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The Expression of Citrulline and other Members of the Arginine Metabolic Family in Developing Watermelon Fruit

Wayne W. Fish
Wes Watkins Agricultural Laboratory
USDA/ARS, 911 Highway 3, P.O. Box 159, Lane, Oklahoma 74555, U.S.A.
Email: waynefish@rocketmail.com

Abstract – **L-citrulline is a naturally occurring amino acid that functions in detoxification of catabolic ammonia (urea production) and is a key element in production of the vasodialator, nitric oxide. Because watermelon flesh and rind possess large quantities of citrulline (1-3 mg/g fresh tissue), watermelon is viewed as a potential natural source for citrulline. Objectives were to measure expression levels of citrulline and other members of the arginine metabolic family in developing watermelon fruit. Physiological amino acids were extracted and quantified from two cultivars of watermelon produced over three growing seasons. Glutamine was the predominant amino acid in flesh and rind the first 14 days of development, but it rapidly fell in concentration while citrulline rapidly rose during the same interval. At 21-28 days postanthesis, citrulline reached its maximum concentration of 100-300 mmoles/kg dry weight and remained at that level. Variability of citrulline levels among individual fruit of a cultivar age group was much greater in the rind than in the flesh. Citrulline levels were markedly influenced by growing conditions.**

Keywords – **Arginine Metabolic Family, L-Citrulline, Physiological Amino Acids, Watermelon, Watermelon Fruit Development.**

I. Introduction

In mammalian physiology, L-citrulline (cit) is a naturally occurring amino acid that functions in detoxification of catabolic ammonia (urea production) and is a key element in production of the vasodialator, nitric oxide. Moreover, L-arginine (arg), the amino acid for which cit is an immediate precursor, is essential to several critical physiological functions such as immunostimulation and blood pressure control, in addition to its role as one of the 20 amino acids that make up proteins. However, almost all ingested free arg is cleared by the liver and does not reach the bloodstream. In contrast, cit is not cleared from portal circulation and is converted to arg in the kidney where it is then circulated to other organs in the body [*1*]. Significant attention has recently been given to use of cit as an effective agent for arg supplementation. The potential therapeutic applications of cit include short bowel syndrome, protein-energy malnutrition in aging, immunostimulation, and blood pressure control [*2*]. These therapeutic uses are currently under clinical investigations [*3*]-[4]- [*5*].

Given its close metabolic relationship with arg, cit can be found in at least small amounts in almost any living organism. In plants, cit is present in high levels in some *Cucurbitaceae*, especially the watermelon (*Citrullus lanatus*). Both the watermelon rind and flesh contain citrulline concentrations of 100-300 mmoles cit/g dry wt (1-3 mg/g fresh tissue) [6]-[7]-[8]. The role of cit in watermelon is believed to be for protection of the plant against oxidative stress, especially during periods of drought [*9*].

Current industrial production of cit is by bacterial fermentation [10]. Given the ever-growing consumer demand for 'natural' foods and food products, watermelon is viewed as a natural source for cit, but little is known about the expression of cit in watermelon fruit as a function of cultivar, growing conditions, or fruit development. The purpose of this research was to examine the expression level of cit and other members of the arg metabolic family in watermelon fruit as it developed from the flowering stage to an over-ripe state. Influences of cultivar type and growing season were also examined over three years. A preliminary report of this study was published [11].

II. Materials and Methods

A. Watermelon cultivars and their culture

Watermelons were grown at the Wes Watkins Agricultural Research Center, Lane, OK in 2009, 2010, and 2011. The soil was a Bernow fine-loamy, siliceous, thermic, Glossic Paleudaf. Two cultivars of watermelon were used for the test: Dixie Lee (Willhite Seed, Poolville, TX), a diploid, and Super Seedless SS7177HQ (Abbott and Cobb, Feasterville, PA), a triploid. The cultivars were arranged in a randomized complete block design with four replicates.

Daily high and low temperatures and maximum and minimum relative humidities, rainfall amounts, wind velocities, and soil temperatures were recorded by an Oklahoma Mesonet station on-site 300 m from the experimental plots. In 2010 and 2011, three moisture potentiometers were placed in each 45 m row of experimental watermelons and read daily. Plant management was provided as outlined by Motes and Cuperus [*12*]. Transplants were placed at 1 m intervals into bare, 2 m wide raised beds 45 m long and on 3 m centers. Plants were irrigated as needed (at the first signs of water stress) and fertilized monthly.

B. Sample preparation

Watermelon flesh tissue for analysis was removed from the center of the heart; rind tissue came from the center top

of the fruit (opposite the ground spot). The peel was removed from the rind, and no red or pink flesh was left on the inner portion of the rind. Tissues were frozen at -20 °C (or -80 °C for long term storage) for 24 hr and thoroughly homogenized for 3 min with a Polytron™ PT 10-35 grinder (Kinematica AB, Lucerne, Switzerland) set at the highest speed. The sample tube was kept in an ice bath while grinding. After grinding, samples were centrifuged at 10,000× *g* for 10 min at 15°C to pellet insoluble debris. An aliquot of the supernatant was removed for derivatization with dabsyl chloride followed by amino acid analysis. Tissue dry weight was determined by placing tissue in a forced air drying oven at 105-108°C (Precision Scientific, Chicago, IL) until the weight was constant (~24-36 hr).

C. Amino acid separation and quantification

The method of Sethuraman *et al.* [13] for the determination of physiologic amino acids and biogenic amines by reversed phase HPLC separation of their dabsyl derivatives as applied to fruit and vegetables [14] was employed to quantify watermelon physiologic amino acids. All reagents and their concentrations were as described by Sethuraman *et al.* [13]. The only departure from that procedure was that dabsyl chloride was dissolved in acetonitrile rather than acetone to prepare the dabsyl reagent. Reactions were carried out using ten times the volume of sample and reagents as were those of Sethuraman *et al.* [13] (i.e., hundreds of □L instead of tens of □L). Twenty-five □L of dabsyl derivatized sample or standards were injected onto the HPLC column. Separation/quantification was with a Varian ProStar ternary solvent HPLC system equipped with an autosampler and diode array detector (Varian, Walnut Creek, CA, USA). A 250 x 4.6 mm i.d. 5□m Luna™ C18 reversed phase column was used (Phenomenex, Torrance, CA, USA) at 50°C and at a flow rate of 1.0 ml/min. Because the ratio of flow rate to column volume for the system used was within 5% that of the system of Sethuraman *et al.* [13], an elution program was employed that was identical to theirs with no apparent impairment of resolution. The dabsyl derivatives eluting from the column were monitored by absorbance at 468 nm, the apparent maximum for a majority of dabsyl amino acid derivatives. The suspected identity of each unknown peak in a derivatized extract was confirmed by co-chromatography of the extract with the derivatized authentic individual amino acid and by comparison of the absorption spectra of the two individual dabsyl derivatives. Calibration curves for each amino acid ranged between 0.1 and 1.6 nmoles of the amino acid injected onto the column, and this was the range to which unknowns were diluted for analysis. Under the method parameters described, the lowest level of amino acid in fresh tissue that could be detected and quantified was approximately 50 nmoles/g fresh weight of tissue.

D. Other analyses

Quantification of carotenoids by HPLC was performed as generally outlined by Craft [15]. Runs were conducted on an Agilent Technologies Model 1100 system fitted with a diode array detector. Separation was carried out on a 3 □m 250 x 4.6 mm C30 column (Waters Corp., Milford, MA) at 25° C. Carotenoids were eluted with the ternary gradient program described by Craft [15]. *Trans*-□-carotene standard was prepared from cantaloupe chromoplasts, and *trans*-lycopene standard prepared from watermelon chromoplasts [16]. Standards were prepared by extraction into hexane, the concentration determined spectrophotometrically using published absorptivities, and a purity correction applied from the HPLC chromatograms as outlined by Craft [15]. The purity correction for cantaloupe chromoplast *trans*-□-carotene was 97.2 %, and the purity correction for watermelon *trans*-lycopene was 91.8 %. *Cis*-isomers of lycopene were quantified using the same integration constant as for all-*trans* lycopene. Total dissolved solids (TDS) were estimated refractometrically with sucrose solutions in H_2O serving as the reference.

Statistical analyses were performed with the aid of Statistica software version 6 (StatSoft, Tulsa, OK). The experimental uncertainty in amino acid determinations was expressed as the coefficient of variation about the mean in order to more clearly compare the experimental variation about both large and small means. Graphical plots for individual amino acids are presented as ± 1 standard deviation about the mean.

III. RESULTS

A. Growing conditions in each crop year

In 2009, watermelon plants were transplanted into the field on June 1. Rainfall for June was 11.8 cm, for July 15.1 cm, and through August 15, it was 5.8 cm. Most of the rain in July fell at the end of the month; overhead irrigation was applied June 30, July 13, and July 22. Average daily high temperature was 30.0 ± 3.6°C, and average daily low temperature was 20.7 ± 2.2°C between fruit-set and harvest. Average daily maximum humidity was 93.0 ± 4.0 % and the daily minimum humidity was 43.4 ± 15.3%. Average bare soil temperature at 10.2 cm depth was 26.2 ± 0.8°C. Average daily wind speed and direction were 2.6 $msec^{-1}$ from the Southeast. Tagging of blooms began on July 6, and the last watermelons (SS7177HQ at 42 days postanthesis) were harvested on August 17.

In 2010, watermelon plants were transplanted into the field on May 18. Between then and July 9, from 0.79 to 7.42 cm of rain fell each week. Moisture potentiometers yielded an average daily reading of 21 ± 11 kPa during this period. Because of the frequency of rainfall during this period, spray applications for foliar disease could not be made, and a moderate to severe leaf spot and stem canker caused by *Myrothecium roridum* occurred [17]. When weather permitted, spraying with a fungicide for foliar disease controlled the infection. Drip irrigation was initiated on July 19 and applied as needed through the rest of the sampling period (up to August 23). Plants were side-dressed with 2.5 kg N/ha on May 25, 28 kg N/ha on June 4, and 27 kg N,P, K/ ha on June 21. Average daily high temperature was 32.5 ± 2.7°C, and average daily low temperature was 22.7 ± 1.1°C between fruit set and

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harvest. Average daily maximum humidity was 96.7 ± 2.4% and average daily minimum humidity was 55.0 ± 12.6%. Average bare soil temperature at 10.2 cm depth was 28.8 ± 1.6°C. Average daily wind speed and direction were 2.7 msec^{-1} from the Southeast. Tagging of blooms began on June 22, and the last watermelons (Dixie Lee at 56 days postanthesis) were harvested on August 23.

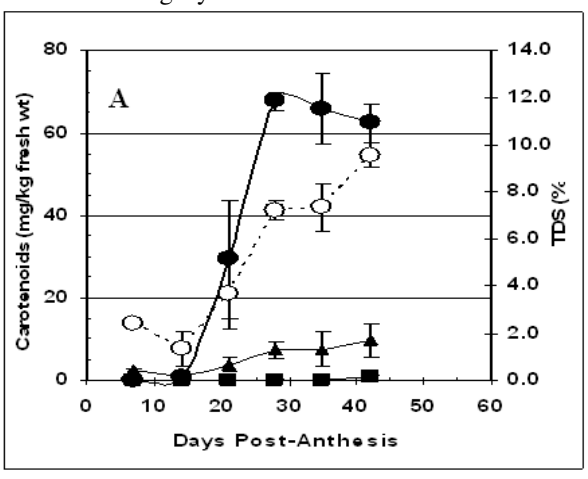
In 2011, watermelon plants were transplanted into the field on May 26. Rainfall for June was 1.5 cm, for July 0.9 cm, and through August 15, it was 1.1 cm. The paucity of rainfall required weekly drip irrigation during the entire growing season. Fertigation, pesticides, and fungicides were applied according to the plant management schedule being followed or as the need arose. Average daily high temperature was 39.0 ± 1.1°C, and average daily low temperature was 23.8 ± 1.5°C between fruit set and harvest. Average daily maximum humidity was 82.0 ± 5.5 % and daily minimum humidity was 26.5 ± 3.0%. Average bare soil temperature at 10.2 cm depth was 34.4 ± 1.4°C. Average daily wind speed and direction were 3.0 msec^{-1} from the Southeast. Tagging of blooms began on June 23, and the last watermelons (SS7177HQ at 56 days postanthesis) were harvested on August 18.

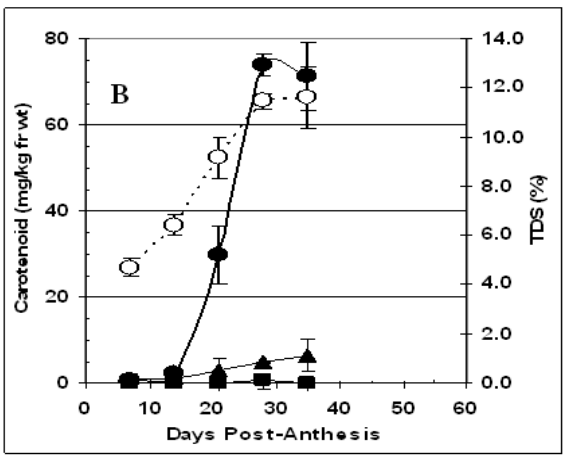
B. Carotenoids and total dissolved solids (TDS) during fruit development

Levels of carotenoids and TDS were affected by developmental stage of the fruit (Fig. 1 A-F). *Trans*-lycopene began appearing between day 14 and 21 postanthesis, and depending on growing conditions, reached maximal levels between day 28 and 42 postanthesis. The lycopene precursor, phytoene, was not detected in the 7, 14, or 21 day old fruit. In general, the *cis*-lycopene and *trans*-□-carotene began to increase slightly at or near fruit ripeness and *trans*-lycopene had reached its maximum level. The level of *trans*-lycopene produced by a given cultivar was dependent on growing conditions. SS7177HQ ranged from 70 mg/kg fresh weight in 2009 down to 52 mg/kg fresh weight in 2010. Dixie Lee was highest in 2009 and 2011 at ~75 mg/kg fresh weight and lowest in 2010 at ~42 mg/kg fresh weight. For all three years, the flesh quality of the triploid remained good for 14-21 days after achieving optimum ripeness while the flesh of the diploid began to deteriorate 7 days after optimum ripeness as gauged by fruit firmness and locule integrity. TDS increased to a maximum level near ripeness and remained at that value through over-ripeness. As expected, TDS varied with crop year and cultivar.

C. Expression of the arginine metabolic family in watermelon flesh during fruit development.

Levels of gln, cit, arg, and orn in watermelon flesh were affected by growth and development of the fruit (Fig. 2 A-F). The average coefficient of variation for estimates of amino acids among 5 watermelons at each sampling date over the length of the study was 15.1 ± 9.2%. For both cultivars in all three growing seasons, gln was the predominant free amino acid during the first two weeks of development. Its concentration rapidly fell to a constant level of 1-10 mmoles/kg dry weight. Cit rapidly increased from ~50 mmoles/kg dry weight at 7 days to become the predominant amino acid at 21 days and remained as such through the over-ripe stage. Arg and orn levels rose during the increase in cit levels, diminished somewhat, and then remained more or less constant. Cit reached its maximum level near optimum ripeness of the watermelon fruit. Once assimilated in the flesh, cit appeared to diminish only slightly with over-ripening. Proline was present at significant levels in both cultivars (15-20 mmoles/kg dry weight) at 7 and 14 days postanthesis. At 21-28 days postanthesis, the level of pro fell while the level of an unidentified dabsyl-reacting ompound eluting ~0.2 min later than pro rapidly increased. At 28 days, levels of these two species were about equal. Pro levels remained constant at 5-8 mmoles/kg dry weight in 2010 while rising to 20-25 mmoles/kg dry weight in 2011 before declining to ~10 nmoles/kg dry weight in the over-ripe phase (Fig. 2C-F). The peak eluting 0.2 min after pro broadened with over-ripeness of the watermelons. This behavior suggested further modification of a single compound or synthesis of new compounds of similar chromatographic behavior. The nature of the growing season had a marked effect on the level of the individual amino acids in the flesh (Fig. 2 A-F). For example, cit in SS7177HQ varied from ~100 mmoles/kg dry weight in 2009 to ~180 mmoles/kg dry weight in 2010 to ~250 mmoles/kg dry weight in 2011. The cultivar, Dixie Lee ranged from ~130 mmoles/kg dry weight in 2009 to ~260 mmoles/kg dry weight in 2010 to ~280 mmoles/kg dry weight in 2011.





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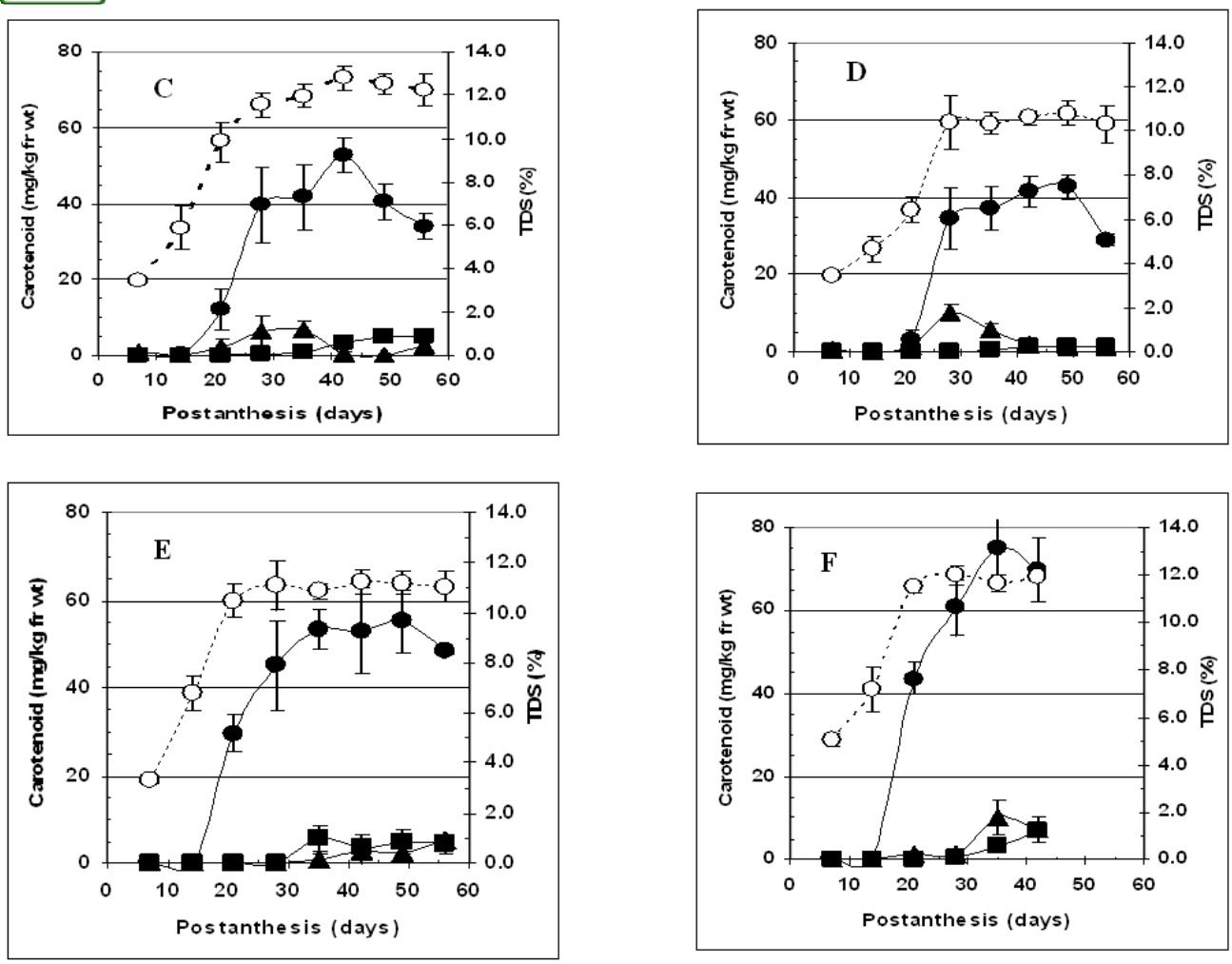
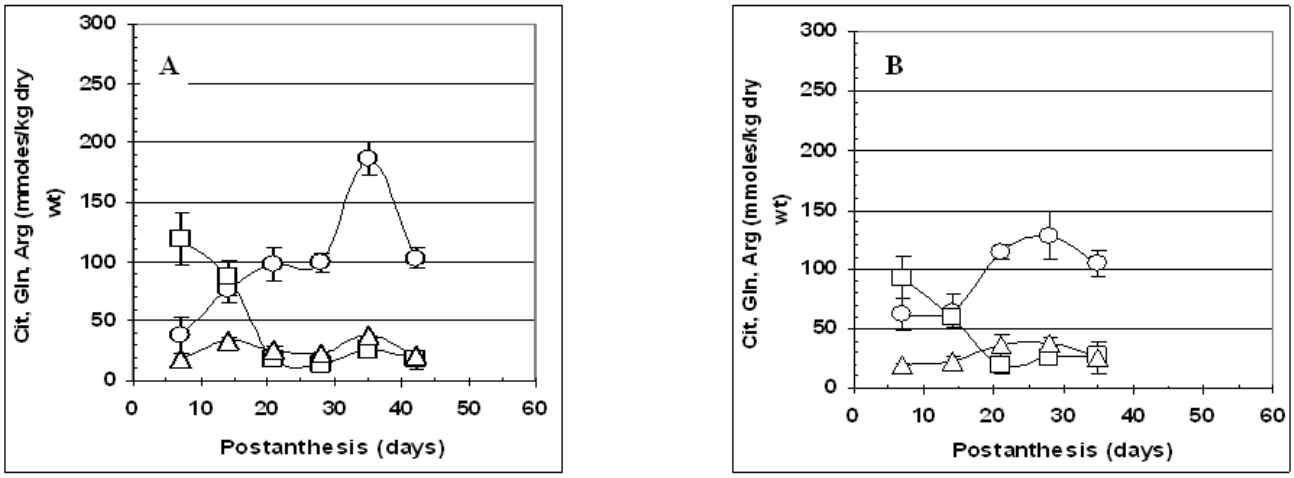


Fig.1. Variation of carotenoids and total dissolved solids (TDS) levels in watermelon flesh as affected by fruit development. Filled circles = *trans*-lycopene. Filled squares = *cis*-lycopenes. Filled triangles = *trans*-□- carotene. Open circles = TDS. A. 2009 crop of SS7177HQ. B. 2009 crop of Dixie Lee. C. 2010 crop of SS7177HQ. D. 2010 crop of Dixie Lee. E. 2011 crop of SS7177HQ. F. 2011 crop of Dixie Lee. Each point is the average of five watermelons, and the error bars are ± 1 standard deviation from the mean.



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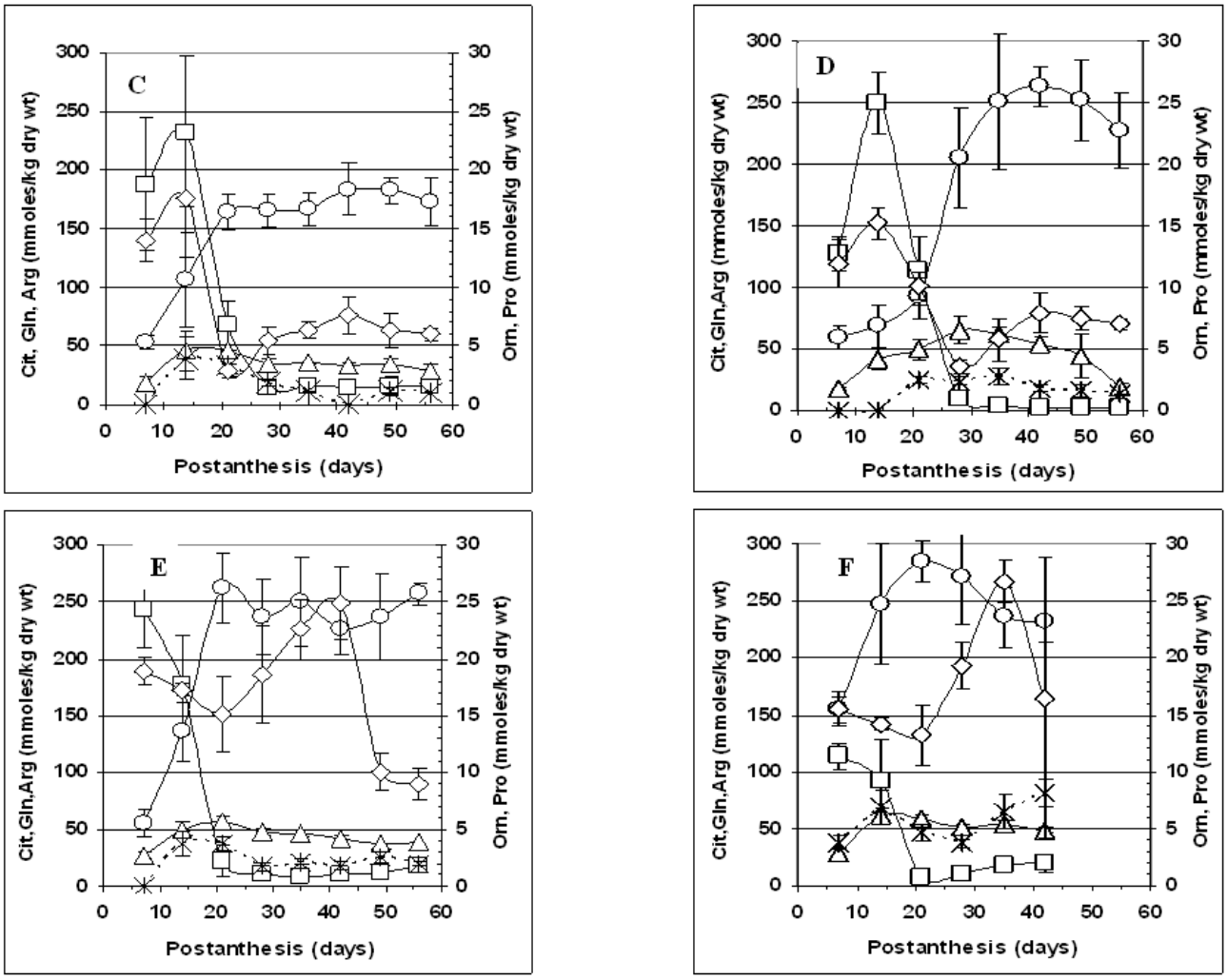


Fig.2. Variation of amino acid members of the arginine metabolic family in watermelon flesh as affected by fruit development. Open squares = gln, Open circles = cit, Open triangles = arg, Xs = orn, Open diamonds = pro. A. 2009 crop of SS7177HQ, B. 2009 crop of Dixie Lee, C. 2010 crop of SS7177HQ, D. 2010 crop of Dixie Lee. E, 2011 crop of SS7177HQ, F. 2011 crop of Dixie Lee. Each point is the average of five watermelons, and the error bars are ± 1 standard deviation from the mean.

D. Expression of the arginine metabolic family in watermelon rind during fruit development

Levels of gln, cit, arg, and orn in watermelon rind were affected by fruit growth and development (Fig. 3A-F). As with flesh tissue, gln was the predominant amino acid during the first 7-14 days of fruit development. Although cit then became the free amino acid in greatest concentration, it was impossible to obtain average values worthy of making cultivar or growing conditions comparisons. The average coefficient of variation for estimates of amino acids over all sampling dates was 47.1% ± 21.1%. The fruit to fruit variation in rind cit values ranged from ± 25% coefficient of variation to as much as ± 100% coefficient of variation. These rinds were from the same watermelons from which flesh samples had been collected and analyzed. In general, from this study and other studies (data not shown) rind from Dixie Lee exhibited greater variation than rind from SS7177HQ, but rind data in general exhibited much larger variation than flesh data. Arg and orn concentrations trended the same as in the flesh, but variability of orn values precluded establishing meaningful interpretations. Pro levels changed little during fruit development, but appeared to rise and fall in a manner similar to that of cit. Average cit values for rind tissue were generally less than, and sometimes equal to those of the flesh. The cit value in rind was greater than that of the flesh for individual watermelons only about 10-20% of the time (data not shown).

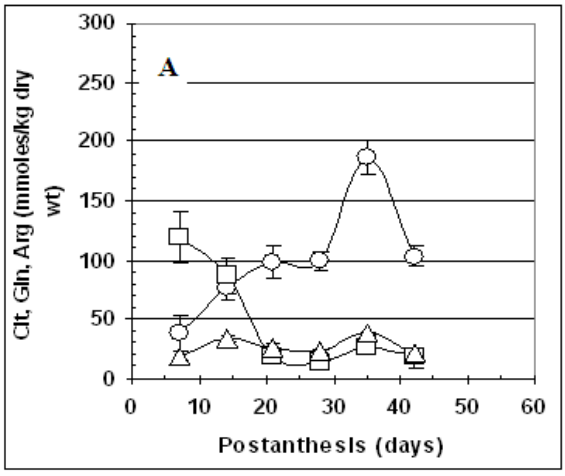
IV. Discussion

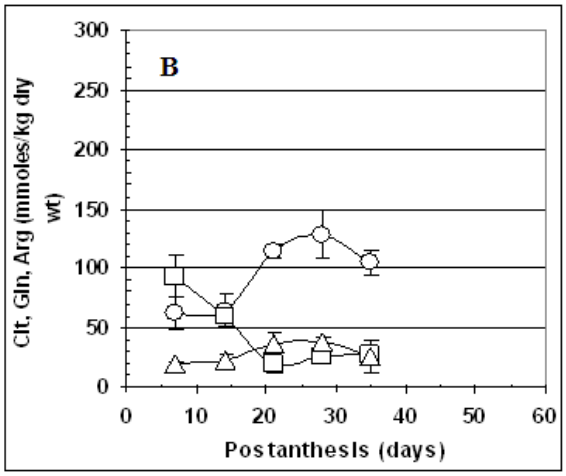
High levels of cit in watermelon flesh are well documented in the literature [*6*]-[7]-[8]-[14]-[18]. Each report except one [8] measured the cit content of ripe watermelons grown under similar growing conditions and at the same locale. The purpose of this study was to build on what is known about cit in ripe watermelon flesh and learn more about its and its metabolic relatives' expressions in developing and ripening watermelon fruit.

To accomplish this, it was necessary to employ an analytical method that quantified all of the amino acids, not just cit. Although fruit to fruit variation of amino acids in watermelon flesh was moderate throughout the fruit development process, ±15.1% coefficient of variation, interesting changes in several amino acids' levels were documented. In an earlier study where a large number of cultivars were compared from two separate locations, a sizeable intra-cultivar variation, ~30%, was observed among ripe fruit from a given locale [8] so that the variations observed in this study are not without precedent.

The extreme variability of amino acid values, especially cit, in watermelon rind tissue in this study, ±47.1% coefficient of variation, can not be explained in light of the small variability among cit values in rind of ripe watermelons reported by others [7]. Such a great variation in cit content among different fruit of the same cultivar, stage of development, and growing season suggests that the extraction process was not quantitative. In the present study, rind samples were removed from the same location on each watermelon to minimize locational variation on the fruit. Additionally, earlier work with spiking experiments and combinatorial experiments, both utilizing watermelon rind, were consistent with an 89 to 101% recovery of cit from rind tissue [11]-[14]. Earlier reports routinely observed higher levels of cit in rind of ripe watermelons than in flesh and up to twice the level of cit in the rind as in the flesh for certain cultivars [6]-[7]. This was not the case for the cultivars employed in this three year study; average flesh values for cit were observed to be equal to, or greater than in rind, although in a small percentage of individual watermelons, cit levels were higher in rind than in flesh. Preliminary evidence suggests that growing conditions strongly influence relative amounts of cit between rind and heart tissues from the same watermelon (unpublished results).

By employing a method that quantified all free amino acids rather than focusing only on cit, this study allowed additional information to be simultaneously gathered on the other amino acid members of the arg metabolic family. Several interesting metabolic possibilities present themselves. First, cit accumulation may occur as a result of a tightly down-regulated arginosuccinate synthase (E.C. 6.3.4.5) (citrulline to arginosuccinate) [19] and a concomitantly unregulated or poorly regulated ornithine transcarbamoylase (E.C. 2.1.3.3) (orn and carbamoylphosphate to cit) [20]. This would result in a buildup of cit and maintenance of only low levels of orn, arginosuccinate, and arg. Second, the high levels of gln in the initial stages of fruit development would provide substrate for carbamoylphosphate synthase II (E.C. 6.3.5.5) to produce carbamoyl phosphate [21]. In turn, carbamoylphosphate together with orn could be converted to cit by ornithine transcarbamoylase. The glu formed as a co-product with carbamoylphosphate could then serve as starting material for ornithine synthesis. Levels of glu in watermelon flesh more or less followed the cit response to fruit development except levels of glu grew from near zero at 7 days to 5-8 mmoles/kg dry weight at ripening (data not shown). Third, pro which is also synthesized from glu directly or via orn may be produced in watermelon fruit at substantial levels because its synthetic starting materials and intermediates are shared with arg. Proline, like cit, is thought to act as an osmotic protectant in plants subjected to drought or high salt stress [22]. Since it shares a common biosynthetic pathway with cit, it is not unreasonable that somewhat elevated levels of pro are observed in most cucurbits [14].





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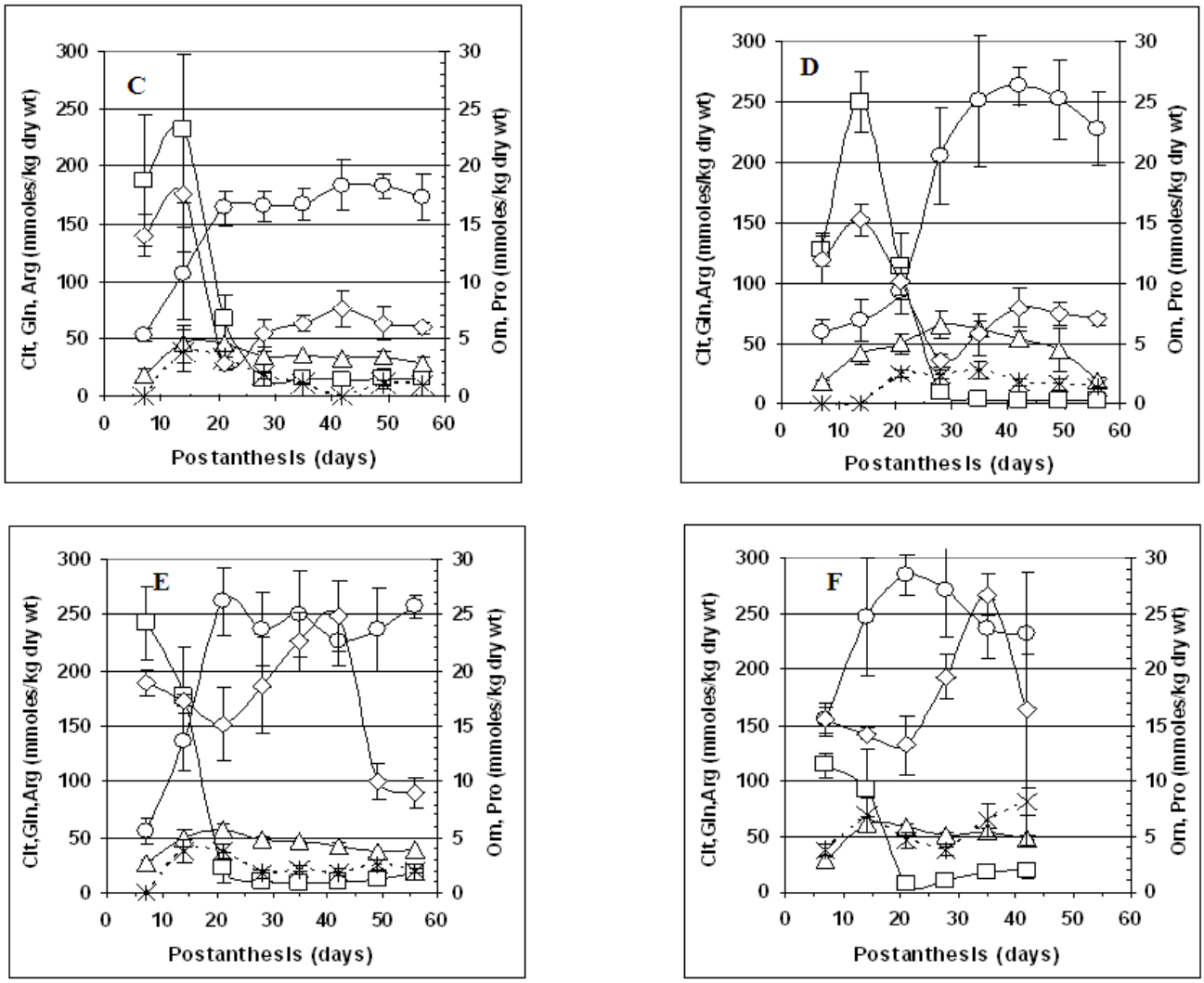

Fig. 3. Variation of amino acid members of the arginine metabolic family in watermelon rind as affected by fruit development. Open squares = gln, Open circles = cit, Open triangles = arg, Xs = orn, Open diamonds =pro. A, 2009 crop of SS7177HQ,. B, 2009 crop of Dixie Lee. C, 2010 crop of SS7177HQ. D, 2010 Crop of Dixie Lee. E, 2011 crop of SS7177HQ, F, 2011 crop of Dixie Lee. Each point is the average of five watermelons, and the error bars are ± 1 standard deviation from the mean.

V. Conclusion

Because of its potential as a therapeutic in human health for such maladies as high blood pressure and protein – energy malnutrition in aging, L-citrulline is receiving clinical attention as an effective agent for arginine supplementation. Watermelon, as one of the few sources of substantial endogenous levels of cit, is viewed as a likely source for 'natural' cit.

This study was undertaken with the objective to better understand the influences of cultivar type and growing conditions on expression levels of cit and members of its metabolic family in commercial watermelon production.

The results of this investigation demonstrate that growing conditions can affect the levels of cit produced in a given cultivar by as much as two-fold, a response similar to that concomitantly observed for carotenoid expression. Furthermore, cit was observed to remain at or near maximal levels after over-ripening of the fruit. This suggests that over-ripe and cull watermelons could serve as acceptable sources for cit production [23].

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AUTHOR'S PROFILE

Wayne W. Fish

was born in Oklahoma, U.S.A. He received his Bachelor degree in agricultural chemistry in 1963 and PhD degree in biochemistry in 1967 from Oklahoma State University, Stillwater, Oklahoma. These were followed by postdoctoral studies in physical biochemistry during 1967-1970 at Duke University, Durham, North Carolina.

He was Professor of Biochemistry at the Medical University of South Carolina from 1970-1986 and Senior Research Associate in Biosciences at Phillips Petroleum Company from 1986-1999. From 1999 to present, he has been a Research Chemist with the Agricultural Research Service branch of the United States Department of Agriculture in Lane, Oklahoma. He has authored or coauthored more than 100 publications in physical biochemistry, enzymology, structure-function in proteins, plant-pathogen interactions, and physical, chemical and biological properties of naturally occurring compounds in fruit and vegetables.

Dr. Fish is a long time member of the American Chemical Society and the American Society for Biochemistry and Molecular Biology. He was the 1986 American Chemical Society's Outstanding Chemist for South Carolina and was a co-awardee of the Horticulture Society Southern Region's L.M. Ware Research award in 2004.