Thesis, Chapter 1

Citrulline in Watermelon: A Review

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Introduction

Watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai) is a major vegetable crop in the U.S., consistently ranking among the top four vegetable crops in production volume since 1978 (as data were available, USDA 2015). In the last century, breeders have developed new cultivars with various desirable traits, such as high fruit quality, excellent post-harvest characteristics, and disease resistance. Successful innovations include uniform hybrids, seedless triploids, high soluble solids content, dark red flesh, 6 kg. seedless watermelons, 3 kg. mini seedless, and 1 kg. palm-sized. Another area with potential involves exploiting existing compounds found in watermelon and breeding to further augment the bioactive content of the fruit.

The health properties of watermelon have earned it a recommendation by the American Heart Association as a heart-healthy food. In addition to lycopene, watermelon is the cucurbit crop containing the highest concentration of L-citrulline (citrulline). Citrulline is a non-proteinaceous, non-essential, physiologically active amino acid has relevance in mammalian metabolism. It is an intermediate metabolite that has sparked much human health research in the past forty years. This spike in citrulline-human health interest in concert with a consumer desire for functional foods has pointed scientists to watermelons as a natural source of citrulline (Perkins-Veazie et al., 2012).

Useful for breeding improved citrulline content in watermelon is to determine the importance of genetics and environment on the trait. Factors affecting citrulline content include the quantification method, cultural practices, production environment, and genetic regulation of the biochemical pathway. Difficulties with citrulline quantification include the fruit tissue type selected for extraction (heart, locular, interlocular, rind), tissue processing (fresh or dry, sample storage conditions), and analytical instrumentation. Cultivation and environmental variables include location, year, drought or salt stress, and grafting. Genetic and developmental sources of variation may include ripeness stage, fruit development, ploidy (diploid, triploid, tetraploid), genotype, lycopene content, and concentration of arginine, for which citrulline is a precursor.

In order to understand the relevance of citrulline in plants and animals, the history of citrulline discovery and research trends will be discussed, followed by metabolic roles in both human and plant metabolism, and its potential in applied science, including product development. After this background information, studies contributing to understanding effectors on citrulline concentration will be discussed.

History of citrulline: Discovery and research trends

Citrulline was first isolated from the juice of watermelon by Koga and Ohtake (1914), and again, in the same lab, by Wada (1930), who characterized and named it (Fragkos and Forbes, 2011). Wada (1932) extracted citrulline from a tryptic digest of casein, the same year that Krebs discovered the urea cycle, in which citrulline serves as an intermediate metabolite (Krebs 1932). Until the 1980s, citrulline was not of much interest because 1) it was non-proteinaceous and 2) it was thought of as an intermediate metabolite, and thus insignificant. As of the 1980s, however, citrulline was found to play a larger role in nitrogen homeostasis, when it was discovered by Windmueller and Spaeth that citrulline is continually produced and released by enterocytes of the small intestine into circulation (Windmueller and Spaeth, 1981; Moinard and Cynober, 2007; Bahari et al., 2013). This was the first demonstration of inter-organ metabolism of citrulline.

Few natural foods are high in L-citrulline, but watermelon is the richest natural source known (Tedesco et al., 1984). Citrulline is also found in most cucurbits, including bitter melon, cucumber, muskmelon, pumpkin, bottle gourd, dishrag gourd, and wax gourd (Inukai et al., 1966). Because of the health benefits of citrulline (and lycopene, the red pigmented carotenoid), and because they can be obtained from a natural food source, watermelon offers a natural and cheap alternative to citrulline health supplements, which are currently produced by bacterial fermentation (mutant *B. subtilis* auxotrophs) (Fish 2014b, Shinji et al., 1966).

Citrulline is also found in high amounts in the rind, which could add utility to cull watermelons and waste. In the United States, approximately 20% of watermelons are discarded from the field because of blemishes (Fish 2014b). Citrulline from these fruits can be used in commercial products as corrosion inhibitors (Odewunmi et al., 2015), in green chemistry applications (dye degradation; Lakshmipathy et al. 2017), and for watermelon juice and other function food products including the (edible) use of the rind (Perkins-Veazie et al., 2012 Egbuonu, A.C.C.; 2015).

Citrulline and human health

Citrulline offers several pharmacokinetic advantages over arginine, for which it is a precursor (Tarazona-Diaz et al., 2013; Collins et al 2007; Wijnads et al., 2012). Its specific metabolism allows it to escape splanchnic extraction, as it is neither hepatically nor intestinally absorbed. (Bahri et al., 2013). Arginine-family metabolites are studied for their role in nitrogen (N) homeostasis. Citrulline also has higher bioavailability than arginine, with 80% of the ingested amount absorbed rapidly into the blood (Mandel et al., 2005). Of the arginine family of amino acids, it is the only one that does not cause adverse effects (i.e. diarrhea) when administered at high doses (Oketch-Rabah et al., 2016). As the amino acid with the highest N:C ratio, arginine's role in nitrogen homeostasis is important to understand in order to elucidate the mechanisms of N homeostasis.

Mammals generate citrulline directly via an enzymatic reaction of L-glutamine in the nitric oxide cycle (Tomlinson et al; 2011, Van De Poll et al., 2007a; Van De Poll et al., 2007b) and indirectly from L-arginine in the urea cycle (Lameu et al., 2009; Flam et al., 2007). Citrulline can be found in proteins, but rather than being added during mRNA translation, arginine moieties are enzymatically converted to citrulline prost-

translationally, a process called citrullination (Clancy et al., 2016). Citrulline has been administered orally in the form of citrulline malate and was found to affect many areas of human health, including skeletal and muscle performance (Perez-Guisado and Jakeman 2010), diabetes (Wu et al., 2007), pharmacology (Rouge et al., 2007; Thibault et al., 2011; Levillain et al., 1997), immunology (Sureda et al., 2009), and neurology (Sase et al., 2013). Acute consumption of watermelon increases citrulline and arginine plasma levels, while chronic consumption increasing fasting arginine plasma levels, but not citrulline levels (Mandel et al. 2005; Collins et al. 2007)

As a member of the L-arginine metabolic family, roles of citrulline include aiding in muscle recovery during exercise (Tarazona-Diaz et al., 2013), and benefiting vascular health, such as improving blood pressure (Figuoroa 2011), and increasing vasodilation in many tissues of the body. Vasodilatory properties of citrulline have been shown to improve erectile dysfunction (Drewes et al., 2003; Shiota et al., 2013) and vasospastic angina (Morita et al., 2013), a symptom of coronary heart disease.

To date, three studies have been conducted examining watermelon juice and exercise performance (Tarazona-Diaz et al., 2013, Cutrufello et al., 2015, Shanely et al., 2016). Neither of them found significant effects of watermelon juice supplementation on aerobic exercise performance, although effects of citrulline malate were observed (Cutrufello et al., 2015). Endurance athletes are particularly interested in finding new carbohydrate sources that enhance performance in addition to providing calories. Shanely et al. (2016) found that when given watermelon juice during exercise (cycling), the athletes were equally energetically sustained compared to the equivalent in carb-rich sports beverages. Additionally, the watermelon treatment group experienced elevated plasma antioxidant capacity, though this did not seem to improve exercise performance (Shanely et al. 2016). It seems that sort-duration, high-intensity exercise is not improved by a single dose of L-citrulline or 24-hour supplement of citrulline (Shanely et al., 2016). Other studies suggest that citrulline supplementation over a 1-week period can increase performance for approximately 10 minutes (when at a high percentage of VO_{2max}) (Hickner et al., 2006; Suzuki et al., 2016). Citrulline has also been investigated for use in other muscle-related health studies, such as prevention of sarcopenia (age-related muscle loss), in which watermelon juice added to parboiled rice was considered in a preliminary study as a cheap and effective treatment for people constrained by poverty or without access to pharmaceutical supplements (Sadji et al., 2015).

Citrulline in plant metabolism

Citrulline metabolism is much more characterized in prokaryotes, and yeast, and humans than in plants. Plants employ physiological amino acids to serve many functions, including buffering effects of abiotic stress in various ways. In several cucurbits including watermelon, citrulline concentration changes dynamically in the foliage in response to drought, salinity, and high light stress, but is of unknown relevance. Probably, cucurbits employ citrulline for osmotic adjustment as a compatible solute, and for intercepting oxidative stress as a radical oxygen species (ROS) scavenger (Akashi et al., 2001; Yokota et al., 2002; Kawasaki et al., 2000). Higher plants tend to co-accumulate citrulline and arginine under salinity stress (Mansour, 2000; Ashraf and Harris, 2004). Even though arginine is the final metabolite in the biosynthetic pathway,

watermelons accumulate citrulline, an intermediate, in the leaves, fruit rind, and mesocarp (Rimando and Perkins-Veazie 2005). Underlying mechanisms of this accumulation, especially in the fruit, have not been fully characterized, but they seem to relate to abiotic stress.

Wild watermelon, found in the Kalahari Desert of Botswana, can maintain its photosynthetic apparatus during periods of drought with high light intensity. Kawasaki et al. (2000) reported that in severe drought and high light conditions in the Kalahari Desert, after all C4 plants had died, wild watermelon, C3 plants, were able to survive. In the desert, leaves of wild watermelon can reach temperatures up to 60°C, which can encourage photo-oxidation and accumulation of reactive oxygen species (Xue and Liu 2008; Kusvuran et al., 2012; Larcher 1995).

Common osmoregulators, such as proline, mannitol and glycine betaine, are highly regulated via activation of biosynthesis and/or suppression of catabolism. Citrulline, whose regulation is less well studied, is a more powerful hydroxyl radical scavenger (OH) than glycine betaine, mannitol and proline (Smirnoff and Cumbes, 1989, Akashi et al., 2001). In *Cucumis melo*, citrulline has been shown to accumulate almost ten times more than proline in drought tolerant accessions (Dagsen et al., 2009). In a study of wild watermelons in the Kalahari, citrulline accumulated up to 50 percent of the leaf amino acid content after five days of water withholding (Kawasaki et al., 2000). Kusvuran et al. (2012) reported that of 65 accessions of mostly Turkish watermelons, the salt tolerant and the drought tolerant accessions accumulated at least twice the amount of citrulline of the sensitive accessions. It is possible that citrulline accumulates in response to stress to improve survival, or that it is merely a metabolic consequence of evolving in dry or saline environments. Akashi et al. (2001) reported that watermelon plants subjected to salt stress accumulated gamma-aminobutyric acid, proline, and glutamine, but not citrulline. Despite these conflicting observations, more work has shown that citrulline seems to accumulate in watermelon in response to drought and salt pressure, though it is still unclear how and why. In contrast, citrulline is maintained at low concentrations due to intense feedback inhibition and transcriptional regulation in prokaryotes.

Citrulline differs from arginine at only one molecular position (an "O" instead of "NH_2" in the -R group) and thus serves similar metabolic roles. Citrulline is a precursor of arginine, which is apparently only synthesized via ornithine in chloroplasts, and the rate of which is tightly regulated by feedback mechanisms, in response to changes in nutritional status (Winter et al., 2015). Winter (2015) reviewed progress in the understanding of plant arginine biosynthesis in great detail. Here, the role of arginine will be considered only in the context of citrulline.

Regulation of arginine and citrulline has been studied extensively in other models, including various prokaryotes, *Saccharomyces cerevisiae,* and *Arabidopsis thaliana*. These systems can provide foundations to help inform hypotheses about analogous pathways in plants. In watermelon, several proteins, like drought-induced proteins (DRIPs) and glutamine acyl transferases (GATs), have been investigated because drought induces increased gene expression of these. DRIP-1, whose expression increases significantly, shares sequence homology to glutamine acetyl transferase (GAT) in *Arabidopsis*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*, suggesting that it belongs

to the arginine homologue family of proteins. Other gene families in the arginine pathway and ornithine cycles are expanded in watermelon as well.

Arginine pathway: Overview

Glutamine is acylated into N-acetylornithine via three enzyme-catalyzed steps: phosphorylation, reduction, and transamination. The fifth step generates ornithine from N-acetylornithine. This last step requires catalysis by two enzymes, one known as acetylornithine deacetylase (AOD, EC 3.5.1.16), which catalyzes deacetylation of N-acetylornithine, yielding ornithine and acetate. This linear pathway is regulated by feedback inhibition of the first step (N-acetyl-glutamate synthase) via arginine, but has yet to be observed in plants (Takahara, 2005). However, it has been observed in *Bacillus subtili*s and *Saccharomyces cerevisiae*.

Novel Polypeptides: Drought-Induced Polypeptides (DRIPs)

Takahara et al. (2005) have investigated two prominent enzymes in watermelon that may function in the arginine pathway. One of these, glutamine acyl transferase (GAT), exhibits both thermotolerance and insensitivity to inhibition by downstream products, citrulline and arginine.

Kawasaki et al., (2000) investigated the relationship between drought tolerance in wild watermelon and citrulline accumulation in leaves by inducing drought stress. From the ~1,000 spots observed from two-dimensional electrophoresis of the polypeptides, seven were found to increase in concentration (Kawasaki et al., 2000). These seven proteins were isolated and named drought induced protein (DRIP) 1-6. Of these, DRIP-1a and DRIP1-b accumulated immensely, amounts second only to ribulose cisphosphate carboxylase (RuBisCO) large subunits.

DRIP-1a and 1b were targeted for cloning using cDNA, and were found to have sequence homologies with the arginine E family. One of the sequences contained histidine, an amino acid that binds to metal cations, which typically aid in hydrolysis of peptidic or N-acetyl bonds to release free amino acids (Kawasaki et al., 2000).

The genome project found both glutamine acyltransferase (GAT) and AOD-homologous genes in *Arabidopsis thaliana* (Genome Project, 2000). Kawasaki et al., (2000) have isolated a novel protein from watermelon that accumulates heavily in drought and high-light conditions: drought-induced polypeptide 1 (DRIP-1) that shares sequence homology with bacterial AOD. Thus, when inducing watermelon with such stress, they expected DRIP-1 to function as AOD. However, it was GAT activity, *not* AOD activity, that was detected in wild watermelon leaves in which DRIP-1 had been strongly induced. Takahara et al., then purified and characterized GAT from wild watermelon leaves.

During citrulline biosynthesis, N-acetylornithine is converted into ornithine by AOD and/or GAT. An examination of these enzyme activities under varying levels of drought/ strong-light stress induction revealed that AOD was below the detection limit (less than 0.02 nmol/(min*mg protein) (Takahara et al., 2005). In bacteria, this enzyme activity is highly upregulated by the presence of a divalent cation, namely cobalt or zinc, but when assayed with a metal, the enzyme was not active in watermelon leaves (they tested the activity assay using *E. coli* as a positive control—and it worked). GAT activity

was detected in watermelon leaves, though its activity did not increase from unstressed leaves compared to stressed (Takahara et al.,, 2005).

Purification of GAT and subsequent cDNA analysis revealed amino acid sequence homologies of this watermelon-derived GAT to At2g37500 from *A. thaliana* (70% identity), B. subtilis GAT (38%), and *S. cerevisiae* GAT (26%). This enzyme was dubbed CLGAT (*Citrullus lanatus* glutamate acyltransferase). Two copies of the GAT gene are present in wild watermelon, transcribed into one type of mRNA, which is believed to encode an auto-cleaving peptide, forming alpha- and beta- subunits (Takahara et al., 2005).

Is the precursor CLGAT polypeptide imported into chloroplasts?

Additionally, Using the CHLOROP program, the first 26 amino acid sequence at the N-terminus of CLGAT was predicted to function as a chloroplast transit peptide (Takahara et al., 2005). Takahara et al. (2005) fused the 26-amino acid cDNA to the coding sequence of green fluorescent protein (GFP), which was transiently expressed in tobacco leaves. Confocal microscopy allowed for observation of GAT expression relative to chloroplasts and their overlap. When GAT transit-sequence-GFP fusion protein was expressed in leaves, chlorophyll autofluorescence was yellow, but no green was detectable in the cytosol. In contrast, when non-fusion GFL was introduced, fluorescence was detected in the cytosol and nucleus but was not seen in chloroplasts.

Those observations suggest that the CLGAT precursor can be transited to the chloroplast by the N-terminus sequence. Enzyme assays including physiologically-relevant amounts of citrulline (30mM) and arginine (7mM) showed that CLGAT is not inhibited by those downstream products. CLGAT activity was maximum at pH 7.0 and 70°C, yielding support for its predicted role in mediating drought/high-light stress.

Genomics data

In comparing pathway expression differences in domestic and wild watermelon, the only pathways enriched more in the wild species in comparison to domestic species were the arginine biosynthesis and alanine, aspartate, and glutamine metabolism (Table 2, Zhu et al.,, 2017). This is consistent with the idea that wild watermelon produces more citrulline than domestic watermelon. Additionally, In annotating a draft genome of watermelon, Guo et al., (2013) identified 14 genes in the citrulline metabolic pathway in watermelon, and compared expression levels to the same pathway in *Arabidopsis*. Gene expression in watermelon had undergone expansion in the arginosuccinase and arginosuccinate synthase families, both of which are involved in converting citrulline to arginine. An arginosuccinase and two arginosuccinate synthase genes were found to be highly down regulated as watermelon fruit development progressed, suggesting a decrease in citrulline degradation with ripening (Guo et al., 2013).

Citrulline in watermelon: Correlations of cultivar and environment

Genetic and developmental sources of variation in citrulline fruit content may include ripeness stage of fruit development, ploidy (diploid, triploid, tetraploid), genotype, lycopene content, and concentration of arginine, for which citrulline is a precursor. Fish (2014a) was the first to publish citrulline data in developing watermelon fruit and in

relation to other arginine-family amino acids. Of the arginine family amino acids, citrulline is present in the highest amount.

Ripeness and flesh color

Watermelons boast a variety of bioactives with antioxidant properties, including carotenoids, physiological amino acids (arginine, citrulline), phenols, flavonoids, and vitamin C (Tlili et al., 2011). Of these classes of bioactives, carotenoid content most directly correlates with the stage of fruit development and ripeness; carotenogenesis increases during development in a sigmoid fashion, while changes in phenols, flavonoids, and vitamin C were conflicting or inconclusive (Tlili, 2011). Flesh color, as in most fruits, is an important index of watermelon ripeness and quality, so understanding the endogenous and exogenous effects on the contributing compounds—carotenoids—is paramount to maintaining high quality in transport and storage, but also potentially important in understanding citrulline content. Whether there is a correlation or causative relationship between citrulline and carotenoids, this may be worth investigating.

Rimando and Perkins-Veazie (2005) looked at citrulline content considering several factors beyond and within cultivar, including citrulline range in domestic watermelon, ploidy level, genotype, flesh color, and fresh weight versus dry weight. In watermelon, red-fleshed fruit had significantly less citrulline than orange or yellow-fleshed fruit on both a fresh and dry weight basis. The two yellow cultivars had similarly high citrulline, but there was significant variation in the other types: red flesh ranged from 70-350 mg/100g FW citrulline (mean 100), while orange flesh cultivars, Tendersweet Orange Flesh and Orange Sunshine, had 50 and 300 mg/100g FW citrulline, respectively (Rimando and Perkins Veazie, 2005). Despite this variation, carotenoids are still considered a potential predictor of the amount of citrulline in watermelon, especially yellow flesh type.

Ploidy

Most eukaryotic genomes contain duplicate genes generated from polyploidy (genome multiplication) resulting from either allopolyploidy or autopolyploidy. Merging and doubling of sets of genomes can create extensive modifications of the genome, and can cause cascading changes in phenotype via modifications at the level of transcriptome, proteome and metabolome. These changes can result in changed or novel agricultural characteristics that did not exist in their diploid parent, such as disease resistance, increased metabolite production, or new secondary metabolites (Soltis and Soltis, 2000). Several traits of 4X watermelon changed compared to the 2X parent, such as increased leaf guard cell and epidermal cell size, reduced pollen production and pollen germination, and increases in chlorophylls a and b, carotenoids, vitamin C, sugar content, salt tolerance, and disease resistance (Liu et al., 2003a, Liu et al., 2003b, Liu et al., 2003c, Liu et al., 2005; Cheng et al., 2008).

In a study of diploid and triploid watermelons, the seeded genotypes had less citrulline than seedless cultivars, with 180 mg/g FW (six cultivars) and 240 mg/g FW (eight cultivars) (Davis et al., 2013). In order to estimate effects of ploidy on citrulline content, six experimental diploids and their triploids and tetraploids were investigated for citrulline content. Of the six families, only one showed significant differences in citrulline content (3X, 4X > 2X), but there were no significant differences when averaged

over family. Those results were similar to a study by Liu et al. (2010) on greenhouse-grown watermelon from nine autotriploid hybrids, also observing no statistically significant differences by ploidy. These data contradict a previous study where 3X watermelons were reported higher in citrulline content than 2X (Perkins-Veazie et al 2012, Tarazona-Diaz et al 2011).

Localization of citrulline within the fruit/plant

Akashi et al., (2017) investigated the spacial accumulation pattern of citrulline in immature and mature watermelon fruit; concurrent with Fish (2012), citrulline was generally correlated with ripeness. Six spatial anatomies were defined and investigated for citrulline content: peel, inner rind, outer- mid- and inner-flesh, and heart), Of these, the peel and heart had the greatest abundance of citrulline, with 4.4 ± 0.8 g/kg and 2.4 ± 0.99 g/kg FW respectively, with rind almost the same as heart (2.1± 0.94 g/kg FW). This observation of a "bipolar" accumulation pattern should be investigated.

Citrulline: Other Sources of Variation

Quantitation

Until development of HPLC methods for citrulline quantification by Jayaprakasha et al. (2011) and Fish (2012), citrulline identification and quantification had to be done using amino acid analyzers or liquid chromatography and mass spectrophotometry (LC-MS), or thin layer chromatography. Variation among experiments with watermelon include tissue type (heart, locular, interlocular, rind, peel), tissue processing (dry vs. fresh weight, sample storage temperature), and analytical instrumentation.

As methods for citrulline analysis were originally developed for animal and human tissues, complete hydrolysis of tissues using strong oxidizers such as 0.1 to 1 M HCl were often done. In watermelon, this harsh treatment oxidizes citrulline and gives erroneously low citrulline values. Amino acid analysis of protein hydrolysates (Berenner and Niederwieser 1960; Davis et al., 2010-2011) in contrast, generally overestimates citrulline because of the presence of glutamine. Ripe watermelon contains from 3 to 10 times more citrulline than glutamine, which poses an unknown and unpredictable measurement bias (Fish and Bruton, 2010). Values of the same cultivars across two studies varied by up to 4-fold when comparing high performance liquid chromatography and thin layer chromatography (Rimando and Perkins-Veazie, 2005; Davis et al., 2010-2011), possibly due to environment.

Use of colorimetric methods may have over-estimated citrulline content (Inatomi et al., 1969). Analytical fee for service labs do quantification only for essential amino acids. Assorted methods for analysis are found in the literature not all of which have been optimized or verified against other methods. In watermelon, citrulline is found in both liquid (80%) and solid (20%) portions of the fruit (Perkins-Veazie, unpublished). Citrulline will be preserved in watermelon held at -20°C for a year but is lost within a week once tissue is homogenized and refrigerated.

Trait stability over environment

Quantitative traits often vary based on environment. Citrulline, produced in leaves in response to environmental conditions, may also accumulate variably in the fruit. Davis et al., (2010, 2011) investigated response to environment by growing five cultivars in

three locations (Cream of Saskatchewan, Red-N-Sweet, Tendersweet Orange Flesh in Clinton and Kinston NC; Black Diamond and Dixielee in Lane, OK). It was determined that there is a wide range of values for citrulline found within cultivar, but no significant differences among the cultivars tested, nor across environments tested. Thus, breeding for high citrulline content in watermelon across different environments may be possible.

Future Directions and Conclusions

From an investigative approach, many of the enzymes in the arginine pathway should be purified and characterized to understand roles in arginine and citrulline accumulation. Specifically, CLGAT function in translocation can be expanded, as well as DRIP1 and AOD function in plants. Further investigation into DRIP1 function is necessary, since its activity did not parallel with the analogous AOD.

Additionally, citrulline accumulates in greatest concentration in the peel and heart. This should be investigated; since the fruit exodermis is photosynthesizing, it may experience more oxidative stress than inside the fruit. There is no documentation on incidence of translocation within the plant and fruit, but this would give interesting insight to physiological sources of variation.

Citrulline content in wild watermelon would be interesting to investigate, including protein homology with domesticated watermelon for biochemical and phylogenetic analyses, but also for plant breeders to use for selection of interesting germplasm for backcrossing single genes into elite lines. If citrulline proves heritable in populations, we could select lines with high citrulline to use in the development of new cultivars.

Additionally, though it is known that citrulline accumulates in high amounts in the leaves due to water withholding, it is unknown whether fruit respond similarly. Accumulation of more citrulline in fruit would provide a new platform for stress-response research via differential gene expression and regulation. That could aid growers in producing watermelon with more bioactive compounds. Use of water and saline stress is a production method in watermelon and tomato to increase soluble solids content (°brix). High-citrulline watermelon could be used to make juice and related products. Concentration in the leaves relative to the fruit in water withholding versus non-withholding may inform new hypotheses about translocation, anabolism, and catabolism of arginine family amino acids.

Wild watermelon is the only known plant species to accumulate citrulline in leaves during drought stress, and watermelon is the only known plant species to accumulate citrulline in the fruit. Evidence suggests that the arginine pathway is induced in the chloroplasts of leaf tissue when water potential, and to a lesser extent, osmotic potential changes. The major contributing regulators are unclear, although there are several candidate proteins. One group may be the ArE homologues, which release free amino acids from acyl moieties, and are expressed due to induction of drought stress. Additionally, two enzymes downstream of citrulline synthesis (ASS and AS) in domesticated watermelon have expanded gene families, with two genes and one gene

differentially expressed during fruit development, respectively and may also regulate citrulline synthesis or accumulation.

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Citrulline Content of Species and Cultivars of the Cucurbitaceae

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Abstract

Watermelon is the most significant, natural source of L- citrulline, a non-proteinaceous amino acid praised for its ability to benefit cardiovascular health and increase vasodilation in many tissues of the body. Other cucurbits may also contain significant amounts of citrulline. XX genotypes were evaluated in triplicate in two environments to estimate citrulline amounts and variation due to genotype, replication, environment…..

Introduction

Watermelon is considered a functional food, owing its benefit to cardiovascular health, reduction of risk to several cancers, and increase of vasodilation in many tissues of the body (sources here). These properties are due to its high concentrations of lycopene and citrulline. Of particular interest in this study is citrulline, and its abundance in fruit of other genera and species in the Cucurbitaceae.

Citrulline is a physiological amino acid with a high nitrogen to carbon ratio that benefits both plant and human health. In human health, citrulline offers many health benefits, with particular relevance in exercise supplementation for muscle performance and recovery (Perez-Guisado and Jakeman 2010, Tarazona-Diaz et al. 2013). Citrulline has been investigated in human diet more exhaustively to this end, but also in other human-health fields, including pharmacology (Rouge et al., 2007; Thibault et al., 2011; Levillain et al., 1997), immunology (Sureda et al., 2009), and neurology (Sase et al., 2013). Citrulline is a precursor to the semi-essential amino acid arginine, and pharmacological study shows that in diseases related to arginine deficiencies, supplementation with citrulline is often more effective than arginine itself. This is due to its more targeted metabolism, which contributes to increased bioavailability, better absorption into the bloodstream and reduced side-effects when administered orally (Tarazona-Diaz et al., 2013; Collins 2013; Bahri et al., 2013; Mandel et al., 2005; Oketch-Rabah et al., 2016).

Metabolism and function of citrulline are well-studied in human and prokaryote systems. However, its production and role in plants is both less studied and more complex. Several studies considering physiological and environmental variables in watermelon suggest potential influences on citrulline abundance. Some research groups report a coaccumulation of both citrulline and arginine under salt stress in higher plants (Mansour, 2000; Ashraf and Harris, 2004).

The few studies found in literature are, most appropriately, performed on watermelon and related species because of the high concentration in the fruit and leaves (cite). These studies show that in response to drought and high-light stress, citrulline accumulates dramatically in the leaves of *Cucumis melo* and some *Citrullus* species, suggesting its role in osmotic adjustment, radical oxygen species scavenging, and potential use as a biomarker for selecting tolerant cultigens (Akashi et al., 2001; Smimoff

and Cumbes, 1989; Yokota et al., 2002; Kawasaki et al., 2000). Several genes are upregulated in watermelon during drought stress (including glutamine acyl transferases), further supporting these ideas (Kusvuran et al., 2012; Winter et al., 2015, Guo et al., 2013). Variation in citrulline content in domestic watermelon cultivars was studied by Rimando and Perkins-Veazie (2005), considering cultivar-level traits, like ploidy, genotype, and flesh color; and experimental variables, such as fresh weight versus dry weight.

Considering both fresh- and dry-weight, citrulline was most abundant in red watermelon fruit compared to orange- and yellow-fleshed fruit. Yellow fruit had similarly high citrulline, while there was dramatic variation in red-fleshed cultivars (0.7-3.5 mg/g FW citrulline (mean 1.0)), while orange-fleshed cultivars, Tendersweet Orange Flesh and Orange Sunshine, had 0.5 and 3.0 mg/g FW citrulline, respectively (Rimando and Perkins Veazie, 2005). Though not apparently causal, it seems carotenoids may prove predictive of citrulline content in some watermelons, especially yellow-fleshed cultivars.

Davis et al. (2013) found that six seeded genotypes yielded significantly less citrulline than seedless cultivars, with 1.8 mg/g FW (six cultivars) and 2.4 mg/g FW (eight cultivars) respectively. Another estimation involved six experimental red flesh 2x lines and their autotetraploids (4x) and triploids (3x). Only one of the six families showed significant differences in citrulline (3x and 4x > 2x), and when averaged by ploidy, no significant differences were observed (SOURCE). These data echo a greenhouse study by Liu et al., (2010) but contradict a study that did not use autoploid lines, showing that instead, 3n watermelons had higher citrulline content than 2x (Perkins-Veazie 2006). This may be due to more intensive cultural practices required of triploid watermelons.

Akashi et al., (2017) investigated citrulline accumulation in watermelon fruit considering both physiological space and time in fruit development; concurrent with Fish (2014a), citrulline was correlated to ripeness, peaking at optimal percent soluble solids content. Spacial analyses revealed a “bipolar” accumulation pattern, given that the heart, rind, and peel had the greatest concentrations of citrulline (FW), respectively.

A study on environmental effect on citrulline content (Davis et al., I2010-2011) used five cultivars across three locations (Cream of Saskatchewan, Red-N-Sweet, Tendersweet Orange Flesh in Clinton and Kinston NC; Black Diamond and Dixielee in Lane, OK). Variation in citrulline content was found within cultivar, but not among cultivars or environments. Since location did not seem to impact within-cultivar variation, high citrulline content in watermelon may be genetic in nature, which warrants investigation in genetically-related relatives in hopes of finding other high- citrulline and arginine fruit and vegetables.

With the exception of watermelon, literature is sparse concerning citrulline and arginine content and function in fruit and vegetables. Fish (2012) investigated the relative amino acid content of several cucurbits, which revealed the highest abundance in Botswana watermelon (279.4 mmoles per kg dry weight), followed closely by domestic watermelon (279.4 mmol $kgDW^{-1}$), with pumpkin, cantaloupe, cucumber, and buffalo gourd falling far behind (26.2, 21.6, 18.9, and 14.1 mmol $kgDW^{-1}$ respectively), and undetected in yellow squash (Fish 2012). However, Fish aimed to assess the reliability of a quantitation method, and thus appropriately sampled single fruit multiple times, to evaluate technical variation instead of biological variation. The present study, however,

was designed to evaluate abundance and biological variation across cultigens representing different cucurbit types, species, and genera.

Materials and Methods

Germplasm, Cultivation and Field Design

Cucurbits were selected to include cultivars common in the United States and Asia. Germplasm spanned seven species representing five genera, including *Citrullus lanatus, Cucumis melo, Cucumis metuliferus, Cucumis sativus, Cucurbita pepo, Melothria scabra, and Momordica charantia* (Table 1).

Cucurbits were harvested in groups: summer squash, winter squash, and all other cultivars. Winter squash, including butternut and pumpkin, were difficult to measure in the HPLC runs, so were excluded from the data summaries. Cultigens were evaluated in 2016 in two locations, the Horticultural Crops Research Station in Clinton, NC and the Cunningham Research Station in Kinston, NC. The experiment was a randomized complete block design with three replications. Plants were grown using five-plant plots with 2-ft spacing in 12-ft. long plots and 5-ft. row spacing. The vines were turned to prevent them from growing into adjacent plots until fruit set**.** Plots were grown using horticultural practices recommended by the North Carolina Extension Service (Sanders, 2004).

Sample Collection

Harvest

For **watermelons**, fruit were harvested when ripe (brown tendril, largest fruit, full seeds, red flesh). **Squash** were harvested unripe, before seed development, according to typical market preference. **Cantaloupe** were harvested when fruit began to turn yellow on the outside and at half-slip or later, according to cultivar recommendation. **Pickling melons** were harvested at approximately three inch diameter. **Cucumbers** were harvested at 50 mm diameter. **Mouse melons** when easily detached from the peduncle and spotting began to fade. Horned melons were harvested when flesh turned completely orange. **Bittermelons** were harvested at two stages: an unripe, green stage before seeds matured, and again once the fruit had ripened and the end of the fruit began to dehisce, exposing the inner, bright-red arils.

Sampling methods

All samples were collected and stored individually in polyethylene bags. Samples were immediately transferred to ice-filled coolers and stored on ice (up to six hours) before being transferred to -18ºC freezer (up to 5 months) until blending. Portion of fruit sampled varied with fruit type, and is detailed here. For methods used for each cucurbit, see Table 1.

Sample Processing

Blending

Samples were left to thaw at 4ºC overnight, or longer if needed (squash and pickling melons took up to 48h). After thawing, samples were blended, some individually, some after being combined by weight, and some after being combined by size (middle third of fruit). For fruit considered "ripe" at marketable stage (harvest time), SSC and pH data were taken to ensure ripe fruit were sampled and combined (watermelon, most melons). Samples were blended until homogenized into a consistent slurry, taking at least 45 sec (Waring Laboratory 7010S 1L 2 Speed Blender w/Timer, using an MC-3 Mini container; 50 to 250 mL capacity). Variations or details for the specific fruit type are described in the next section.

Blending and ripeness qualification

Samples were half-thawed in water, and seeds were removed before blending. Fruit were blended individually for 45 seconds or until homogenized into a consistent slurry (Waring Laboratory 7010S 1L 2 Speed Blender, using an MC-3 Mini container; 50 to 250 mL capacity).

For parents, individual samples were analyzed for percent soluble solids and pH. For offspring, percent soluble solids and pH data were recorded, and purees of qualifying fruit (%SS >7.9, pH 5.0-6.5) were pooled within plot by weight (within 0.1g) and quality data were recorded, percent soluble solids and pH again, in addition to lycopene, citrulline, and arginine. Percent soluble solids and flesh pH were measured using a digital refractometer (Atago PAL-1, Atago, Inc., Bellevue WA) and digital pH meter (Hach, Model (H260G; Hach, Loveland, Colorado) equipped with a stainless steel rounded electrode (PH77-SS; Hach), respectively.

Individual purees (parents) or pooled homogenates (offspring) were further processed on a polytron for 15 seconds (Polytron PT 10-35 GT) and aliquoted into 1.5mL tubes for freezing at -18°C. Aliquots were transported on ice to Kannapolis, NC where there were stored at -80°C until extraction.

Citrulline and arginine extraction and quantification

Extraction

Frozen watermelon purees stored at -20°C were thawed at room temperature and weighed out in 0.2 g +/- 0.01g aliquots. To these aliquots, 0.03M H_3PO_4 (1.2mL) was added before vortexing (1 min). Purees were then sonicated (30 min), left at room temperature to rest (10 min), and then centrifuged (14000 rpm, 4ºC, 20 min; centrifuge 5417 R, Eppendorf). Supernatants (1 mL) were filtered into amber HPLC vials (17 mm nylon syringe filter, F2513-2, Thermo Scientific) and frozen at -80ºC until HPLC analysis.

Quantification

Citrulline and arginine concentrations were determined using a modified method of Jayaprakasha et al (2011). HPLC was performed using an Elite LaChrom, Hitachi system with a Gemini 3u C18, 110 A, 250X4.6mm. 00G-4439-EO, phenomenex column

and C18 4 x 2.0; AJO-4286, phenomenex SecurityGuard Cartridges. Injection volume was 5 ul of filtered supernatant using a mobile phase of 0.015M H_3PO_4at 0.5ml/min at room temperature (25°C) with a runtime of 30 min.

Data Analysis

LS means were simultaneously compared via the procedure for general linear models (PROC GLMMIX) using SAS (SAS Institute, Cary, NC). Locations, replications, and genotypes were all analyzed as random effects. Analysis of variance (ANOVA) was used to determine significance of each variance component and their interactions for each of the five traits. Correlations between quality traits were calculated using the SAS procedure for correlations (PROC CORR).

Results

All analyses were performed assuming unrelated cultigens (i.e. no grouping by genus, species, kind, or type). For citrulline, the interaction between location and cultigen was significant (P<0.0001), along with cultigen (P<0.0001), though location alone was not significant (*P*=0.861). For arginine, the location x cultigen interaction was not significant (P= 0.061), as was location (P=0.2359), while cultigen was significant (P<0.0001). The same was true when citrulline and arginine were combined ($P_{loc*clt}$=0.383; $P_{loc*clt}$=0.531; P_{clt}<0.0001). For SSC, only clt was significant (*P*<0.0001) Cultigen was by far the largest mean square for all four traits. **Table 2**. pH was used only for correlations.

Considering citrulline, cultigens containing the highest amounts (based on LS means) (in mg$(100g)^{-1}$ fresh weight) were 'Crimson Sweet' watermelon (284.9), 'Dixielee' watermelon (236.3), 'Golden Beauty' melon (86.3), Mouse melon (64.4), and horned melon rind (45.2). Least squares means revealed eight groups, with only watermelon being distinctly separated. Considering arginine, cultigens containing the highest amounts (in mg$(100g)^{-1}$ fresh weight) were 'Crimson Sweet' watermelon (147.2), 'Dixielee' watermelon (132.0), Bitter melon arils (65.9), horned melon rind (39.2), and horned melon flesh (38.4). Grouping by least means revealed three lettered groups, again, with only one distinctly separated group (watermelons). Considering the combined concentrations of citrulline and arginine, cultigens containing the highest amounts (in mg/100g) fresh weight were 'Crimson Sweet' watermelon (432.6), 'Dixielee' watermelon (369.3), 'Golden Beauty' melon (104.3), mouse melon (99.0), and horned melon rind (84.6). Six groups were found using multiple comparisons, watermelons being the only distinct group. Considering cit and cit+arg amino acid concentrations, watermelons had the greatest LS means (ranked 1st and 2nd) , at least twice as large compared to the next highest cultigen, which was 'Golden Beauty' (ranked 3rd). Bitter melon aril ranked 4th for citrulline (65.9) but last for arginine (2.7). That was the greatest difference between citrulline and arginine rankings.

Correlations were performed between SSC, pH, citrulline (cit), arginine (arg), and citrulline plus arginine. Significant correlations (other than arg to cit+arg and cit to cit+arg) included cit and arg (0.173), SSC and cit+arg (-0.558), and SSC and cit (-0.22).

Discussion

There are several cucurbits that have significant bioactive and nutrient profiles. For example, watermelon has a high concentration of citrulline and lycopene that impart health benefits including increased vasodilation, cardiovascular health, and reduced risks for stroke and several cancers (Collins et al., 2006; Perkins et al., 2012). In addition to benefiting human health, citrulline is also thought to help mediate drought and salt stress in plants, while also serving as a radical oxygen species scavenger (Akashi et al. 2001; Kusvuran et al., 2013; Wang et al., 2014). However, to date, it is not known whether watermelon is the cucurbit with the highest amount of citrulline. A study by Fish (2012) revealed relative amino acids among several cucurbit types, but was mostly focused on methods development, not biological variation. This study was designed to compare citrulline and arginine content across several cucurbit types, each represented by up to two cultivars. Factors accounted for include cultivar, cultivar type, location, replication, soluble solids content (°Brix), acidity (pH), arginine, and citrulline.
interaction between location and cultigen was significant for arginine ($P>0.0001$), main effects cannot be considered reliable of variation. Should I investigate simple effects here?

High quality watermelon cultivars are usually characterized by vibrant red flesh color, high SSC, and slightly acidic flesh pH (~5.5). Watermelons have an increased reputation as a functional food with consumers. Moderate correlations of SSC and arginine to citrulline, coupled with positive but weak correlation of lycopene to citrulline, suggests that simultaneous breeding for increases in lycopene, citrulline, and SSC may be possible. Understanding inheritance of quality attributes with bioactives such as lycopene and citrulline should encourage breeding efforts to increase these traits of interest.

From this study, watermelons contain the greatest concentration of citrulline and arginine, at least twice as all other cultigens tested. 'Golden Beauty' melon is a good source of citrulline. Additionally, mouse melon and horned melon may be interesting sources of the arginine family of amino acids, perhaps because of their large seed and aril content relative to mesocarp.

Negative correlations of SSC to citrulline and cit+arg contradict moderate positive correlations in a watermelon heritability study (unpublished data). Given the wide array of cultigens, correlations here may be affected by our choice of cucurbit species to test. Investigation of specific genera, species, and types may be warranted to pursue meaningful relationships between arginine family amino acids and ripeness parameters.

Further studies may elucidate other sources of variation, including method of extraction, water activity, and physiological ripeness. The extraction method employed for all cucurbits was optimized for extraction using watermelon tissue, which is low in cellulose and easy to homogenize. Tissues like horned melon rind, bitter melon, and zucchini may not yield full recovery due to their toughness. Additionally, physiological ripeness may be an important factor to incorporate. In watermelon, it is thought that citrulline content peaks at physiological ripeness (Fish 2014). The same may be true for other cucurbits.

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Species and Type	Cultigen	Source	Blending[w]
Citrullus lanatus			
Red-flesh seeded watermelon	'Dixielee'	Syngenta	M1
	'Crimson Sweet'	Syngenta	M1
Cucumis melo			
Golden Beauty type	NC BL[v]	NC BL[v]	M2
Honeydew	'Dulce Nectar'	Park Seeds	M2
	'Snow Mass'	Park Seeds	M2
Muskmelon	'Aphrodite'	Syngenta	M2
	'Athena'	Syngenta	M2
Pickling Melon	Green-Striped	Kitazawa Seed	M3
Sprite Melon	White-skinned	NC BL	M2
	Yellow-skinned	NC BL	M2
Cucumis metuliferus			
Horned melon	Horned melon, rind	Kitazawa Seed	M4
	Horned melon, flesh		*

Cucumis sativus					
Pickling-type		‘Expedition’		Seminis	M3
		‘Vlaspik’		Seminis	M3
Slicer-type		‘Dasher II’		Seminis	M3
		‘Intimidator’		Seminis	M3
Cucurbita pepo					
Squash, yellow		‘Enterprise’		Park Seeds	M4
		‘Goldstar’		Park Seeds **0**	M4
Zucchini, green		‘Payload’		Stoke Seeds	M3
		‘Payroll’		Stoke Seeds	M3
Melothria scabra					
Mouse Melon		Mouse Melon		Park Seeds	M4**
Momordica charantia					
Indian bitter melon		Unripe		Kitazawa Seed	M4
		Ripe, rind			M4
		Ripe, arils			+

Source	df	SSC	Arginine	Citrulline	citarg[w]
Location	1	***14.94**	204.15	3.4163	154,75
Rep(Loc)	4	2.00	160.63	111.78	531.45
Cultigen	23	***69.93**	***3400.69**	***8048.00**	***19736.2**
Loc x Clt	20	0.95	240.71	***431.66**	425.26
Error	60	0.84	142.38	111.68	390.12

Table 1. Cultivar names are included in alphabetical order by species, type, and cultivar.

[v]NCBL= North Carolina Breeding Line

[w]Processing methods: **M1**: Fruit were sampled by cutting in half at the equator of the fruit and scooping flesh from the innermost portion of the fruit; samples were combined by weight; **M2**: Fruit were sampled by cutting a section of flesh approximately 2in x 2in opposite the groundspot. Rind was cut away from ripe flesh before bagging and freezing; samples were combined by weight. **M3**: Whole fruit were harvested and frozen. After thawing, the middle third of the fruit was cut and if present, peels were removed using a hand-peeler; Samples were combined by weight. **M4**: Whole fruit were sampled and frozen. After thawing, samples were blended whole and combined by weight, except for mouse melons and bitter melon arils.

*=Flesh was blended to loosen seeds from arils; samples were centrifuged to remove seed debris from sample. Supernatant was tested.

**= after freezing, outer cuticle was removed

[+]= all ripe arils were quickly removed from the seeds while sample was still frozen.

Table 2. Survey ANOVA of four quality traits considering location (loc), replication (rep) within location, cultigen, location and cultigen (clt)interaction, and error terms random.

Species and Type	Cultigen Information	DP(ct.)[W]	SSC (°Brix)	Arginine mg(100g)$^{-1}$	Citrulline mg(100g)$^{-1}$	Cit+Arg[x] mg(100g)$^{-1}$
Citrullus lanatus						
Red-flesh seeded	'Crimson Sweet'	2(6)	10.6±0.2	147.4±11.8	285.3±27.5	432.6±15.7
	'Dixielee'	3(9)	11.2±0.6	132.2±11.6	237.8±45.4	370±38.8
Cucumis melo						
Golden Beauty	NC BL[v]	2 (20)	11.0±0.6	18.13±4.5	86.3±4.4	104.4±8.9
Honeydew	'Dulce Nectar'	6 (17)	11.5±1.3	16.3±1.0	16.3±1.0	41.5±8.8
	'Snow Mass'	5 (14)	12.9±2.3	17.7±5.0	17.7±4.9	45.6±14.4
Muskmelon	'Aphrodite'	5 (15)	11.5±1.3	12.8±2.1	34.0±6.9	46.8±8.3
	'Athena'	6 (18)	7.22±1.5	13.5±1.8	41.0±7.3	54.5±8.5

Pickling Melon	Green-Striped	5(14)	3.4±0.5	10.4±2.6	16.7±6.2	36.5±24
Sprite Melon	White-skinned	2 (20)	10.7±0.4	9.6±0.6	35.4±18.0	45±18.6
	Yellow-skinned	2 (20)	9.7±0.4	9.4±1.4	40.7±16.8	50.2±18.2
Cucumis metuliferus						
Horned melon	rind	3 (9)	4.4±0.05	41.2±20.6	49.3±34.4	90.4±54.7
	flesh	3 (9)	7.3±1.2	39.0±12.1	9.0±5.1	48.1±17.2
Cucumis sativus						
Pickling-type	‘Expedition’	6 (18)	2.9±0.3	20.0±4.9	21.1±9.2	45.5±17.1
	‘Vlaspik’	6 (18)	3.0±0.2	27.3±3.3	25.2±8.4	40.3±10.7
Slicer-type	‘Dasher’	6 (18)	3.2±0.4	26.3±6.0	29.1±11.7	55.5±16.8
	‘Intimidator’	5 (13)	3.4± 0.3	29.8±7.3	22.8±6.0	52.6±11.2
Cucurbita pepo						
Squash, yellow	‘Enterprise’	6 (16)	4.3±0.4	24.0±3.5	7.12±4.8	31.1±7.8
	‘Goldstar’	6 (18)	4.5±0.2	25.2±4.4	5.6±4.5	27.4±9.3
Zucchini, green	‘Payload’	6 (11)	3.1±0.4	34.7±31.0	8.5±6.2	20.2±7.8
	‘Payroll’	6 (17)	2.9±0.5	15.7±7.6	4.2±2.8	43.2±36.3
Melothria scabra						
Mouse Melon	Mouse Melon	4 (120)	3.1±0.1	34.6±7.6	66.3.6±24.0	100.9±31.4
Momordica charantia						
Bitter melon	Unripe	6 (18)	2.0±0.3	27.9±17.8	11.4±11.5	39.2±29
	Ripe, rind	5 (15)	3.2±0.2	17.0±13.2	5.1±1.5	22.1±14.7
	Ripe, arils	5 (15)	16.6±2.3	68.0±23.7	3.±1.8	70.9±22.1

V= NCBL: North Carolina Breeding Lines

W=DP(ct): Data points (plots) followed by total fruit count sampled in parentheses.

X= the combined concentrations of citrulline and arginine.

Table 4. LS means of citrulline and arginine content of 24 cucurbit cultigens, grouped based on significant differences.

Beauty Mouse Melon	64.4	BC	Horned M Rind	39.2	BC	Beauty Mouse Melon	99	B
Horned M Rind	45.2	BCD		38.4		Horned M Rind	84.6	BC
Athena	40.7	BCD	Horned M flesh		BC	BMRA	69.2	BCD
Sprite, Yellow	39.8	BCDE	Mouse Melon	34.3	BC	Dasher	54.3	BCDE
	34.2	BCDE	Payroll	30.3	C		54.2	
Sprite, White		F		29.5		Athena		BCDE
	33.7	BCDE	Intimidator		C		52.2	BCDE
Aphrodite		F		26		Intimidator		F
	28		Dasher		C		49.3	BCDE
Dasher		CDEF		25.9		Sprite, Yellow		F
	27		Bitter Melon		C	Horned M	47	BCDE
Snow Mass		CDEF		24		flesh		F
	24.9		Goldstar		C		46.5	BCDE
Dulce Nectar		DEF		23.8		Aphrodite		F
	22.9		Enterprise		C		44.7	BCDE
Pickling Melon		DEFG		21.1		Snow Mass		F
	22.6		Expedition		C		44	BCDE
Expedition		DEFG		18		Sprite, White		F
	22.5		Golden Beauty		C		43.9	BCDE
Intimidator		DEFG		17.4		Expedition		F
	22.5		Snow Mass		C		41	BCDE
Vlaspik		EFGH		17.2		Dulce Nectar		F
	9.4		Vlaspik		C		39.9	BCDE
Bitter Melon		EFGH		15.9		Vlaspik		F
Horned M flesh	8.5	EFGH	Dulce Nectar		C		38.1	BCDE
				15.3		Payroll		F
Payroll	7.2	FGH	BMRR		C	Bitter Melon	35.7	CDEF
	6.3		Payload	15	C	Pickling Melon	33.8	CDEF
Enterprise		FGH		13.4		Enterprise	30.7	DEF
BMRR	5	GH	Athena		C	Goldstar	28.7	EF
Payload	4.3	H	Aphrodite	12.8	C	BMRR	20.5	EF
Goldstar	4.3	H	Pickling Melon	10.2	C	Payload	19.7	F
BMRA	2.7	H	Sprite, White	9.6	C			
			Sprite, Yellow	9.4	C			

Table 5. Pearson correlation coefficient of traits.

Trait	Cit+Arg[w]	Citrulline	Arginine	Flesh pH
Citrulline	0.666*	-		
Arginine	0.006	0.173*	-	
Flesh pH	0.342	0.110	-0.216	
SSC[v]	-0.558*	-0.22 *	0.104	-0.290

[w]combined citrulline and arginine concentrations.
*Indicates significant at p=0.05.
[v] Soluble solids content.

Narrow-Sense Heritability and Genetic Variance Component Estimates for Citrulline And Lycopene In Two North Carolina Watermelon Populations

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Abstract

Watermelon fruit (*Citrullus lanatus*) is a natural source for phytonutrients including lycopene, citrulline, and arginine. Two segregating, highly outcrossed North Carolina watermelon populations, NC High Yield (NCHYW) and NC Small Fruit (NCSFW), were evaluated for these traits, pH, and SSC. Parents tested in 2015 (N_{SF} = 300, N_{HY} = 300) were sampled for citrulline, arginine, lycopene, pH, and soluble solids content (SSC). Their offspring were tested in 2016 if the sampled fruit of the parents were of qualifying ripeness (SSC≥8, pH 5.5-6.5), resulting in 251 families (N_{SF} = 72, N_{HY} = 175). Narrow-sense heritability was estimated in each of the populations using two methods: 1) parent-offspring regression and 2) variance of half-sibling family means. Heritability for citrulline NCHYW was moderate in both parent-offspring and half-sibling estimations (38% and 43%), as was arginine (40% and 44%) and lycopene (46% and 47%, respectively). Estimates for these traits in NCSFW were considerably different. In NCHYW, moderate correlations were found between SSC and citrulline (0.40), arginine (0.40), their combination (0.45), and lycopene (0.30) all of which were significant, except lycopene. Lycopene was weakly but significantly correlated to citrulline (0.22) but not correlated to arginine (0.06). Similar correlations were found in NCSFW, though weaker. Based on these heritabilities and correlations, selection for high lycopene and citrulline content may be accomplished efficiently, especially if the NCHYW population is used.

Introduction

The American Heart Association endorses watermelon as a heart-healthy food (American Heart Association) due to its low sodium and fat content. Additionally, watermelon contains both lycopene and citrulline, bioactives with implications in both plant and human health (Fish, 2012; Tedesco et al., 1984). Lycopene, a red-pigmented carotenoid, serves endogenously as a strong antioxidant endogenously in plants and dietarily in humans. In plants, it is an intermediate in carotenoid biosynthesis, and in watermelons, it is found in greatest abundance in red fleshed cultivars (70-90% of total carotenoids) (Tomes et al. 1963; DiMascio et al., 1989). Mutations in genes up- and down-stream in its synthesis generate other flesh colors, including orange-, canary yellow-, and salmon yellow-fleshed watermelons (Kang et al., 2010; Tadmor et al. 2004). Lycopene and total carotenoid abundance increase rapidly at 10 to 12 days after pollination in diploid watermelons, and continue to increase as the fruit matures (Lv et al., 2015).

Epidemiological studies have found that diets including high-lycopene fruit and vegetables (tomato, watermelon, grapefruit, guava) can reduce risk of stroke and

cardiovascular diseases (Steinmetz and Potter, 1996, Strandhagen et al., 2000). Lycopene scavenges radical oxygen species and quench DNA chain breaking agents (Stahl et al., 1997). Lycopene is most known for its ability to reduce cancer cell growth and induce cell death in malignant leukemia, mammary, endometrial, lung, and prostate cancer cells (Amir et al., 1999; Kotake-Nara et al. 2001; Levy et al., 1995; Muller et al., 2002). Lycopene plays a role in cardiovascular health by slowing the development of atherosclerosis through attachment to low-density lipoproteins (LDLs) in blood plasma and protect against lipid peroxidation and foam cell production (Matos et al., 2000; Gianetti et al 2002; Arab and Steck, 2000).

Citrulline, a physiological amino acid with a high N:C ratio, has implications in both plant and animal health. Citrulline has been administered orally (citrulline malate) in both human and mouse studies to affect certain areas of human health, including skeletal and muscle performances and muscle loss prevention (Perez-Guisado and Jakeman 2010; Sadji et al., 2015), pharmacology (Rouge et al., 2007; Thibault et al., 2011; Levillain et al., 1997), immunology (Sureda et al., 2009), and neurology (Sase et al., 2013). Citrulline supplementation offers several pharmacokinetic advantages over arginine, including a more targeted role in human metabolism, increased bioavailability and absorption into the blood, and decreased side-effects when administered orally (Tarazona-Diaz et al., 2013; Collins 2007; Bahri et al., 2013; Mandel et al., 2005; Oketch-Rabah et al., 2016). Health areas of greatest interest include muscle recovery during exercise (Tarazona-Diaz et al., 2013), and vascular health. Vascular health benefits include increasing vasodilation, which is exhibited by decreased systolic and diastolic blood pressure (Figuoroa et al., 2011) correcting erectile dysfunction (Drewes et al., 2003; Shiota et al., 2013), and decreasing incidence of vasospastic angina (Morita et al., 2013), a symptom of coronary heart disease.

The specific function and metabolism of citrulline are considerably less articulated in plants, compared to its characterization in humans, prokaryotes, and yeast. Several studies suggest that citrulline serves abiotic stress abating roles, as citrulline accumulates dramatically in the foliage of watermelon and related species in response to drought, salinity, and high light. Citrulline may act as a compatible solute for osmotic adjustment, and as a radical oxygen species scavenger during times of extreme oxidative stress (Akashi et al., 2001; Smimoff and Cumbes, 1989; Yokota et al., 2002; Kawasaki et al., 2000). A coaccumulation of both citrulline and arginine under salt stress has been reported in higher plants (Mansour, 2000; Ashraf and Harris, 2004). Several genes are upregulated in watermelon during drought stress, including glutamine acyl transferases, further supporting these ideas (Kusvuran et al., 2012; Winter et al., 2015).

Of the bioactive metabolites found in watermelon fruit, carotenoid content most directly correlates with the stage of fruit development and ripeness. Carotenogenesis increases during development in a sigmoid fashion, while reports on phenolic compounds, and vitamin C relative to fruit development are conflicting or inconclusive (Tlili, 2011). Understanding carotenoid metabolism, the primary contributor to watermelon flesh color, is paramount to maintaining high quality in transport and storage, and may also relate to citrulline content.

Rimando and Perkins-Veazie (2005) studied variation in citrulline content in watermelon, considering ploidy level, cultivar, flesh color, and fresh weight versus dry weight. Red watermelons had significantly less citrulline than orange and yellow-fleshed

fruit on both a fresh and dry weight basis. The two yellow cultivars had similarly high citrulline, but there was significant variation within red and orange colors; red flesh ranged from 70-350 mg/100g FW citrulline (mean 100), while orange flesh cultivars, Tendersweet Orange Flesh and Orange Sunshine, had 50 and 300 mg/100g FW citrulline, respectively (Rimando and Perkins Veazie, 2005). Despite this variation, carotenoids are still considered a potential predictor of the amount of citrulline in watermelons, especially in yellow flesh watermelons.

Considering seedlessness, six seeded cultivars yielded less citrulline than eight seedless cultivars, with 180 mg/100g FW (seeded) and 240 mg/100g FW (seedless) (Davis et al., 2013). To estimate effects of ploidy on citrulline content, six experimental lines (2x) and their autotetrapoids (4x) and triploids (3x) were investigated for amount of citrulline produced. Of the six families, only one showed significant differences in citrulline content (3x and 4x > 2x), but when averaged by ploidy, no significant differences were observed. These data parallel a results of Liu et al., (2010), using watermelon from nine triploid hybrids grown under greenhouse conditions. In contrast, 3x watermelons had a higher citrulline content than 2x fruit in field grown watermelons lacking autoploid lines (Perkins-Veazie 2006).

Citrulline accumulation in watermelon fruit appears to be correlated to ripeness. Akashi et al., (2017) investigated spacial and temporal citrulline accumulation in watermelon fruits; concurrent with Fish (2014a), citrulline content peaked at optimal soluble solids content. Akashi et al. described a "bipolar" accumulation pattern, with peel (440 ± 80 mg/100g) having the highest content on a fresh weight basis compared to heart (240 ± 99 mg/100g FW) and rind (210 ± 94 mg/100g FW). Citrulline content in the rind varies within cultivar, between cultivar, and depending on the fruit stage. Despite reports of much variation between cultivars (±47.1% coefficient of variation in Fish 2014), rind has consistently higher citrulline content than the flesh in most studies that compare tissue types (Fish 2014; Rimando and Perkins-Veazie 2005; Jayaprakasha et al. 2011).

Davis et al., 2010-2011) investigated response to environment using five cultivars in three locations (Cream of Saskatchewan, Red-N-Sweet, Tendersweet Orange Flesh in Clinton and Kinston NC; Black Diamond and Dixielee in Lane, OK). There was a wide range of values for citrulline within cultivar, but no significant differences among cultivars, nor across environments tested. Based on this study, since location did not appear to significantly affect within-cultivar variation, breeding for high citrulline content in watermelons across widely different environments may be possible. Other findings suggest that citrulline is significantly affected by environment; when grown in two locations, Oklahoma and Texas, citrulline content varied widely within the same cultivars (Fish and Bruton, 2010).

Breeding for plant metabolites proves difficult when environmental effects predominate. Citrulline concentrations (or the perception of them) change significantly with many factors including: cultural practices (grafting, planting densities, harvest date); environmental effects (growing season, location, year, drought and salt stress); fruit ripeness; cultivar-level variation (genotype, ploidy, lycopene content, arginine content); and analytical methods (tissue type, tissue processing, sample storage, extraction method, analytical instrumentation). Understanding this variation is important to consider when making breeding decisions. The objective of this research was to estimate genetic and environmental variance for citrulline content, along with other fruit quality traits.

Methods

Cultivation and Field Design

Summer 2015: Parents

In 2015, two North Carolina watermelon populations, NC Small-Fruit (NCSFW) and NC High-Yield (NCHYW), (N_{SF} = 300, N_{HY} = 300) were grown in Castle Hayne, NC. Plots were planted on raised, shaped beds with 3.1 m centers and single hills 1.2 m apart. Single fruit were harvested and seeds extracted from single-plant plots for planting in 2016.

Summer 2016: Offspring

Seeds from 2015 parents of qualifying ripeness SSC ≥8, pH 5.5-6.5) were planted in 2016 (N_{SF} = 72, N_{HY} = 175). Offspring were tested at two locations, The Horticultural Crops Research Station in Clinton, NC and the Cunningham Research Station in Kinston, NC. The experiment was performed using randomized complete blocks with two replications. Replicates of each population were planted in a randomized complete block design. Field layout was identical to the parent population, except offspring were grown using six-plant plots 3.7 m long, instead of single-plant hills.

Single-plant hills and 6-plant plots were grown using horticultural practices recommended by the North Carolina Extension Service (Sanders, 2004). Irrigation was using drip tubes in beds covered with black polyethylene mulch. Soil type was a Norfolk fine sand at Castle Hayne, Orangeburg loamy sand at Clinton, and a Norfolk sandy loam at Kinston. Each plant was manually trained each week in a spiral by turning all the vines in a clockwise circle around the crown until 70% of the plants in the field had set fruit**.** Training vines may introduce environmental variation resulting from altered light intensity on the canopy, but this allowed for efficient sampling later in the season, thus reducing experimental error.

Germplasm

NC Small Fruit

This population was created in 2005 and included cultivars New Hampshire Midget, Minilee and Allsweet, which contributed yield, earliness, quality, disease resistance, and small fruit size. This population was intercrossed every year, while selecting for yield, earliness, quality, disease resistance and small fruit size**.**

NC High Yield

This population was created in 2005 from crosses of Calhoun Gray, Dixielee, Mt. Hoosier, Big Crimson, Starbrite, Legacy, Red-N-Sweet, Sangria, and Early Arizona**..** F2s were planted in summer 2006 and were intercrossed five times between 2006 and 2015.

Sample Collection

For parents, single, ripe fruit were harvested (brown tendril, largest fruit, full seeds, red flesh*) from each plot for quality analysis. Watermelons were cut transversely between blossom- and stem-ends. Samples of ~100g in size were scooped from the center of the watermelon (ice cream scoop) and bagged individually in custom polyethylene bags of 4 ml thickness (Uline, GA, US). Small Fruit samples were mixed tissue samples

(heart, locule, interlocule); High Yield samples were from heart only. Samples were kept on ice for no longer than 6 hours, after which they were frozen at -18C until blended.

For the offspring, the same sampling procedure applied using four fruit per plot, of which three ripest were sampled for quality analysis using the (ripeness indicators above. Samples were bagged (and later blended) individually.

*Fruit in NC-High Yield were segregating for flesh color; some fruits had swirled red-and-yellow flesh.

Blending and ripeness qualification

Samples were half-thawed in water, and seeds were removed before blending. Fruit were blended individually for 45 seconds or until homogenized into a consistent slurry using a blender (Model Waring Laboratory 7010S 1L and an MC-3 Mini container).

For parents, individual samples were analyzed for SSC and pH. For offspring, Fruit SSC and pH data were recorded, and purees of qualifying fruits SSC ≥8, pH 5.0-6.5)were pooled within plot by weight (within 0.1g) and quality data were recorded, including SSC, pH, lycopene, citrulline, and arginine. SSC and flesh pH were measured using a digital refractometer (Atago PAL-1, Atago, Inc., Bellevue WA) and digital pH meter (Hach , Model (H260G; Hach, Loveland, Colorado) equipped with a stainless steel rounded electrode (PH77-SS; Hach), respectively.

Individual purees (parents) or pooled homogenates (offspring) were further processed on a Polytron for 15 seconds (Polytron PT 10-35 GT, Kinematica, NY), aliquoted into 1.5mL tubes, and frozen at -18C. Aliquots were transported on ice to Kannapolis, NC where they were stored at -80˚C until extraction.

Lycopene quantification

Lycopene concentration was measured using 5mL thawed aliquots diluted in 15mL deionized water. Sample absorbance at 560 and 700nm was measured using an UltraScan PRO colorimeter (Hunter Lab, Reston, VA, USA). Total lycopene content was calculated using the formula (Abs560-Abs700)*DF(wt/volume)* slope (31.8), where slope is calculated by plotting values from the colorimeter versus the same sample when analyzed with hexane extraction using a spectrophotometer and standardized with an external lycopene standard (Davis et al., 2003). Units are expressed as $\mu g*g^{-1}$ fresh weight (FW).

Citrulline and arginine extraction and quantification

Extraction

Frozen watermelon purees stored at -20^{O}C were thawed at room temperature, 0.03M H_3PO_4 (1.2mL) added to 0.2g +/- 0.01g aliquots of puree, and vortexed for 1 min. Purees were then sonicated (30 min), left at room temperature to rest (10 min), and then centrifuged (14 000 rpm, 4˚C, 20 min; centrifuge 5417 R, Eppendorf). Supernatants (1 mL) were filtered into amber HPLC vials (17 mm nylon syringe filter, F2513-2, Thermo Scientific) and frozen at -80˚C until HPLC analysis.

Quantification

Citrulline and arginine concentrations were determined using a modified method of Jayaprakasha et al (2011). HPLC was performed using an Elite LaChrom, Hitachi system with a Gemini 3u C18, 110 A, 250X4.6mm. 00G-4439-EO, phenomenex column and C18 4 x 2.0; AJO-4286, phenomenex SecurityGuard cartridges. Injection volume was 5 ul of filtered supernatant using a mobile phase of 0.015M H_3PO_4at 0.5ml/min at room temperature (25˚C) with a runtime of 30 min.

Data Analysis

Data were analyzed with general linear models (PROC GLM) in SAS v9.1 (SAS Institute, Cary, NC). Location, replication, and genotype were considered random effects. Analysis of variance (ANOVA) was used to determine significance of each variance component and their interactions for each of the six traits. Variance components were estimated using PROC VARCOMP using SAS software.

Narrow-sense heritability (h^2) was calculated for each trait using two methods. First, a parent-offspring regression was calculated using the SAS mixed procedure (PROC MIXED), which included parent data (one location) and offspring data (averaged over 2 replications each at 2 locations). Second, h^2 was calculated using the variance of half-sibling family means, using PROC VARCOMP. Variance components were estimated for additive and environmental, additive x environment, and other components of interest. The equation used here was specific to heritability calculations for half-siblings, where σ^2_F is family (cultigen)-wise variance, $\sigma^2_{P\_HS}$ is phenotypic variance of half-sibling values, σ^2_{LF} is family by location interaction variance, σ^2_e is error variance, s = number of locations, b= number of blocks, and n= number of samples per plot (Isik et al. 2017):

$$\frac{\sigma^2_F}{\sigma^2_{P\_HS}} = \frac{\sigma^2_F}{\sigma^2_F + \frac{\sigma^2_{LF}}{s} + \frac{\sigma^2_e}{sbn}}$$

Correlations between quality traits were calculated using the SAS procedure PROC CORR.

Results

Since offspring were replicated in two locations, we were able to run family analyses using them. In all traits for both populations, with the exception of lycopene in NCHYW, the location by cultigen interaction was not significant.

For citrulline content, location (P_{HY} =0.0008; P_{SF} < 0.0001) and cultigen (P_{HY} < 0.0001; P_{SF} < 0.0001) were significant in both populations, while replication was not significant (P_{HY} =0.308; P_{SF} < 0.195). For arginine content, location (P_{HY} < 0.0001) was significant in NCHYW but not in NCSFW (P_{SF} = 0.181). Location (P_{HY} < 0.0001; P_{SF} < 0.0001) and cultigen (P_{HY} < 0.0001; P_{SF} = 0.0499) were significant in both populations, though cultigen only barely so in NCSF. For combined citrulline and arginine, location (P_{HY} = 0.0099) was significant only in NCHY. Replication (P_{HY} =0.0003; P_{SF} < 0.0001) and cultigen (P_{HY} < 0.0001; P_{SF}< 0.039) were significant in both populations.

For lycopene, the location, replication, and cultigen effects were all significant (P_{HY} < 0.0001), including the location by cultigen interaction (P_{HY} =0.0017). In NCSFW, only location (P_{SF} < 0.0001) and cultigen (P_{SF} < 0.0001) were significant. For pH,

cultigen ($P_{HY} < 0.0001$) was significant in NCHY while location ($P_{HY} < 0.0001$ $P_{SF} < 0.0001$) was significant in both populations. For SSC, location ($P_{HY} < 0.0001$) was significant in NCHY, while replication ($P_{HY} = 0.0005$; $P_{SF} = 0.0002$) and cultigen ($P_{HY} < 0.0001$; $P_{SF} < 0.0001$) were significant in both populations.

Analysis of variance revealed that in NCHYW, location was the largest variance for citrulline, arginine, lycopene, pH, and SSC, while for citrulline plus arginine, replication within location was the largest variance. For NCSFW, location was the largest variance for pH and lycopene, but replication within location was largest for citrulline, arginine, citrulline plus arginine, and SSC (Table 2).

Variance component estimates suggest that error was the greatest amount of variation for all traits of interest in the NCHYW population, with cultigen as the next-most significant component. For NCSFW, however, this was true only for citrulline plus arginine, SSC, and pH. Error was greatest for citrulline, followed by location x cultigen and replication within location. Proportions were similar for arginine, but five-fold less. Variance for lycopene came primarily from location, followed by error and cultigen (Table 3).

Narrow-sense heritability (heritability) varied greatly among the traits evaluated. In NCHYW, parent-offspring regression found moderate heritability in lycopene (46%), citrulline (38%) and arginine (40%), and moderate to low heritabilities for arginine plus citrulline (29%), pH (30%), and SSC (17%). Heritability using half-siblings found similar heritabilities for citrulline (43%), arginine (44%), their combination (29%), lycopene (47%), and pH (40%). Half-sibling heritabilities were much higher for SSC (60%). (Table 4). In NCSFW, heritabilities estimated using parent-offspring regression revealed a moderately high heritability for citrulline (65%) and SSC (60%), moderate for lycopene (44%) and citrulline plus arginine (33%), and low for arginine, pH, and SSC (9.4%, 9.8%, and ≈0%, respectively). Estimation using half-siblings revealed some dramatically differing values. Citrulline had low heritability (22%) compared to its parent-offspring estimate (65%). Arginine was low (14%), along with pH (15%). Lycopene was more heritable in NCSFW considering half-siblings (68%) over parent-offspring (44%). (Table 4).

Both populations had weak, positive correlations for lycopene with citrulline (r_{HY}=0.22 ; r_{SF}=0.15) and with Cit+Arg (r_{HY}=-0.20 ; r_{SF}=0.13). Citrulline and arginine were positively correlated in both populations, with NCSF having a much stronger correlation (r_{HY}=0.43 ; r_{SF}=0.73). In both populations, SSC was correlated significantly with citrulline (r_{HY}=0.40 ; r_{SF}=0.24), arginine (r_{HY}=0.40; r_{SF}=0.18), and their combination (r_{HY}=0.45; r_{SF}=0.23). While pH was negatively correlated with all other traits and was significant for each trait in at least one population, significance was not consistent between populations (significant for Cit+Arg and citrulline in NCHY, and significant for arginine and lycopene in NCSF) (Table 5).

Discussion

Watermelon is an economically significant crop globally as well as in the US. has important nutritive and bioactive profiles and properties, with the highest, raw-fruit concentrations of both lycopene and citrulline in addition to potassium and vitamins C and A.. In mammals, these phytonutrients together have implications in vasodilation, cardiovascular health, and reduced risks for stroke and several cancers (Collins et al.,

2006; Perkins et al., 2012). With recent national attention as a heart-healthy food due to its high lycopene and citrulline content, breeders are interested in increasing their concentrations in watermelon, a new focus for breeding. It might improve the marketability of watermelon as a functional food, while also increasing stress tolerance in the plant (Akashi et al. 2001; Kusvuran et al., 2013; Wang et al., 2014).

However, there have been no studies reporting heritability of lycopene, citrulline, or arginine in watermelon. Genetic variance was present for all six quality traits in both the parents and the offspring of the two populations studied (Table 1). For citrulline, there are several studies that report variation among and within cultigen in different environments, including that of Davis et al. (2011), which prompted this study.

Lycopene abundance varies greatly in red, orange, and yellow-fleshed watermelon, with red-fleshed cultigens containing lycopene as the major carotenoid, followed by orange- and yellow-fleshed cultigens. Perkins-Veazie et al. (2001) evaluated 13 watermelon cultigens for lycopene and found a range of variation from 37.9 to 71.2 mg/kg. Davis et al. (2006) found great variation among 50 watermelon cultivars, ranging from 33 to 100 mg/kg. Both Yoo et al. (2012) and Nagal et al. (2012) also reported high variation in lycopene among cultigens.

Heritability estimates are used to plan efficient breeding strategies to improve trait value. This is now available to improve citrulline and arginine. Zhang et al. (2010) reported on the general combining ability and heritability of lycopene, which were relatively high, suggesting a potential additive effect due to dominant genes. In the present study, environment was a significant contributor in all traits for NCHYW and had the largest mean squares, although error and cultigen had the largest variance components, except for lycopene, where location was the largest. Despite that, heritability for lycopene was moderate (47%). In NCHYW, heritability was essentially the same for citrulline and arginine, considering both parent-offspring regression (38% and 40%, respectively) and half-siblings (43% and 44%, respectively). In NCSFW, heritabilities were considerably different and fewer significant variance contributors were revealed, suggesting a potentially insufficient sample size. Variance components for all traits were more consistently distributed in the NCHYW population, with error predominating, followed by cultigen. This may be true because of the significantly larger sample size, as compared to NCSFW (N_{HY}≈650; N_{SF}≈230).

Davis et al. 2013 determined that SSC and lycopene content were slightly and positively correlated, using a study of six diploid cultigens, their autotetraploids, and their triploid progeny. No correlation was found between citrulline and SSC. In contrast, moderate correlations were found between SSC and citrulline (0.40), arginine (0.40), their combination (0.45), and lycopene (0.30) in the present study, which involved solely diploids. Lycopene was weakly but significantly correlated to citrulline (0.22) and not correlated to arginine (0.06). Similarly directional and significant correlations were found in NCSFW, though correlations were generally weaker.

High quality watermelon cultivars are usually characterized by vibrant red flesh color, high SSC, and slightly acidic flesh pH (~5.5. Watermelons have an increased reputation as a functional food with consumers; Moderate correlations of SSC and arginine to citrulline, coupled with positive but weak correlation of lycopene to citrulline, suggests that simultaneous breeding for increases in lycopene, citrulline, and SSC may be

possible. Understanding inheritance of quality attributes with bioactives such as lycopene and citrulline should encourage breeding efforts to increase these traits of interest.

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Table 1. Count, mean, low value, and high value for citrulline, arginine, lycopene, and fruit quality traits for NC High Yield watermelon (NCHYW) and Small Fruit (NCSFW) populations.[z]

Statistic	SSC[w] (°Brix)	pH	Lycopene (μg*g^{-1})	Citrulline (mg*100g^{-1}FW)	Arginine (mg*100g^{-1}FW)	Cit+Arg[v] (mg*100g^{-1}FW)
NCHYW Parent Population						
N	175	175	175	175	175	175
M	9.4 ± 0.9	5.49 ± 0.26	45.9 ± 9.0	98.7 ± 21.4	161.3 ± 60.23	260.0 ± 76.0
X_L- X_H	8-11.8	5.04-6.29	29.3-93.1	44.6-154.8	21.8-357.0	66.4-487.1
NCHYW Offspring Population						
N	645	646	653	642	642	642
M	10.3 ± 0.7	5.8 ± 10.0	42.8 ± 8.5	237.5 ± 56.8	124.5 ± 20.8	362.0
X_L- X_H	8.1-11.5	2.9-9.3	19.7-82.3	17.4-528.7	10.1-221.3	37.7-671.3
NCSFW Parent Population						
N	72	72	72	72	72	72
M	9.54 ± 1.0	5.56 ± 0.23	64.25 ± 14.76	105.05 ± 26.86	126.78 ± 64.04	231.86 ± 81.40
X_L- X_H	8.0-12.4	5.03-6.33	40.3-115.3	34.0-202.3	16.1-287.2	51.7-443.3
NCSFW Offspring Population						
N	263	258	271	263	263	263
M	10.0 ± 0.7	5.62 ± 0.26	52.3 ± 12.2	228.6 ± 90.3	142.6 ± 48.0	371.2 ± 129.6
X_L- X_H	8.1-11.7	5.00-6.46	27.6-84.5	27.2-562.1	14.5-300.7	152.5-838.8

[w]Soluble solids content.
[v]combined citrulline and arginine concentration in mg/100g FW
[z] parent values are of single watermelons planted in 2015; offspring values are averaged over two locations and two replications, with up to three fruit sampled per plot, planted in 2016

NCSHY Offspring Population

Source	df	cit+arg[w]	Citrulline	Arginine	Lycopene	Flesh pH	SSC
Location	1	24783.755*	5508.692*	63008.888*	3715.559*	3435.278*	17.239*
Rep(Loc)	2	31308.433*	416.331	26687.822*	403.268*	215.233	2.719*
Cultigen	175	6340.699*	622.088*	4584.363*	108.365*	136.302*	0.884*
Loc x Clt	171	4324.161	383.428	2660.713	61.965*	82.983	0.377
Error	289	3678.387	351.656	671776.840	41.9134	73.706	0.353

NCSFW Offspring Population

Source	df	cit+arg[w]	Citrulline	Arginine	Lycopene	Flesh pH	SSC
Location	1	40374.375	29056.011*	928.757	14166.710*	8126.308*	0.917
Rep(Loc)	2	165400.089	71659.270	19333.523*	93.925	215.654	2.371*
Cultigen	71	18140.470	9301.122*	2620.941*	187.654*	164.092	0.722*
Loc x Clt	71	16074.329*	7507.168	1356.356	73.556	140.147	0.328
Error	262	12561.765	5947.171	1859.208	56.695	136.761	0.262

Table 2. Analysis of variance (degrees of freedom and mean squares) for 6 fruit quality traits in the NC High Yield watermelon (NCHYW; 175 cultigens) and NC Small Fruit watermelon (NCSFW; 69 cultigens) populations across two locations, and two replications.

[*]significant at 0.05 probability.
[v]degrees of freedom
[w]combined citrulline and arginine concentrations.

Make heritability to just 2 decimal places

Table 3. Means, heritabilities and variance components for six quality traits in two watermelon populations (NCHYW and NCSFW) using the variance of half-sibling family means.

Statistic	SSC[w]	pH	Lycopene	Citrulline	Arginine	Cit+Arg[x]
NCHYW variance component estimates						
Location	0.045	10.96	10.36	115.97	11.74	11.19
Rep (Loc)	0.016	1.01	2.33	147.39	-0.547	172.79
Cultigen	0.14	14.49	13.14	513.96	66.82	533.93
Loc x Clt[v]	0.013	5.91	8.60	114.39	-13.15	161.83
Error	0.35	73.07	43.08	2426.4	374.92	3847.8
NCHYW quantitative genetic calculations						
Additive	0.57	57.94	52.55	2055.85	267.30	2135.71
Genetic	0.57	57.94	52.55	2055.85	267.30	2135.71
Dominance	0	0	0	0	0	0
Phenotypic	0.67	79.17	67.62	2719.64	354.45	3178.58
h^2	0.60	0.406	0.466	0.434	0.436	0.339
NCSFW variance component estimates						
Location	-0.0195	61.25	106.88	238.89	-119.42	11.19
Rep (Loc)	0.0463	-1.40	0.165	1168.3	256.01	172.79
Cultigen	0.119	6.55	34.73	565.58	139.00	533.93
Loc x Clt[v]	0.0256	5.78	3.155	1379.9	252.26	161.83
Error	0.265	135.81	57.96	5457.0	1786.6	3847.8
NCSFW quantitative genetic calculations						
Additive	0.47	26.20	138.92	2262.30	556.01	2715.31
Genetic	0.47	26.20	138.92	2262.30	556.01	2715.31
Dominance	0	0	0	0	0	0
Phenotypic	0.55	63.04	154.98	4316.52	1128.78	7077.02
h^2	0.600	0.151	0.684	0.216	0.195	0.135
Statistic	**SSC[w]**	**pH**	**Lycopene**	**Citrulline**	**Arginine**	**Cit+Arg[x]**

[w]Soluble solids content.

[v]Combined citrulline and arginine concentrations.

Make heritability to just 2 decimal places

Table 4. Estimates of narrow-sense heritability of 6 quality traits in two populations, NC High yield (NCHYW) and NC Small Fruit (NCSFW) using a parent-offspring regression and also by using the variance of half-sibling family means.

Estimation:	h^2 by Parent-Offspring		h^2 by Half-Siblings	
Population / Trait	**NCHY**	**NCSF**	**NCHY**	**NCSF**
SSC[w]	0.173	-0.343	0.604	0.600
pH	0.295	0.098	0.406	0.151
Lycopene	0.458	0.436	0.466	0.684
Arginine (arg)	0.396	0.094	0.436	0.195
Citrulline (cit)	0.379	0.653	0.434	0.216
arg + cit[v]	0.294	0.334	0.339	0.135

[w]Soluble solids content.

[v]Combined citrulline and arginine concentrations.

Table 5. Pearson correlation coefficient of traits for n=175 offspring from two

Trait	Cit+Arg[w]	Citrulline	Arginine	Lycopene	Flesh pH
NCHYW					
Citrulline	0.962*	-			
Arginine	0.663*	0.432*	-		
Lycopene	0.204*	0.222*	0.066	-	
Flesh pH	0.204*	0.189*	0.160	0.291	-
% SS[v]	0.450*	0.396*	0.403*	0.304	0.384
NCSFW					
Citrulline	0.967*	-			
Arginine	0.879*	0.730*	-		
Lycopene	0.125*	0.146*	0.063	-	
Flesh pH	0.105	0.077	0.137*	0.492*	-
% SS	0.233*	0.236*	0.183*	0.310*	0.401*

populations (175 offspring in NCHYW and 72 offspring in NCSFW).

*Indicates significant at *P*=0.05.

[v] Soluble solids content.

[w]Combined citrulline and arginine concentrations.