sulfur (o---, quadratic $P \leq 0.001$), S contributed from sulfate (●—, ns), and organic S (▼····, quadratic $P \leq 0.001$) in 'Granex 33' onion (*Allium cepa* L.).


g kg-1 Dry Weight
0
1
2
3
4
20
32
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80
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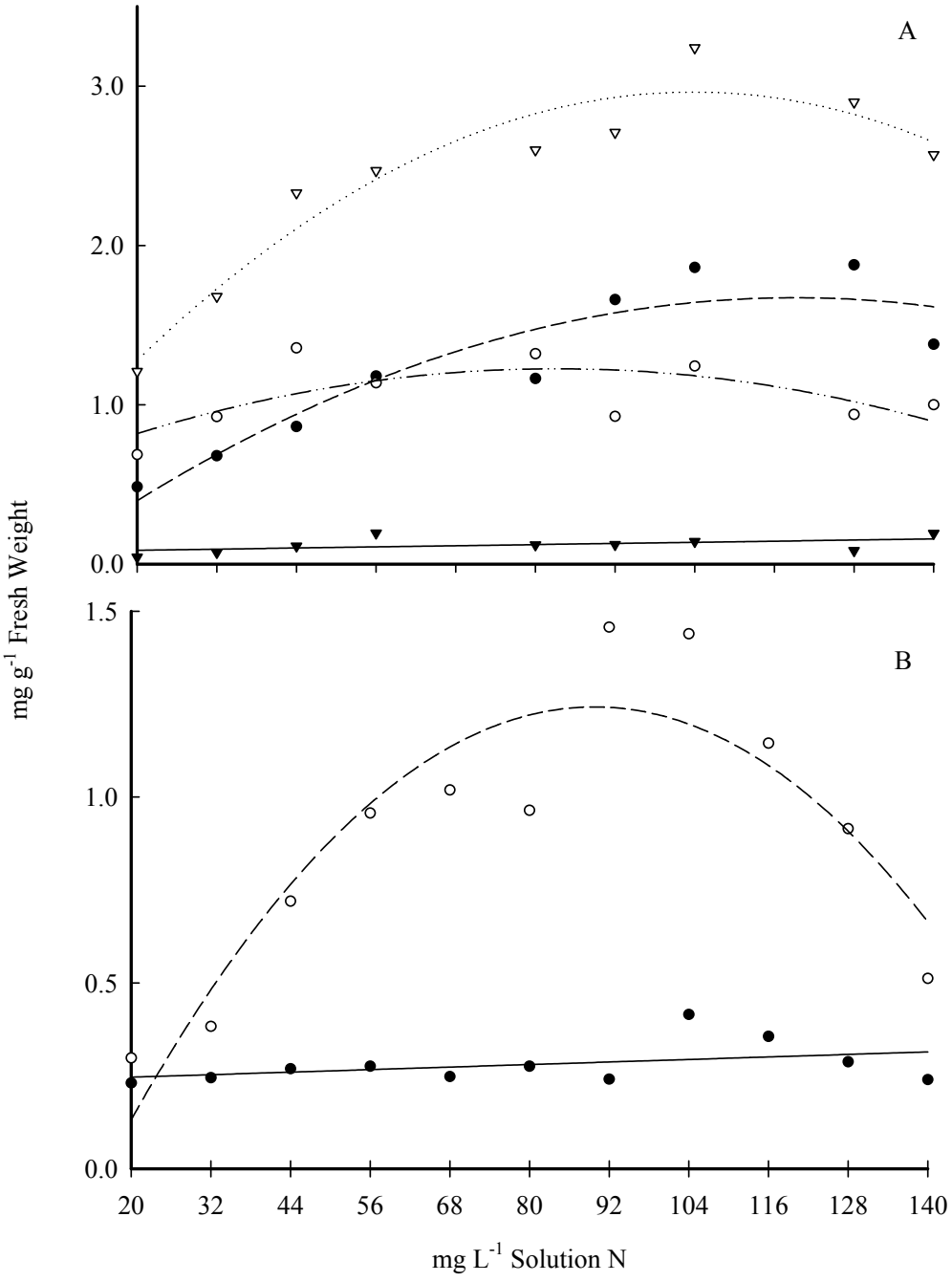
mg L-1 Solution N

Figure 6 A. The effect of nitrogen solution levels on the flavor precursors in 'Granex 33' onion (*Allium cepa* L.) bulbs. Total S-alkenyl cysteine sulfoxides (▽····, $P \leq 0.001$), (+)-S-methyl-L- cysteine sulfoxide (● ---, $P \leq 0.07$), and *trans*-(+)-S-(1-propenyl)-L-cysteine sulfoxide (○··–, $P \leq 0.03$) responded quadratically to N levels. A linear increase was observed for (+)-S-propyl-L-cysteine sulfoxide (▼—, $P \leq 0.02$).

B. N levels influenced bulb peptide intermediates in the flavor biosynthetic pathway. γGlutamyl propenyl cysteine sulfoxide (○---, $P \leq 0.001$) responded quadratically to N concentration, but 2-Carboxypropyl glutathione (●—, $P \leq 0.001$) responded linearly to N solution levels.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mg g-1 Fresh Weight
mg L-1 Solution N

CHAPTER 5

NITROGEN AND SULFUR AVAILABILITY INTERACT TO AFFECT THE FLAVOR BIOSYNTHETIC PATHWAY IN ONION[3]

[3] Coolong, T.W. and W.M. Randle. To be submitted to J. Amer. Soc. Hort. Sci.

Additional index words: *Allium cepa*, methyl cysteine sulfoxide, 1-propenyl cysteine sulfoxide, propyl cysteine sulfoxide

Abstract

To determine the extent to which sulfur (S) and nitrogen (N) fertility interact to influence the flavor biosynthetic pathway in onion (*Allium cepa* L.), 'Granex 33' onions were grown in hydroponic solution culture with varying levels of S and N availability. Plants were grown at 5, 45 or 125 $mg \cdot L^{-1}$ sulfate (SO_4^{2-}), and 10, 50, 90 or 130 $mg \cdot L^{-1}$ N, in a factorial combination. Bulb FW, total bulb S, total and individual flavor precursors and their peptide intermediates in intact onion tissue were measured. To measure the effect of S and N on alliinase activity, flavor precursors were also measured in onion macerates. S and N availability in the hydroponics solution interacted to influence all flavor compounds except *S*-methyl-L-cysteine sulfoxide. Levels of *S*-methyl-L-cysteine sulfoxide were influenced by N and S levels in the solutions; however, no interaction was present. At the lowest SO_4^{2-} or N levels most precursors and peptides measured were present in very low concentrations. When SO_4^{2-} or N availability was adequate, differences among flavor compounds were small. Results indicated that S fertility had a greater influence on *trans*-*S*-1-propenyl-L-cysteine sulfoxide accumulation, while N availability had a greater influence on S-methyl-L-cysteine sulfoxide levels. Flavor precursors remaining in the onion macerates revealed that the percentage of intact precursors hydrolyzed by alliinase were not significantly influenced by either SO_4^{2-} or N levels in the solutions, except for 1-PRENCSO, which was affected by N levels. N and S fertility interacted to influence the flavor biosynthetic pathway and may need to be considered together when manipulating onion flavor compounds.

Introduction

Onions, (*Allium cepa* L.) have been cultivated since antiquity primarily for their flavoring properties. Onion flavor is dominated by the presence of organosulfur compounds, in particular the *S*-alk(en)yl-L-cysteine-sulfoxides (ACSO). During consumption onion vacuoles lyse allowing the enzyme alliinase to hydrolyze the individual ACSOs in the bulb, giving rise to the compounds responsible for onion's characteristic flavor and aroma. As such, onions accumulate large quantities of S. Randle (1992) reported bulb sulfur (S) concentrations in excess of one-percent on a dry weight basis in plants grown with high S fertility. Much of the S taken up by onions is partitioned into organosulfur compounds as part of the flavor biosynthetic pathway.. Randle et al. (1995) reported that up to 95% of the organically bound S in onion bulbs grown in low S fertility could be accounted for in compounds from the flavor biosynthetic pathway.

S enters the plant actively as sulfate (SO_4^{2-}). SO_4^{2-} is transported to the leaves, where it can be stored in the vacuole or be reduced to sulfide and assimilated into cysteine in the chloroplast (Hell, 1997). Cysteine is quickly incorporated into proteins or used in the synthesis of methionine and glutathione (Leustek et al., 2000). In onion, cysteine and glutathione serve as precursors to a number of gammaglutamyl peptides (γGP), which are utilized in the flavor biosynthetic pathway (Randle and Lancaster, 2002).

Because the ACSOs and their breakdown products are organosulfur compounds, S fertility has been thoroughly investigated as a means of altering onion flavor. Onion pungency increased in response to enhanced S fertility (Freeman and Mossadeghi, 1970;

Hamilton et al., 1997; Randle 1992; Randle and Bussard, 1993). Changes in S fertility also resulted in alterations in the concentration and composition of the ACSOs in onion, which ultimately could influence flavor quality in the bulb (Randle et al., 1995). As a result it is generally accepted that S fertility is a primary factor by which one can greatly alter onion flavor. The influence of most other mineral nutrients on onion flavor, on the other hand, are less understood.

Most investigations on the effects of N fertility and onion have focused on developmental and yield aspects (Brewster and Butler, 1989; Brown et al., 1988; Drost et al., 2002; Hussaini et al., 2000). However, Randle (2000) reported that very high levels of nitrogen (N) could influence onion pungency as well as the composition of ACSOs in the bulb. Additionally, N form in a hydroponic solution was shown to affect onion pungency (Gamiely et al., 1991). Because high-N fertility has affected onion flavor, further inquiries using lower N levels are warranted.

Several investigations have shown a relationship between nitrate (NO_3^-) and SO_4^{2-} reduction in plants. A 50-70 % decrease in adenosine 5'-phosphosulfate reductase (APR), a key enzyme in SO_4^{2-} assimilation, in response to the absence of N fertility in *Arabidopsis thaliana* was reported (Koprivova et al. 2000). Decreases in APR as well as adenosine triphosphate sulfurylase were also observed in *Lemna minor* when plants were exposed to decreasing NO_3^- levels (Brunold and Suter, 1984). Restricted NO_3^- fertility repressed another enzyme in the SO_4^{2-} reduction pathway, ferredoxin-sulfite reductase, in leek (*A. tuberosum*) (Takahashi et al., 1996). S and N fertility interacted to influence, S values in *Brassica napus,* a high-S accumulating plant (Pinkerton, 1998). Additionally, S deprivation reduced NO_3^- uptake in curly kale (*B. oleracea* L.) (Stuiver et al., 1997).

Our purpose here was to investigate the interaction of changes in N and S availability on the flavor biosynthetic pathway in onion. Previous investigations reported on either N or S alone, with no chance to observe possible interactions.

Materials and Methods

PLANT CULTURE. On 25 Jan. 2002, 'Granex 33' onions (Asgrow Seeds, Kalamazoo, Mich.) were seeded into sheets of 2.54 cm^2 rock-wool cubes (Grodan, Hedenhusene, Denmark). Seedlings were fertilized twice weekly with a full strength Hoagland's #2 nutrient solution and watered as needed (Hoagland and Arnon, 1950). Seedlings were greenhouse grown at day/night setpoints of 25/18 ^oC under natural photoperiods (≈ 34^o N latitude) for five weeks. Radiant flux as measured at noon on a clear sunny day was 1230 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Seedlings received two applications of metalaxyl (Ridomil; Syngenta, Greensboro, NC) at two and four weeks of age. On 3 Mar. 2002 plants were transferred to 37.9-L plastic containers (Rubbermaid, Inc., Wooster, Ohio). Ten plants were placed in 2.54 cm diameter holes spaced 10 x 16 cm on the container lids. Each container was brought to 28 L with deionized water and nutrient salts were added. The study was a three x four factorial design, with three SO_4^{2-} treatment levels and four N treatment levels. The experiment was completely randomized with 12 total treatment levels, each having four replications of with ten plants per replication. The S levels consisted of 5, 45, and 125 $mg \cdot L^{-1}$ SO_4^{2-}, which were achieved by the addition of 12.9, 115.8, and 321.3 $mg \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, respectively. The levels of SO_4^{2-} were so chosen because previous research has shown that onion flavor is most responsive to SO_4^{2-} over a similar range (Freeman and Mossadeghi, 1970; Randle et al., 1995). Magnesium concentrations were balanced through the addition of 253.5 and 168.4 $mg \cdot L^{-1}$

$MgCl_2 \cdot 6H_2O$ to the 5 and 45 $mg \cdot L^{-1}$ SO_4^{2-} treatments, respectively. Chloride concentrations were not balanced, and ranged between 132 and 219 $mg \cdot L^{-1}$ in this study, which are well within concentrations used in previous studies (Randle and Bussard, 1993; Randle et al., 1995). The N treatment levels consisted of 10, 50, 90, and 130 $mg \cdot L^{-1}$ N, which were achieved through the addition of 28.5, 142.7, 256.5, and 371 $mg \cdot L^{-1}$ NH_4NO_3, respectively. The N levels used were based on previous research indicating that onion flavor responded to a wide range of N concentrations (unpublished data). All solutions contained 174.2 $mg \cdot L^{-1}$ K_2HPO_4, 95 $mg \cdot L^{-1}$ KCl, 138 $mg \cdot L^{-1}$ $CaCl_2$, 10 $mg \cdot L^{-1}$ Iron chelate (Sequestrene 330), 2.86 $mg \cdot L^{-1}$ H_3BO_3, 1.81 $mg \cdot L^{-1}$ $MnCl_2 \cdot 4H_2O$, 0.22 $mg \cdot L^{-1}$ $ZnSO_4 \cdot 7H_2O$, 0.08 $mg \cdot L^{-1}$ $CuSO_4 \cdot 7H_2O$, and 0.04 $mg \cdot L^{-1}$ $Na_2MoO_4 \cdot 2H_2O$. Nutrient solutions were brought to volume daily with deionized water and were completely replaced every two weeks. The average pH of initial solutions were 7.3, which and fell to 6.3 over the two week period. Electrical conductivity (EC) of the nutrient solutions was measured at the beginning and end of each two-week period (model 09-326; Fisher Scientific, Pittsburgh, Pa.). Average EC of the initial solutions ranged from ≈ 1100 to 1800 μS and fell to between 1000 to 1600 μS early in the experiment. EC measured near the end of the experiment fell to between 600 to 1200 μS over the two-week period. Solution aeration was provided with a single 14.4 cm aquarium airstone per tub. Foliage was supported at a height of about 25 cm above the tub lid with welded wire mesh.

Plants were greenhouse grown to maturity at day/night set points of 25/18 ^oC under natural photoperiods and light intensities. Plants were considered mature when at least 50% of the plants in a given treatment had soft pseudostems. Plants were harvested

between 15 and 23 June 2002. Plants receiving low S or N treatments matured first, while those receiving high S or N matured last. At harvest, roots and foliage were removed and average bulb fresh weights were obtained. Bulbs were then placed in mesh bags and cured at ambient greenhouse temperatures for one week. The eight most uniform bulbs were selected from each replication for analysis.

Analyses were performed on the combined tissue from each eight-bulb replication. Tissue for the analyses was obtained by cutting three, five mm thick longitudinal wedges from each bulb. One wedge group was used to measure total S , while the second wedge group was used to measure the intact ACSOs and γGPs. A third wedge group was used to measure flavor precursors in the onion macerate.

TOTAL BULB S. Bulb tissue was dried at 65 ^{o}C in a forced air oven (Linberg Blue, Asheville, N.C.) for 5 d. Dried tissue was then ground through a 0.5 mm screen with a Cyclotec Mill (model 1093; Tector, Hoganas, Sweden). Total bulb S was determined using 0.25 g of tissue on a Leco 232 S determinator (Leco Corp., St. Joseph, Minn.). Samples received 0.1 g of vanadium pentoxide accelerant before analysis (Leco Corp.). Total bulb S was determined by averaging the results from three subsamples from each treatment level and replication. Calibration and S quantification were done using certified standard reference materials (Rice Flour; Leco Corp.).

ACSOs AND THEIR PEPTIDE INTERMEDIATES. The ACSO and γGP contents from intact bulb tissue were determined according to Randle (2000). The tissue wedges from each eight bulb replication were weighed and the ACSOs and γGPs were extracted twice (using 5mL$\cdot$g^{-1} fresh weight) in 12 methanol (MEOH): 3 water and once in 12 ethanol (ETOH): 3 water over a 3 d period. Extracts were then combined into one

solution from which 15 mL was analyzed. Gamma-L-glutamyl-L-glutamic acid (Sigma, St. Louis, MO) and (±)-S-1-butyl-L-cysteine sulfoxide, synthesized according to the method of Lancaster and Kelly (1983) and Armstrong and Lewis (1951), were used as internal standards in concentrations of 0.2 and 1.0 $mg \cdot g^{-1}$ FW, respectively. Standards were added to 15 mL of the extract solutions, which were then dried using forced air (Evap-o-Rac; Cole Parmer, Vernon Hills, Ill.). The ACSOs measured were: *S*-methyl-L-cysteine sulfoxide (MCSO), *trans*-*S*-1-propenyl-L- cysteine sulfoxide (1-PRENCSO), and *S*-propyl-L- cysteine sulfoxide (PCSO). The two γGPs measured were 2-carboxypropyl glutathione (2-Carb) and γ-glutamyl propenyl cysteine sulfoxide (γGPECSO).

ACSOs remaining in the onion macerate were extracted using the method of Lancaster et al. (1998). After an incubation period of 10 min at room temperature (25 ^{o}C), a 0.5 mL aliquot of onion juice macerate was placed in a 20 mL scintillation vial to which 10 mL of 12 MEOH: 3 water was added. Internal standards were added as described above and the entire solution was dried using forced air.

The dried ACSO samples were redissolved in 1 mL of 18 MΩ water. A 0.75 mL aliquot of each solution was then subjected to ion exchange chromatography using a 10 x 40 mm column (Bio-Rad, Hercules, Calif.) with 3 mL of Dowex 1 x 8 resin (200 to 400 mesh; Bio-Rad). The ACSOs were separated from the γGPs using four successive concentrations of glacial acetic acid (0.1, 0.2, 2 and 5 M). The ACSOs and γGPs were contained in the 0.1 and 2 M fractions while the 0.2 and 5 M fractions were discarded. The collected fractions were dried under forced air.

Dried sample fractions were redissolved in 1.0 mL of 18 MΩ water, from which 100 µL was pipetted into a 1.5 mL microcentrifuge vial and dried *in vacuo* using a Labconco Centrivap Concentrator (Labconco, Kansas City, Mo.). Upon dryness, 250 µL of 1 ETOH: 1 Triethylamine (TEA): 1 HPLC water was added to each vial and dried again. Samples were then derivitized by adding 7 ETOH: 1 TEA: 1 phenylisothiocyante : 1 18 MΩ water. Vials were immediately flushed with nitrogen gas, capped, and stored at room temperature for 18 min. Vials were then uncapped and dried. Dry samples were redissolved in 1 mL of 7 water: 2 acetonitrile and transferred to 2.0 mL borosilicate vials for HPLC analysis.

Samples were analyzed on a Waters 2690 Separations Module (Waters Corp.) equipped with an autosamper and coupled to a Waters 996 photodiode array detector (Waters Corp.). Fifty micro-liters of sample was injected into a 250 x 4.6 mm, 5 µm column (Spheri- 5 RP-18; Applied Biosystems, Foster City, Calif.) mated to a 15 x 3.2 mm, 7 µm guard column (RP-18 Newgard; Applied Biosystems) for separation. Column temperature was maintained at 30 ^{o}C. Eluents were A) aqueous acetonitrile (60%), B) 0.14 M sodium acetate with 0.05% TEA buffered to a pH of 6.35 using glacial acetic acid. All eluents were filtered through 0.45 µm nylon filters (Millipore, Molsheim, France). The flow rate was set at 1.0 mL$\cdot$min^{-1} for the duration of the run. A gradient run was programmed as follows: 15% A for 1.1 min, 15% - 45% over the next 21.1 min, then 45% -100% A over the next one min, and then hold at 100% A for the next 14 min. The gradient was returned to the initial 15% A: 85% B over the next 1.0 min., and the column was conditioned at that setting for the next 12.9 min until the next sample was injected.

Compounds were detected on a photodiode array detector set at 254 nm. Data were collected and peaks integrated using Millenium Chromatography Software (Version 3.05; Waters corp.). Peaks were assigned by comparing retention times of authentic standards prepared as described in Lancaster and Kelly (1983) and Randle et al. (1995)

Statistical Analysis. Data were subjected to the GLM procedure and linear and polynomial regression analyses when appropriate, using SAS statistical software. (Version 8.2, SAS institute, Cary, N.C.). Percentage data were tested for normality, and arcsin transformations were performed. Contrasts among the treatments were done using Scheffe's method of multiple comparisons.

Results and Discussion

PLANT GROWTH RESPONSE. Early in the experiment, no growth differences were observed between various treatments. As onions entered their tenth week of growth visible differences were observed among the low-N treatments and all others, regardless of S level. Plants grown in 10 $mg \cdot L^{-1}$ N had fewer leaves, and exhibited yellowing. At that time S did not visibly appear to be influencing growth. Observations near the end of the experiment (≈15 weeks) showed that plants exposed to low-N levels matured early and had uniformLy small bulbs, regardless of S level. At this time, plants exposed to low-S (5 $mg \cdot L^{-1}$ SO_4^{2-}) had visibly smaller bulbs across N levels.

BULB FRESH WEIGHT. Bulb FW was influenced by S ($P < 0.001$, F=25.1) and N ($P < 0.001$, F=35.0) availability in the solution (Table 5). Additionally, S and N fertility interacted to influence bulb FW ($P < 0.001$, F=5.1) (Fig. 7A) according to the GLM procedure. Average bulb FW varied from 96 to 206 $g \cdot bulb^{-1}$ and responded quadratically to changes in S and N availability (Table 6). Not surprisingly, plants grown

at the lowest levels of S and N produced bulbs of the smallest FWs. Similarly, Freeman and Mossadeghi (1970) reported that low S fertility in a sand culture system could negatively influence bulb FW. Randle (2000) reported that bulb FW decreased with increasing N availability in solution culture; however, in that study plants were grown at very high levels of N, and it was suspected that high N levels produced excessive foliage at the expense of bulb yield.

TOTAL BULB S. Both S and N levels in the nutrient solution interacted to affect total bulb S ($P < 0.001$, F=28.4) (Fig 7B). Individually, S ($P < 0.001$, F=285.8) and N ($P < 0.001$, F=29.4) levels significantly influenced total bulb S (Table 5). Average total bulb S ranged from 0.14 to 0.47 % dry-weight (Table 6). Similarly, Randle et al. (1999) reported a three-fold increase in total bulb S in response to increasing S fertility. Minimal changes in total bulb S were observed in onions grown over several high N levels (Randle, 2000). Similarly, while plants grown at the lowest S or N levels had uniformly low levels of total bulb S (0.14-0.20%), bulb S rose linearly with S concentrations in the solution, while the response of bulb S to N levels above 10 $mg \cdot L^{-1}$ was more variable. Our data suggests that low-N fertility depressed bulb total S accumulation much in the way that low-S fertility did (Randle et al., 1995), hence the significant interaction. Because bulb FW was closely correlated to total S ($p = 0.72$), and bulbs grown at low-S or low-N were unusually small, it is likely that the lowest S and N levels limited plant metabolism, thus decreasing the need for excess S which is often sequestered in the flavor biosynthetic pathway.

INTACT FLAVOR PRECURSORS AND γGP INTERMEDIATES. Total ACSOs are simply the sum of the three individual ACSOs measured, and are useful in

describing the net effect of treatments on the flavor pathway in onions. Though N and S levels interacted to influence individual ACSOs, only N fertility significantly influenced total ACSO accumulation ($P < 0.001$, F = 50.3) according to the GLM procedure (Table 7). Levels of total ACSOs in the bulb ranged from 1.07 to 2.81 $mg \cdot g^{-1}$ FW, and responded quadratically to N levels (Table 6), increasing sharply between 10 and 50 $mg \cdot L^{-1}$ N and then leveling off. Single degree of freedom contrasts between N levels indicated that plants grown at 10 $mg \cdot L^{-1}$ N, had significantly lower ($P < 0.05$) levels of total ACSOs in the bulb than all other N levels. Total ACSOs did not differ in bulbs grown at 45, 90 and 130 $mg \cdot L^{-1}$ N.

Both S ($P < 0.001$, F= 13.9) and N ($P < 0.001$, F=52.8) fertility individually influenced the accumulation of MCSO in the bulb; however, they did not interact to affect MCSO concentrations (Table 7). Bulb MCSO content ranged from 0.57 to 1.91 $mg \cdot L^{-1}$ FW and generally increased with rising N levels, but fell with increasing S. Similar responses of bulb MCSO concentrations to S or N fertility have been previously reported (Randle, 2000; Randle et al., 1995). Although S was shown to significantly affect MCSO concentrations according to the GLM procedure, it did not contribute significantly to the regression response, which when fitted, showed only a quadratic response to N concentration in the solution (Table 6). Consequently, MCSO concentrations increased nearly 250% across N treatment levels, while similarly spaced changes SO_4^{2-} availability resulted in smaller, 12 to 65%, changes in MCSO (Fig. 8B). Our data suggests that N, and not S availability had the greatest an impact on bulb MCSO concentrations. Because many of the intermediates in the MCSO pathway have not been

routinely identified in vivo, we have not measured the possible changes in the peptides leading to the synthesis of MCSO.

Solution S levels significantly influenced bulb 1-PRENCSO concentrations ($P < 0.001$, $F = 215.8$), but the main effects of solution N were insignificant (Table 7). Furthermore, S and N interacted to significantly influence 1-PRENCSO concentrations in the bulb ($P < 0.001$, $F = 17.55$). For those plants grown at either 50, 90 or 130 $mg \cdot L^{-1}$ N, concentrations of 1-PRENCSO increased roughly seven-fold as SO_4^{2-} levels in the solutions rose from 5 to 45 $mg \cdot L^{-1}$. Levels of 1-PRENCSO were unaffected between 45 and 125 $mg \cdot L^{-1}$ SO_4^{2-}, regardless of N level (Fig. 8C). Interestingly, S levels in the solution did little to change 1-PRENCSO concentrations in bulbs when plants were grown at 10 $mg \cdot L^{-1}$ N. This suggests that while S and N levels do interact to influence 1-PRENCSO in the bulb, the S level in a solution has a much greater affect on 1-PRENCSO than does N level. Randle et al. (1995) similarly reported large changes in 1-PRENCSO concentrations in response to S fertility levels with sufficient N, but noted much smaller changes in 1-PRENCSO in response to N fertility with sufficient S levels (Randle, 2000).

Both S ($P < 0.012$, $F = 5.2$) and N ($P < 0.001$, $F = 16.4$) fertility levels influenced bulb PCSO concentrations (Table 7). Additionally, there was a significant interaction between S and N fertility, affecting bulb concentrations of PCSO ($P < 0.03$, $F = 2.7$). Generally, PCSO levels in the bulb increased as the availability of N increased but fell as S fertility increased (Fig. 8D). Bulb concentrations of PCSO are typically much lower than either MCSO or 1-PRENCSO, (Kopsell et al., 2001; Kopsell and Randle, 1999; Randle, 2000). However, in this experiment PCSO concentrations were higher than those

of 1-PRENCSO for bulbs grown at 5 $mg \cdot L^{-1}$ SO_4^{2-} and either 50, 90 or 130 $mg \cdot L^{-1}$ N (Table 7). Randle et al (1995) reported similar results in plant grown with 0.10 $meq \cdot L^{-1}$ S fertility.

The pathway leading to the synthesis of PCSO in onion is similar to that of 1-PRENCSO as they share several intermediates. Therefore, it is noteworthy that the synthesis of one of these precursors is favored over the other under conditions of low-S and adequate-N availability in the nutrient solutions. Because the ratio of PCSO to 1-PRENCSO changes with S and N fertility, it seems probable that a point of regulation in the synthesis of PCSO versus 1-PRENCSO occurs when γglutamyl-*S*-1-propenyl cysteine, which is reported to be the last common intermediate, diverges to form either PCSO or 1-PRENCSO. A detailed review of the synthesis and chemistry of the ACSOs can be found in Randle and Lancaster (2002) or Block (1992).

Apart from the implications for onion flavor quality, the response of the individual flavor precursors to S and N availability provided some insight into the metabolic regulation of the entire ACSO pathway. Our data suggests that the pathway leading to the synthesis of MCSO is regulated primarily by N availability, whereas the synthesis of 1-PRENCSO is regulated to a greater extent by S availability. Previous research further supports our findings (Randle 2000; Randle et al., 1995). It is an interesting question as to why the individual ACSOs are influenced differently by either S or N nutrition. It is also unclear the advantage conferred to the plant when a particular ACSO is preferred in these different situations.

Two peptide intermediates, 2-Carb and γGPECSO were measured in response to S and N nutrition. Concentrations of 2-Carb were influenced by S ($P < 0.001$, F=31.1)

and N ($P < 0.001$, F=36.1) availability in the solution (Table 7). Additionally, both nutrients interacted to influence levels of 2-Carb in the bulb ($P < 0.002$, F = 3.5), which tended to increase as both S and N concentrations rose (Fig. 2E). Concentrations of 2-Carb in the bulb ranged from 0.43 to 0.97 $mg \cdot g^{-1}$ FW and were highly correlated to total bulb S (r = 0.85). This would be expected as 2-Carb has been hypothesized to be a storage reservoir for N and S in the bulb (Lancaster and Shaw, 1991). Interestingly 2-Carb is an intermediate in the production of both 1-PRENCSO and PCSO; however, correlations between 2-Carb and 1-PRENCSO (r = 0.61) or 2-Carb and PCSO (r = 0.41) were poor.

Bulb γGPECSO levels responded significantly to S ($P < 0.001$, F=49.2) and N ($P < 0.001$, F=23.9) fertility. Similar to 2-Carb, S and N fertility levels interacted to influence levels of γGPECSO in the bulb ($P < 0.001$, F = 6.1). Generally, γGPECSO concentrations increased with N and S fertility, much the way levels of 2-Carb did. However, roughly a ten-fold difference was observed between the lowest and highest levels of γGPECSO in the bulb, whereas 2-Carb concentrations doubled (Fig. 8F). Additionally, concentrations of γGPECSO and 1-PRENCSO were highly correlated (r=0.73), which was expected, as γGPECSO is the penultimate peptide in the synthesis of 1-PRENCSO. Previously it has been reported that γGPECSO, like 2-Carb, functions as a storage reservoir for S and N in the bulb (Lancaster and Shaw, 1991). Levels of γGPECSO remained high in the bulb, even when concentrations of 1-PRENCSO were relatively high and accumulated in large amounts, which supports the claim that γGPECSO functions as S and N reserve in the bulb. Additionally, Kopsell et al., (2002)

reported that γGPECSO likely contributes to production of the lachrymatory factor in onions, and thus changes in γGPECSO content may affect flavor attributes of the bulb.

Because neither 2-Carb or γGPECSO are thought to be involved in the production of MCSO, it therefore could be reasoned that increases in MCSO would lead to a corresponding decrease in either peptide, as a limited amount of S is partitioned into the production of MCSO instead of 1-PRENCSO or PCSO. However, correlations between MCSO and either 2-Carb (r = 0.41) or γGPECSO (r=0.22) were poor, and do not support this assumption.

ACSOs IN THE ONION MACERATE. When onion cells are disrupted and vacuoles lysed, the enzyme alliinase is released and individual ACSOs are hydrolyzed to form a number of sulfenic acids and thiosulfinates. Each ACSO contributes to thiosulfinates that lend unique flavor characteristics upon degradation. Upon hydrolysis, MCSO tends to impart a cabbage-like and fresh onion flavor sensation, whereas the hot burning sensations commonly associated with cooking onions can be attributed to the hydrolysis of 1-PRENCSO. PCSO has been reported to confer fresh onion and chive-like flavor sensations upon hydrolysis by alliinase (Randle et al., 1994). Because it is difficult to quantify the thiosulfinates and lachrymatory factor we have chosen to measure the ACSOs remaining in the onion macerate to gain a better understanding of how the individual ACSOs contribute to the flavor of onions (Kopsell et al., 2002; Randle et al., 1994). If the level of ACSOs remaining in the macerate is subtracted from levels of intact ACSOs we can also determine the extent of alliinase activity.

Levels of total ACSOs hydrolyzed were affected by S ($P < 0.004$, F = 6.6) and N ($P < 0.001$, F = 16.3) fertility levels (Table 8). A significant interaction was not detected.

Levels of degraded ACSOs ranged from 0.41 to 1.69 $mg \cdot g^{-1}$ FW. Not surprisingly, the general response to N concentrations was for levels of hydrolyzed ACSOs to increase as N solution levels increased, similar to the overall trend for intact ACSO accumulation. The effects of S availability on levels of degraded ACSOs were slightly different, as the highest levels of hydrolyzed ACSOs occurred in the macerate from bulbs grown at 45 $mg \cdot L^{-1}$ SO_4^{2-}. The relative percentage of intact ACSOs hydrolyzed varied from 41-62%; however, large variations in the hydrolysis of ACSOs likely led to a non-significant response to either S or N fertility here (Table 8).

Average levels of hydrolyzed MCSO ranged from 0.70 to 0.84 $mg \cdot g^{-1}$ FW, and were significantly influenced by N levels in the hydroponics solution ($P < 0.001$ F = 10.1). Hydrolyzed MCSO was not significantly influenced by S levels or by an S and N interaction. Lancaster et al., (1998), however, did show a significant response of MCSO hydrolysis to S fertility. Generally, those bulbs grown at the lowest level of N had the least amount of MCSO degraded, which was expected, as those bulbs had the least amount of total intact MCSO. The percentage of MCSO hydrolyzed ranged from 37-50%, but again, due to high variability in the hydrolysis of MCSO in the macerate, the effects of either S or N on MCSO hydrolysis were not significant.

Average levels of 1-PRENCSO hydrolyzed by alliinase ranged from 0.12 to 0.60 $mg \cdot g^{-1}$ FW. In this case, S and N availability significantly interacted ($P < 0.001$, F = 6.4) to influence the level of 1-PRENCSO hydrolyzed in the macerate. In addition, as a main effect, S availability had a significant influence ($P < 0.001$, F = 105.5) on levels of 1-PRENCSO hydrolyzed, as was the case with the accumulation of intact 1-PRENCSO (Table 8). Lancaster et al. (1998) reported near complete hydrolysis of 1-PRENCSO in

the onion macerate. Similarly, we observed levels of 1-PRENCSO hydrolysis near 100% for bulbs grown in all treatment combinations other than 10 $mg \cdot L^{-1}$ N. However, the hydrolysis of 1-PRENCSO in the macerate of bulbs grown in the lowest N levels was significantly lower ($P < 0.01$, F = 4.26) ranging from 58 to 82% suggesting that alliinase activity is affected by low N fertility (Fig 8).

Levels of PCSO hydrolyzed in the macerate were influenced by S ($P < 0.01$, F= 5.6) and N ($P < 0.001$, F = 10.0) availability in the nutrient solution; however, a significant interaction was not present. Hydrolyzed PCSO ranged from 0.05 to 0.14 $mg \cdot g^{-1}$ FW, with the overall trend being to increase hydrolysis as N fertility increased and as S fertility decreased, much like the accumulation of intact PCSO in the bulb. The relative percentage of PCSO hydrolyzed averaged 60% and was not significantly influenced by either S or N levels. This percentage degraded was slightly higher than those previously reported by Lancaster et al., (1998).

Previous investigations into alliinase activity in vitro have shown that 1-PRENCSO is preferentially hydrolyzed over both MCSO and PCSO (Nock and Mazelis, 1987; Schwimmer, 1969). Additionally Lancaster et al. (1998) reported that alliinase acts differently on each of the individual ACSOs in vivo. In general 1-PRENCSO was more completely hydrolyzed than was either MCSO or PCSO. Therefore, 1-PRENCSO would tend to contribute more to the actual flavor of an onion than would the other two ACSOs on a FW basis. Our data concurs with these findings, as the percentage of 1-PRENCSO hydrolyzed in the macerate was higher than MCSO and PCSO at all treatment levels except one. However, unlike the other ACSOs, the level of 1-PRENCSO hydrolysis, was influenced by low N fertility (Fig 9). This suggests that low-N availability decreased the

activity levels of alliinase for 1-PRENCSO, which could be explained by the existence of alliinase isozymes (Lancaster et al., 2000; Nock and Mazelis, 1987). Therefore, flavor was influenced by S or N fertility in two ways. One, by changing total levels of intact ACSOs in the bulb, and two, the activity of alliinase may be altered via fertility changes.

Conclusion

Our purpose was to document how S and N availability interacted to influence the biosynthetic flavor pathway in onion. Low SO_4^{2-} or N fertility levels similarly reduced levels of ACSOs and γGPs in the bulb, with the exception of MCSO. At the lowest levels bulb FW was significantly reduced compared to the other treatments. Furthermore, N level had a greater effect on the synthesis of MCSO. Conversely, SO_4^{2-} levels had a greater influence on the synthesis of 1-PRENCSO. Our data also suggests that the largest changes in concentrations of the ACSOs and γGPs in the bulb occurs in plants grown at the lowest levels of SO_4^{2-} and N. Only minor differences appear in bulbs from plants grown that the higher levels of SO_4^{2-} and N. Thus, the effect of each nutrient on the flavor pathway diminishes as each becomes present in adequate of luxuriant levels. Additionally, because each ACSO will impart different flavor sensations, varying S and N levels may have implications for flavor quality in the bulb.

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Table 5. The effects of solution sulfate (SO_4^{2-}) and nitrogen (N) availability levels on the marginal means and standard error for bulb FW and total bulb sulfur (TS) in hydroponically grown 'Granex 33' onions.

Solution	FW	TS
SO_4^{2-} mg·L^{-1}	g·bulb^{-1}	%
5	118 ± 7	0.160 ± 0.006
45	165 ± 12	0.358 ± 0.026
125	170 ± 12	0. 376 ± 0.028
ANOVA[z]	***	***
N mg·L^{-1}		
10	97 ± 2	0.194 ± 0.006
50	173 ± 13	0.307 ± 0.035
90	172 ± 14	0. 335 ± 0.038
130	166 ± 6	0.375 ± 0.046
ANOVA[z]	***	***

[z] ***, Significance at $P < 0.001$

Table 6. The regression responses for the effect of sulfate and nitrogen availability on the average bulb fresh weight (FW), total bulb sulfur (TS), total *S*-alk(en)yl-L-cysteine-sulfoxides (ACSO), *S*-methyl-L-cysteine sulfoxide (MCSO), *trans*-S-1-propenyl-L-cysteine sulfoxide (1-PRENCSO), *S*-propyl-L-cysteine sulfoxide (PCSO), 2 carboxypropyl glutathione (2-Carb), and gammaglutamyl-1-propenyl cysteine sulfoxide (γGPECSO) concentrations in hydroponically grown 'Granex 33' onion bulbs. In the equations: sulfate (S), nitrogen (N), sulfate*nitrogen (SN).

Response	Regression[z] Equation	R^2
FW	$FW = 73.3 + 2.85N - 0.02N^2 + 0.002SN$	0.74
TS	$TS = 0.14 + 0.005S - 0.00004S^2 + 0.00002SN$	0.80
ACSO	$ACSO = 0.74 + 0.04N - 0.0002N^2 - 0.00003SN$	0.80
MCSO	$MCSO = 0.24 + 0.04N - 0.0002N^2$	0.81
1-PRENCSO	$1\text{-}PRENCSO = 0.26 + 0.01S - 0.00009S^2 - 0.00001N^2 + 0.00004SN$	0.78
PCSO	$PCSO = 0.08 + 0.002N - 0.000005SN$	0.53
2-Carb	$2\text{-}Carb = 0.34 + 0.01N - 0.00007N^2 + 0.00002SN$	0.69
γGPECSO	$\gamma GPECSO = 0.004 + 0.005N - 0.00003N^2 + 0.00002SN$	0.58

[z] Significance at $P < 0.001$

Table 7. The effects of sulfate (SO_4^{2-}) and nitrogen (N) availability levels in solution on marginal means and standard errors for total *S*-alk(en)yl-L-cysteine-sulfoxides (ACSO), *S*-methyl-L-cysteine sulfoxide (MCSO), *trans*-S-1-propenyl-L-cysteine sulfoxide (1-PRENCSO), *S*-propyl-L-cysteine sulfoxide (PCSO), 2 carboxypropyl glutathione (2-Carb), and gammaglutamyl-1-propenyl cysteine sulfoxide (γGPECSO) in hydroponically grown 'Granex 33' onions.

Solution	ACSO	MCSO	1-PRENCSO	PCSO	2-Carb	γGPECSO
SO_4^{2-} ($mg \cdot L^{-1}$)			$mg \cdot g^{-1}$ FW			
5	2.13 ± 0.18	1.76 ± 0.19	0.16 ± 0.03	0.21 ± 0.02	0.54 ± 0.02	0.04 ± 0.002
45	2.24 ± 0.19	1.42 ± 0.15	0.64 ± 0.02	0.18 ± 0.02	0.77 ± 0.07	0.25 ±0.04
125	2.02 ± 0.17	1.24 ± 0.13	0.63 ± 0.04	0.15 ± 0.01	0.82 ± 0.05	0.27 ± 0.04
ANOVA[z]	ns	***	***	*	***	***
N ($mg \cdot L^{-1}$)						
10	1.11 ± 0.06	0.57 ± 0.05	0.44 ± 0.03	0.10 ± 0.01	0.44 ± 0.02	0.04 ± 0.003
50	2.32 ± 0.09	1.67 ± 0.11	0.49 ± 0.09	0.18 ± 0.01	0.77 ± 0.05	0.23 ±0.05
90	2.63 ± 0.10	1.91 ± 0.13	0.50 ± 0.09	0.21 ± 0.02	0.83 ± 0.06	0.20 ± 0.04
130	2.50 ± 0.13	1.76 ± 0.14	0.51 ± 0.09	0.23 ± 0.03	0.82 ± 0.07	0.29 ± 0.06
ANOVA[z]	***	***	ns	***	***	***

[z] ns, *, ***, Nonsignificant or significant at P=0.05 and 0.001 respectively

Table 8. The effects of sulfate (SO_4^{2-}) and nitrogen (N) levels on the marginal means and standard errors for intact total *S*-alk(en)yl-L-cysteine-sulfoxides (ACSO), *S*-methyl-L-cysteine sulfoxide (MCSO), *trans*-S-1-propenyl-L-cysteine sulfoxide (1-PRENCSO), *S*-propyl-L-cysteine sulfoxide (PCSO), hydrolyzed by alliinase in the onion macerate. The percentage of the total intact precursor, which was hydrolyzed by alliinase is listed in parenthesis to the right of each precursor.

Solution SO_4^{2-} ($mg \cdot L^{-1}$)	ACSO	MCSO	1-PRENCSO	PCSO
		$mg \cdot g^{-1}$ FW		
5	0.90 ± 0.12 (41%)	0.71 ± 0.11 (37%)	0.12 ± 0.02 (87%)	0.13 ± 0.02 (58%)
45	1.40 ± 0.14 (62%)	0.73 ± 0.09 (48%)	0.60 ± 0.04 (94%)	0.12 ± 0.02 (67%)
125	1.17 ± 0.13 (57%)	0.53 ± 0.09 (39%)	0.60 ± 0.05 (94%)	0.08 ± 0.01 (56%)
ANOVA[z]	** (ns)	ns (ns)	*** (ns)	** (ns)
N ($mg \cdot L^{-1}$)				
10	0.53 ± 0.09 (48%)	0.71 ± 0.11 (37%)	0.32 ± 0.05 (56%)	0.05 ± 0.01 (56%)
50	1.44 ± 0.12 (61%)	0.84 ± 0.08 (50%)	0.48 ± 0.09 (97%)	0.12 ± 0.01 (65%)
90	1.36 ± 0.15 (51%)	0.81 ± 0.12 (41%)	0.49 ± 0.09 (99%)	0.13 ± 0.02 (58%)
130	1.33 ± 0.08 (54%)	0.70 ± 0.08 (39%)	0.49 ± 0.08 (98%)	0.14 ± 0.02 (54%)
ANOVA[z]	*** (ns)	*** (ns)	*** (**)	*** (ns)

[z] ns, **, ***, Nonsignificant or significant at P=0.01 and 0.001 respectively

Figure 7. The effects of solution sulfate (SO_4^{2-}) and nitrogen (N) availability levels on the average bulb FW (A) and total bulb sulfur (TS) (B) in hydroponically grown 'Granex 33' onions.

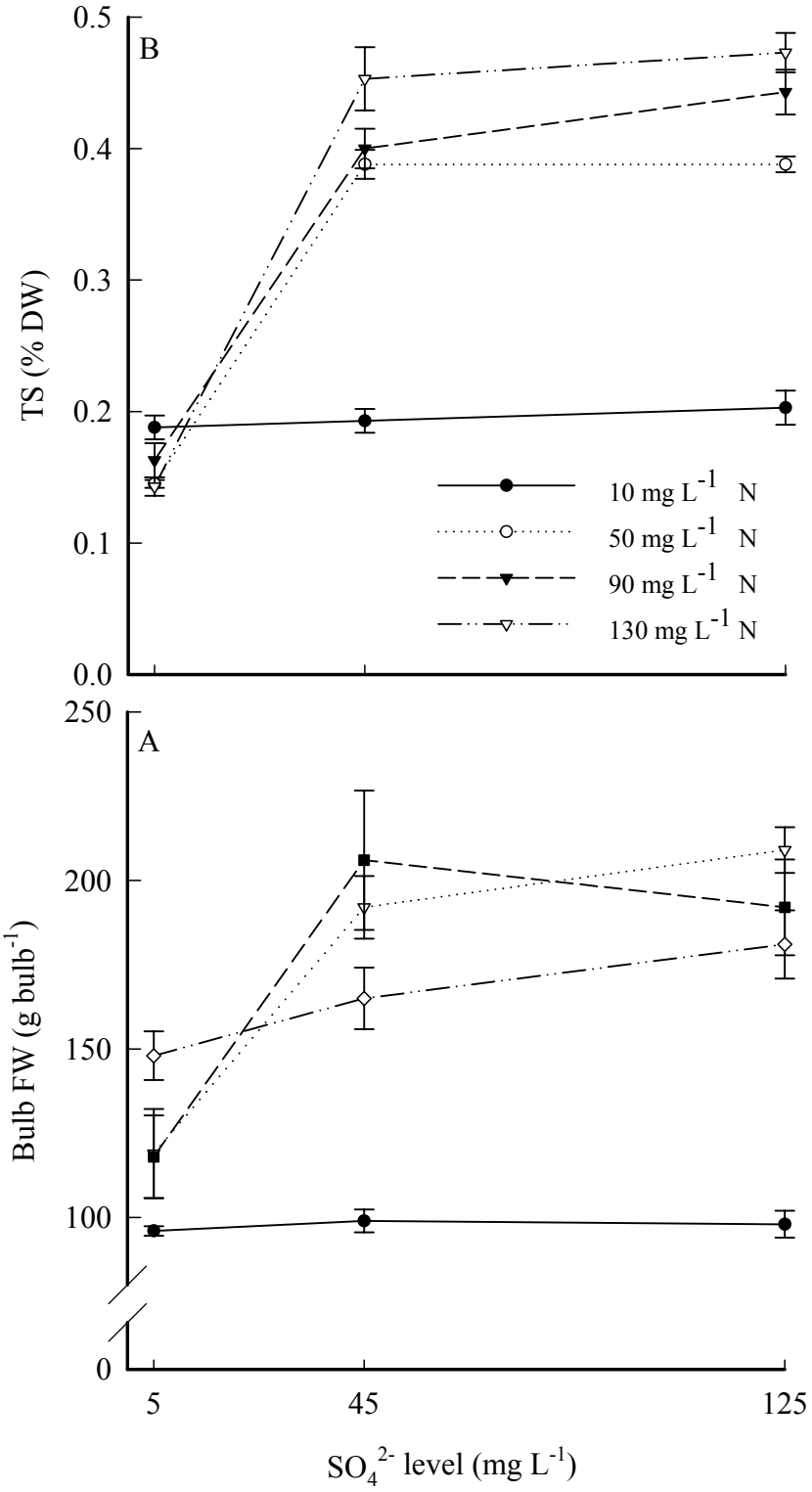

B
A
TS (% DW)
Bulb FW (g bulb-1)
0.5
0.4
0.3
0.2
0.1
0.0
250
200
150
100
0
5
45
125
10 mg L-1 N
50 mg L-1 N
90 mg L-1 N
130 mg L-1 N
SO4 2- level (mg L-1)

Figure 8. The effects of solution sulfate (SO_4^{2-}) and nitrogen (N) availability on the levels of total *S*-alk(en)yl-L-cysteine-sulfoxides (ACSO) (A), *S*-methyl-L-cysteine sulfoxide (MCSO) (B), *trans*-S-1-propenyl-L-cysteine sulfoxide (1-PRENCSO) (C), *S*-propyl-L-cysteine sulfoxide (PCSO) (D), 2 carboxypropyl glutathione (2-Carb) (E), and gammaglutamyl-1-propenyl cysteine sulfoxide (γGPECSO) (F) in hydroponically grown 'Granex 33' onions.

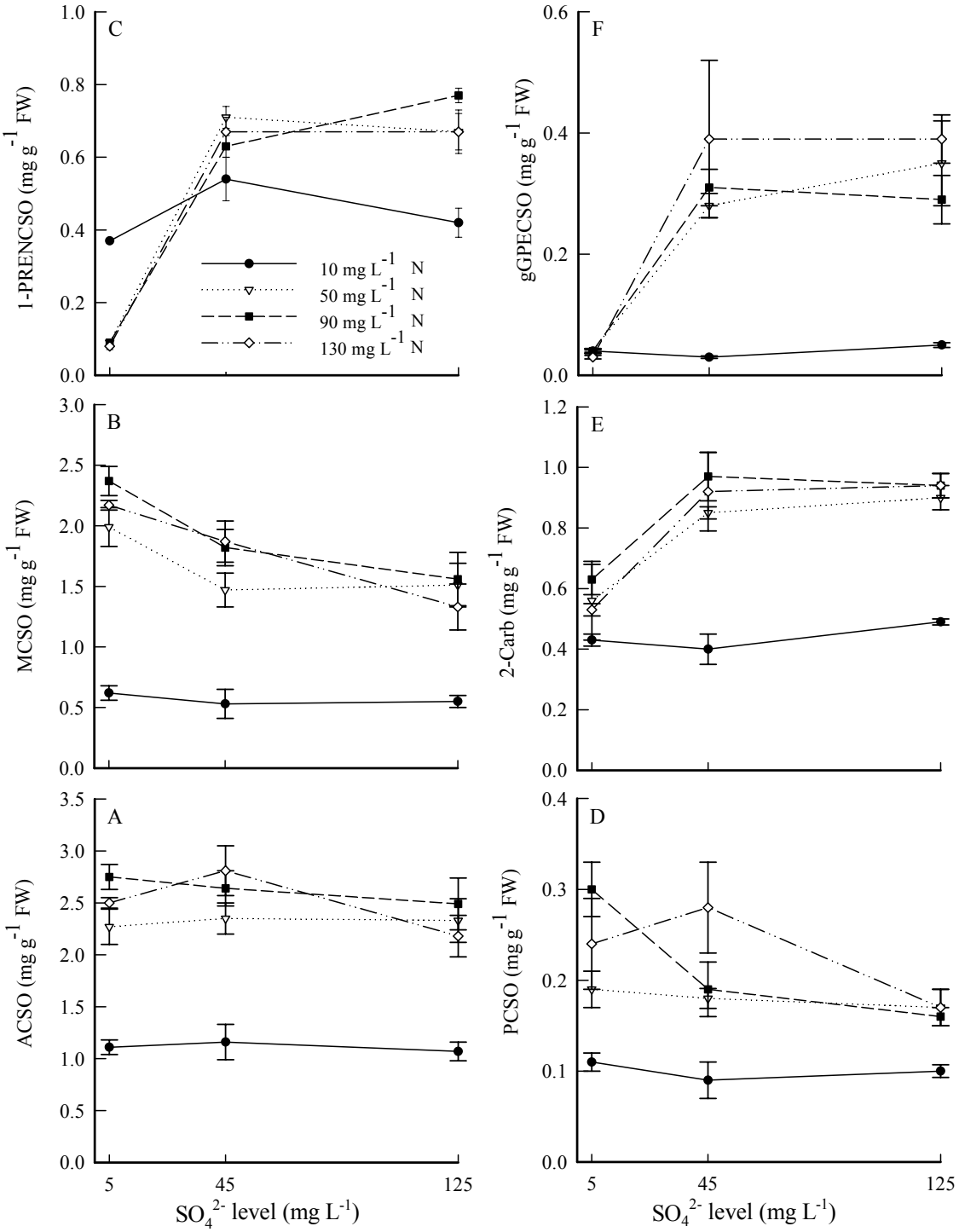
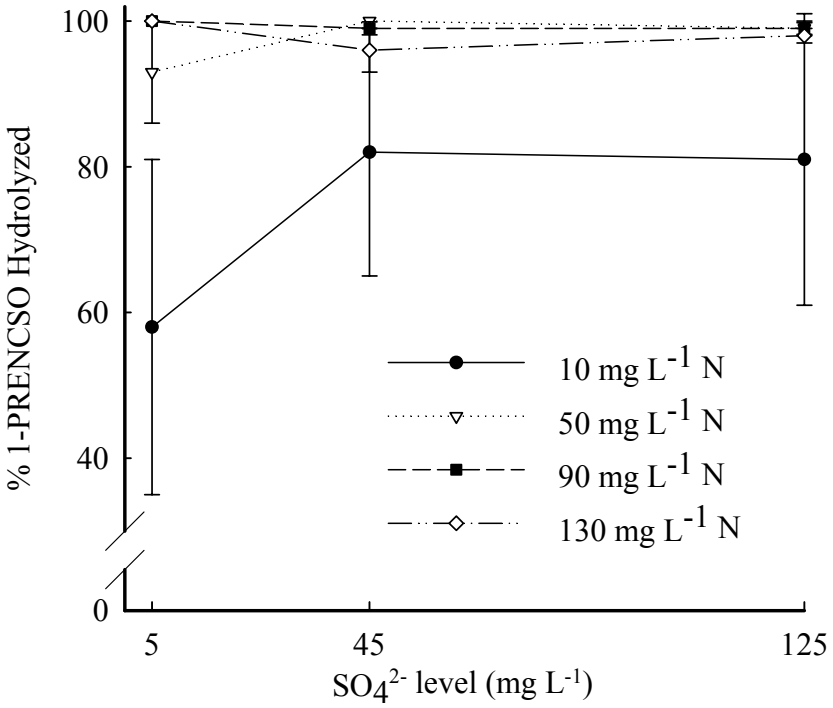

C
1-PRENCSO (mg g -1 FW)
10 mg L-1 N
50 mg L-1 N
90 mg L-1 N
130 mg L-1 N
F
gGPECSO (mg g -1 FW)
B
MCSO (mg g -1 FW)
E
2-Carb (mg g -1 FW)
A
ACSO (mg g -1 FW)
D
PCSO (mg g -1 FW)
5
45
125
SO4 2- level (mg L-1)

Figure 9. Solution sulfate (SO_4^{2-}) and nitrogen (N) levels affected the percentage (%) of intact *trans*-S-1-propenyl-L-cysteine sulfoxide (1-PRENCSO) hydrolyzed by alliinase in the onion macerate.


100
80
60
40
0
% 1-PRENCSO Hydrolyzed
10 mg L-1 N
50 mg L-1 N
90 mg L-1 N
130 mg L-1 N
5
45
125
SO4 2- level (mg L-1)

CHAPTER 6

CONCLUSIONS

Our first study examined the effects of varying growing temperature and developmental age on the flavor pathway in onion. Results concurred with previous investigations into temperature and its effects on flavor intensity, as well as with empirical observations that have been made with the sweet onion industry in Georgia. The large linear increases in total bulb sulfur (S) and flavor precursors suggest that temperature is a primary means by which onion flavor potential is altered. We also observed that developmental age had no effect on the nature of the response of onion flavor to growing temperature. Noteworthy, was the large increases in flavor precursors and total bulb S concentrations observed between bulbs grown at 26.7 and 32.2 ^{o}C. Although changes were evident at lower temperatures, synthesis of precursors and S assimilation nearly doubled for bulbs grown in this range. Bulb fresh weight data, however, indicated that a growing temperature of 32.2 ^{o}C was deleterious for the plants. It remains unclear as to why the plant would not only continue, but increase the assimilation and reduction S and synthesis of the flavor precursors, which is metabolically expensive for the plant, when the overall health and growth of the plant is impaired. It is plausible that the flavor precursors and associated peptides could serve as a reservoir for nitrogen (N) and S. Because bulbs grown at 32.2 ^{o}C were much smaller than bulbs grown at other temperatures, it was necessary to increase the concentration of

precursors and peptides in the bulb in order to have a sufficient reserve of N and S, as well as carbon skeletons to be used in the following growing season, when floral initiation and reproduction occurs. When accounting for fresh weight, the total levels of *S*-alk(en)yl cysteine sulfoxides (ACSO) in bulbs grown at 26.7 and 32.2 ^{o}C, are 277 and 294 mg·bulb^{-1}, respectively. Thus, when considering the total levels of precursors in the bulbs, and not concentrations, differences are much smaller. This indicates that if the ACSOs and related peptides served as a reservoir for N and S, both bulbs would have roughly the same amounts of reserves available for growth the following year.

Nonetheless, the information obtained in our temperature experiment would be useful to those wishing to grow onions with a particular flavor profile. For those wishing to produce pungent, flavorful bulbs, a warmer climate may be desired, yet those wanting to grow milder bulbs would want to have production in a region with a cooler growing season.

Our second experiment was concerned with growing onions hydroponically over a wide range of N levels. Our intent with this study was to determine the N concentration in solution culture, which produced bulbs with a flavor profile similar to that of field grown onions. A previous study growing plants hydroponically with high levels of N, typical of a Hoagland's solution, produced bulbs that had higher levels of *S*-methyl-L-cysteine sulfoxide (MCSO) than *trans*-(+)-*S*-1-propenyl-L-cysteine sulfoxide (1-PRENCSO), which is atypical of a field grown bulb. Our goal was to determine at what N level 1-PRENCSO was present in a higher concentration than MCSO, thus allowing one to produce a bulb in solution culture that was similar to a field grown bulb with regards to the flavor pathway. Our data showed that 1-PRENCSO became the dominant

precursor at N levels near 40 mg· L^{-1} N. Interestingly, changes in N availability led to large changes in MCSO, compared to relatively small concentrations in 1-PRENCSO levels. The observation that N fertility influenced MCSO to a greater extent than 1-PRENCSO drove our next study, which looked at varying N and S fertility together in a factorial design.

By varying S and N fertility together we were able to show and that the two nutrients interacted to influence the flavor pathway, particularly at when they were supplied at low levels. Most notable, however, was that S fertility levels primarily influenced the production of 1-PRENCSO, whereas N fertility levels mainly affected the production of MCSO in the bulb. This suggests that, enzymes or substrates involved in the production of the two precursors were affected differently by S and N fertility. However, because the oxidases and γ-glutamyl transpeptidases involved in the synthesis of 1-PRENCSO and MCSO are similar in the substrates to which they bind, it is not unreasonable to assume that those enzymes would be affected similarly by N and S nutrition. The γ-glutamyl peptide intermediates proposed in the synthesis of MCSO and 1-PRENCSO all contain the same number of N and S atoms, thus there is no single intermediate that would serve as a strong sink for N and S. Furthermore, data exists that indicates methionine may serve as an intermediate in MCSO synthesis. If this proves true, it would indicate the presence of a secondary pathway of MCSO synthesis, very much different from the pathway leading to the synthesis of 1-PRENCSO. This would help explain how the accumulation these two ACSOs would differ in response to N or S fertility.

As horticulturalists, we are constantly trying to find applications for our work. While we have demonstrated that N availability influences the onion flavor pathway in a solution culture system, field tests would have to be performed to before any production suggestions could be made.

The research that I have described in the previous three chapters will serve as a useful starting point for future research. Because the effects of temperature were so dramatic, research is now being conducted to determine how changes in root temperature specifically affect flavor development. And because the results of the S and N fertility on the synthesis of 1-PRENCSO and MCSO were so different, research looking at intermediates in pathway leading to MCSO is now being planned. Now we know how to manipulate onion during growth and development to accentuate the synthesis of MCSO and depress levels of 1-PRENCSO, hopefully making it easier to isolate intermediates in the synthesis of MCSO, which have been difficult to isolate in vivo.