International Journal of Biotechnology and Bioengineering Research
Volume 1 Number 1 (2010) pp. 115–123

http://www.ripublication.com/ijbbr.htm

Analysis of Anthocyanin in *Caesalpinia sappan* Linn. and *Carissa carandas* Linn. using HPLC

Rajeshkannan Chinnasamy[1], Supriya Sasindranathan[2], Murugesan Shourimuthu[3]* and Senthilkumar Natchiappan[4]

[1]Junior Research Fellow, Institute of Forest Genetics & Tree Breeding, Forest Campus, R.S. Puram, Coimbatore-641 002, Tamil Nadu, India
E-mail: rajkhn.c@gmail.com
[2]Institute of Forest Genetics & Tree Breeding, Forest Campus, R.S. Puram, Coimbatore-641 002, Tamil Nadu, India
E-mail: supriya2011@gmail.com
[3]Scientist - F, Head, Division of Bioprospecting, Institute of Forest Genetics & Tree Breeding, Forest Campus, R.S. Puram, Coimbatore-641 002, Tamil Nadu, India.
**Corresponding Author E-mail: murugeshirdi@icfre.org and murugeshirdi@gmail.com*
[4]Scientist – C, Division of Bioprospecting, Institute of Forest Genetics & Tree Breeding, Forest Campus, R.S. Puram, Coimbatore-641 002, Tamil Nadu, India.
E-mail: senthilnk@icfre.org and senthilnj@hotmail.com

Abstract

Anthocyanins are safe to body health, since they are easy to incorporate in food products. Anthocyanins are gaining increasing interest in view of their health properties. *Caesalpinia sappan* Linn., a traditional Indian medicinal plant is used widely in oriental medicine as it is a good source of secondary metabolites and vitamins. *Carissa carandas* L., an important dry land fruit crop exceedingly a hardy shrub generally found in forest has been mentioned to be used as purgative, stomachic, antidote for snake-bite, anthelmintic, spasmolytic, cardiotonic in the old chemical literatures. In the present study, the heartwood of *C.sappan* and the fruit of *C.carandas* were analysed for the presence of anthocyanins using HPLC making use of a gradient mobile phase. The samples were analysed at pH 1.0 and pH 4.5 and further quantified using UV spectrophotometer. Both *C.sappan* and *C.carandas* were found to contain the anthocyanin, Cyanidin 3- glucoside.

Keywords: Anthocyanin, High Performance Liquid Chromatography, *Caesalpinia sappan*, *Carissa carandas*, Cyanidin 3-glucoside.

Introduction

Anthocyanins are members of a class of nearly universal, water-soluble, terrestrial plant pigments that can be classified chemically as both flavonoids and phenolic. They are widely distributed in plants. They are responsible for blue, purple, violet, magenta, red and orange plant coloration which inturn depends on the acylation patterns and various environmental influences (Jackman and Smith, 1992). They are relatively unstable and often undergo degradative reactions during processing and storage. They play a role in reduction of coronary heart disease (Bridle and Timberlake, 1996) and increased visual acuity (Timberlake and Henry, 1988) and also have antioxidant (Takamura, H and Yamagami, 1994; Wang et al., 1997) and anticancer properties (Karaivanova et al., 1990; Kamei et al., 1995). Anthocyanins have also found considerable potential in food industry as safe and effective food colorants interest in this application has increased in recent years. Quantitative and qualitative anthocyanin compositions are important factors in determining the feasibility of the use of new plant materials as anthocyanin based colorant sources.

Caesalpinia sappan is distributed in Southeast Asia (Washiyama et al., 2009). It is being used traditionally for its wide variety of ethnomedicinal properties. Greatest medicinal value is ascribed to its heartwood (Badami et al., 2003). Several triterpenoids, flavonoids, oxygen heterocycles, etc. were isolated from *C.sappan*. The use of heartwood as a colouring agent for wine, meat, fabric, etc. is well established with good medicinal value for food products, beverages and pharmaceuticals (Badami et al., 2004). Recent research confirms its anticancer, antitumor, antimicrobial (Kim et al., 2004), antiviral, immunostimulant (Moon et al., 1992; Mok et al., 1998) antifungal activity (Reddy et al., 2003) and several other activities. The woody part contains brazilin and brasilein and an essential oil consisting of D-a-phellandrene, ocimene, tannin, gallic acid and saponin (Mar et al., 2003). The extract of the dried heartwood has been found to be a potential immunosuppressive agent. The reported main phenolic compounds are brazilin, chalcone, protosappanin and homisoflavonoid (Fu et al., 2008). Heartwood of *C.sappan* is considered to be a potential source of pigments, especially the anthocyanins. *Carissa carandas* Linn. (Syn. *Carissa congesta* Wight) an evergreen shrub, is used in traditional system of medicine, as an anthelmintic, astringent, appetizer, antipyretic, in biliary, stomach disorders, rheumatism and disease of the brain. Studies have shown that the extract of the plant possesses cardiotonic, antipyretic and antiviral activity. Various cardiac glycosides, carissone and sitosterol were reported from the root extract of the plant. Among the tribal groups of India, the decoctions and extracts of the roots of this plant are effective remedies in the management and/or control of convulsions and epilepsy. However, no scientific data are available to validate the folklore claim (Hegde et al., 2009). The fruit is used as an astringent, antiscorbutic and remedy for biliousness. The leaf decoction is valued in cases of intermittent fever, diarrhea, oral inflammation and ear ache. The fruit is sour, acrid, astringent, appetizer, antipyretic, useful in disease of brain and antiscorbutic properties. The leaves have antipyretic activity. The roots are bitter, stomachic and anthelmintic, cardiotonic properties as roots contain carissone, carindone, carinol etc (Rabbani et al., 2010). Fruits were demonstrated to contain a mixture including 2-phenylethanol, linalool, isoamylalcohol, benzaylacetate and novel

triterpenic alcohol. A tincture of fruits is used in skin infection; sand decoctions of wood are employed as a tonic to strengthen the tendons of slim patients. Enzymatic and mild hydrolysis of polar glycosides from *C.carandas* yielded digitoxigenin, oderosiden and sugars: D-glucose & D-digitalose (Rastogi et al., 1966). Measurement of total anthocyanins pigment content along with indices for the degradation of these pigments is very useful in assessing the color quality of these foods. The study was conducted on the above mentioned plant samples to detect the presence of anthocyanin in them using HPLC and to determine the amount present using UV.

Methodology

Collection of the plant material

Different parts of *C.sappan* such as leaves, pods, twigs and heartwood and fruits of *C.carandas* used for the analysis were TNAU Coimbatore.

Sample preparation for HPLC analysis

The extraction was done following the procedure described by Giusti & Wrolstad (1996). Anthocyanin purification was carried out using the method of Fuleki and Francis, (1968). Alkaline and acid hydrolysis was performed by the method of Hong and Wrolstad, (1990).

High performance liquid chromatography (HPLC)

HPLC instrument with L-4000 UV detector, L-6200 Intelligent pump and RP-C18 column from Hitachi with DataAce workstation was used. The analytical system used was a PolyLC ODS C-18 column (5 micron). The mobile phase used in the study was, Solvent A: 100% HPLC grade acetonitrile, Solvent B: 1% phosphoric acid, 10% acetic acid, 5% acetontrile (v:v:v) in water. Flow rate: 1ml/min. For anthocyanins a linear gradient from 0% to 12% A in 13 min and to 20% A in 15 min was used and for saponified anthocyanins a linear gradient from 0% to 30% A in 30 min was used. Purified anthocyanin extracts (20 μl) were injected directly into the system.

Estimation of monomeric anthocyanin content and polymeric color by UV measurement

Monomeric anthocyanin content and polymeric color was determined using the pH differential and bleaching methods suggested by Wrolstad, 1993.

Results and Discussion

HPLC Analysis

The heartwood of *C.sappan* was subjected to HPLC analysis since it exhibited more medicinal property than the leaf which possessed more of anthocyanin. Anthocyanin from heartwood of *C.sappan* and fruit of *C. carandas* was separated by HPLC (Figures 2-5) (Table 1). Cyanidin – 3 – glucoside peak was identified for 91-99% of the total area at 520nm of 1.5 – 1.8 minutes. Information from HPLC profiles showed that the major pigments were cyanidin 3 - glucoside with total area of 99.01% in heartwood of *C.sappan*.

Table 1: HPLC estimation of Anthocyanin in heartwood of *C.sappan* and fruit of *C. carandas.*

S.No	Name of the sample	Retention time (min)	Area (%)	Identified compound
1	Anthocyanin Standard at 520nm	1.794	99.025	Cyanidin 3-glucosides
2	*C.sappan* (Heartwood) extract at 520nm	1.789	99.01	Cyanidin 3-glucosides
3	*C. carandas* fruit extract at 520nm	1.794	99.025	Cyanidin 3-glucosides
4	*C. carandas* fruit extract Saponified sample 520nm	1.787	99.019	Cyanidin 3-glucosides

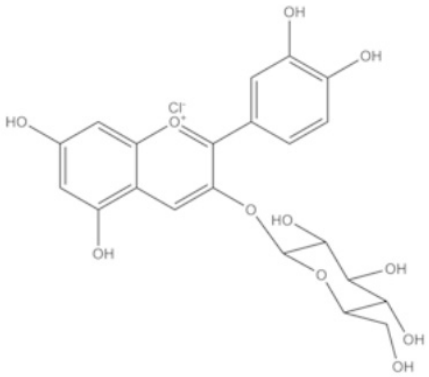

Figure 1: Structure of Cyanidin 3-glucoside.

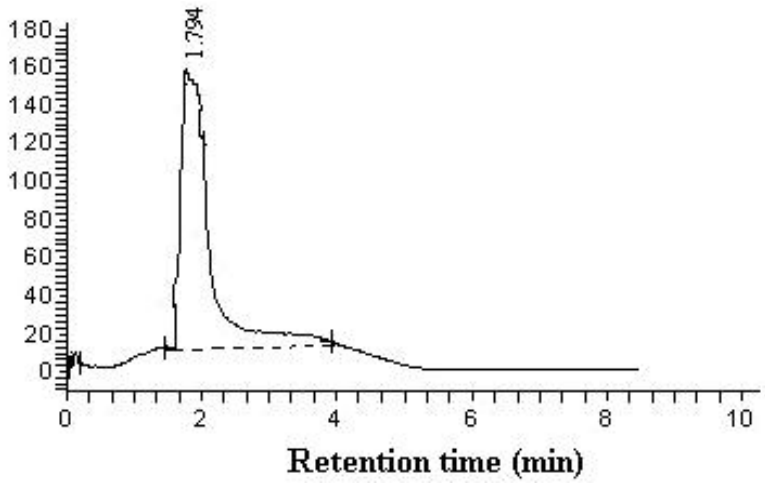

Figure 2: HPLC Chromatogram of Anthocyanin standard at 520nm.

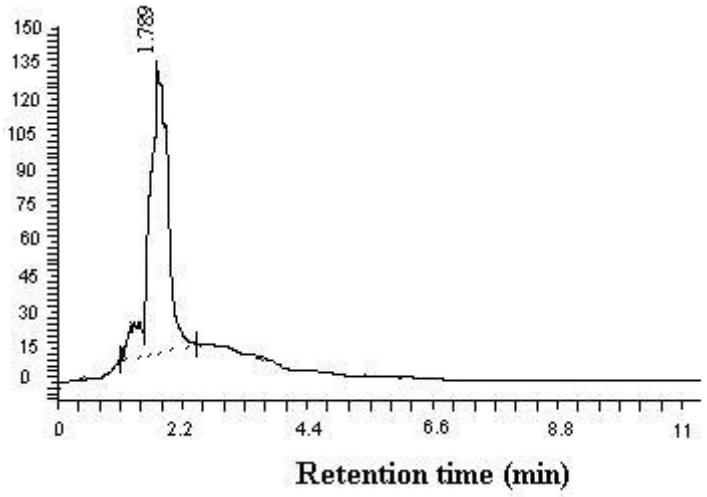

Figure 3: HPLC Chromatogram of *C.sappan* (Heartwood) at 520nm.

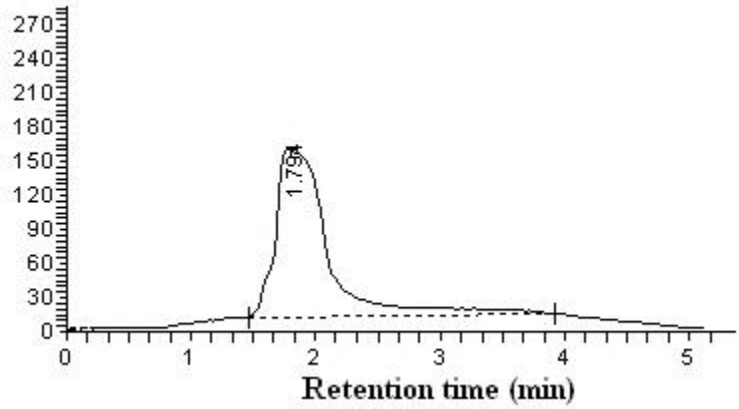

Figure 4: HPLC Chromatogram of *C.carandas* fruit at 520nm.

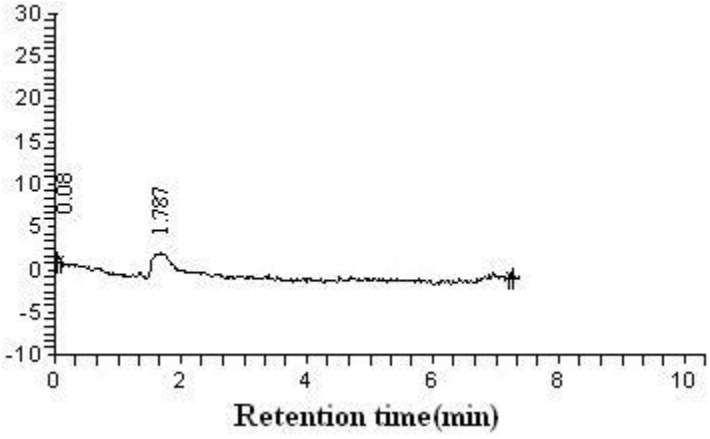

Figure 5: HPLC Chromatogram of *C.carandas* fruit (saponified) at 520nm.

UV Measurement of Total Anthocyanin

The differential method measures the absorbance at two different pH values and relies on the structural transformations of the anthocyanin chromophore as a function of pH. Fuleki and Francis (1968) used pH 1.0 and pH 4.5 buffers to measure anthocyanin content have applied to a wide range of commodities (Wrolstad et al., 1982; Wrolstad et al., 1995). To determine total anthocyanin content, the absorbance at pH1.0 and 4.5 is measured at the $\lambda_{vis\ max}$ 510 nm and 700nm.

Table 2: UV measurement of total anthocyanin.

Sample	pH-1.0		pH-4.5		Absorbance	Anthocyanin (mg/l)
	510nm	700nm	510nm	700nm		
C.sappan						
Heartwood	0.294	0.114	0.228	0.079	0.0312	5.18
Twig	0.524	0.548	0.313	0.230	0.0107	1.79
Leaf	1.583	1.548	1.674	1.674	0.085	14.19
Pod	0.478	0.478	0.233	0.233	0.016	2.67
C.carandas						
Fruit	0.040	0.014	0.050	0.026	0.002	0.330

Discussion

According to Harborne the spectra of anthocyanin peaks can provide information about the presence of acylating groups and reported that the ratio of absorbance at the acyl maximum (340nm) to the absorbance at the anthocyanin (Acyl) maximum wavelength (520 nm) λ max acyl / λ max acyl, is a measure of the molar retention of the cinnamic acid to the anthocyanidin 3-glucosides indicates that *C.carandas* fruit extract was not acylated. Saponification of *C. carandas* anthocyanin confirmed that major pigment cyanidin - 3 - glycoside did not contain acylating groups. The retention time (1.78) of the saponified anthocyanin coincided with those obtained from purified pigment of *C. carandas* and standard {Kuromanin chloride (sigma)} retention time is 1.55 to 1.78. Information from HPLC profiles, saponification and acid hydrolyses of the capulin anthocyanins showed that the two major pigments were cyanidin 3-glucoside (34%) and cyanidin 3-rutinoside (63%) with no acylating group. It was reported that the major anthocyanin present in plums were cyanidin 3-rutinoside (41%) and cyanidin 3-glucoside (31%). Other fruits reported to contain cyanidin 3-glucoside and cyanidin 3-rutinoside are rhubarb, red and black raspberries, boysenberry, loganberry, blackberry and red and black currant. Anthocyanins, particularly cyanidin glycosides, have been found to possess a broad spectrum of biological activities, including scavenging effects on activated carcinogens and mutagens and effects on cell cycle regulation (Feng et al., 2007).

It is evident from Table 2 that the anthocyanins are present in all the tissues of the plant therefore the anthocyanin pigments from *C.sappan* and *C.carandas* can be widely used as food colorant. The amount of anthocyanin was found to be highest in

the leaf extract of *C.sappan* followed by the heartwood. The twig of *C.sappan* possessed the least amount of anthocyanin. The fruit of *C.carandas* was analysed for the presence of anthocyanin and was found to contain 0.33 mg/l of the sample.

Conclusion

Caesalpinia sappan is one of the trees that were found to possess much medicinal value and the extract from *C. carandas* fruit showed high quantity of cyanidin 3-glucosides which exhibits high antioxidant potential. HPLC analysis reveals the presence of anthocyanin in *C.sappan* heartwood and *C. carandas* fruit which is also a rich source of polyphenols and hence it can be widely used in food industry as colouring agent. The present study has provided some basis for the ethnomedicinal values of these plants in the treatment and prevention of various diseases.

Reference

[1] Alejandro Ordaz-Galindo, Wesche-Ebeling, P., Wrolstad,R.E., Luis Rodriguez-Saona and Jamet, A.A., 1999, "Purification and identification of Capulin (*Prunus serotina* Ehrh) anthocyanins," Food Chem., 65, pp. 201-206.

[2] Badami, S., Moorkoth, S. and Suresh, B., 2004, "*Caesalpinia sappan* – A medicinal and dye yielding plant," Nat Prod Rad., 3, pp. 75 – 82.

[3] Badami, S., Moorkoth, S., Rai, S.R., Kannan, E. and Bhojraj, S., 2003, "Antioxidant Activity of *Caesalpinia sappan* Heartwood," Biol. Pharm. Bull., 26(11), pp.1534 – 537.

[4] Barrit, B.H. and Torre, L.C., 1973, "Cellulose thin- layer chromatographic separation of Rubus fruit anthocyanins," J Chromatogr. 75, pp.151-155.

[5] Barrit, B.H. and Torre, L.C., 1975, "Fruit anthocyanin pigments of red raspberry cultivars," J Am Soc Hortic Sci., 100, pp. 98-100.

[6] Bridle, P. and Timberlake, C.F., 1996, "Anthocyanins as natural food colors-selected aspects," Food Chem., 58, pp. 103-109.

[7] Feng, R., Ni, H.M., Wang, S.Y., Tourkova, I.L., Shurin, M. R., Harada, H. and Yin, X.M., 2007, "Cyanidin-3-rutinoside, a Natural Polyphenol Antioxidant, Selectively Kills Leukemic Cells by Induction of Oxidative Stress," J. Biol. Chem., 282(18), pp. 13468–13476.

[8] Fu, L.C., Huang, X.A., Lai, Z.Y., Hu, Y.J., Liu, H.J. and Cai, X.L., 2008, "A New 3-Benzylchroman Derivative from Sappan Lignum (*Caesalpinia sappan*) ," Molecules, 13, pp.1923-1930.

[9] Fuleki, T. and Francis, F.J., 1968, "Quantitative methods for anthocyanins. Extraction and determination of total anthocyanin in cranberries," J Food Sci, 33, pp. 72-78.

[10] Giusti, M.M. and Wrolstad, R.E., 1996, "Radish anthocyanin extract as a natural red colorant for maraschino cherries," J Food Sci, 61, pp. 688-694.

[11] Harborne, J.B., 1958, "The chromatographic identification of anthocyanin pigments," J Chromatogr, 1, pp. 473-488.

[12] Hegde, K., Thakker, S.P., Joshi, A.B., Shastry, C.S. and Chandrashekhar, K.S, 2009, "Anticonvulsant Activity of *Carissa carandas* Linn. Root Extract in Experimental Mice," Trop J Pharmaceut Res, 8(2), pp.117-125.

[13] Hong, V. and Wrolstad, R.E., 1990, "Use of HPLC separation/photodiode array detection for characterization of anthocyanins," J. Agric. Food Chem., 38 (3), pp. 708–715.

[14] Jackman, R.L. and Smith, J.L., 1992, "Anthocyanins and betalins in natural food colorants," (G.A.F. Hendry and J.D. Houghton,eds.), 183-241.

[15] Kamei, H., Kojima, T., Hasegawa, M., Koide, T., Umeda, T., Yukawa, T. and Terabe, K., 1995, "Suppression of tumor cell growth by anthocyanins in vitro," Cancer Invest, 13, pp. 590-594.

[16] Karaivanova, M., Drenska, D. and Ovcharov, R., 1990, "A modification of the toxic effects of platinum complexes with anthocyans," Eksperimentalna meditsina i morfologiia, 29, pp. 19-24.

[17] Kim, K.J., Yu, H.H., Jeong, S.I., Cha, J.D., Kim, S.M. and You, Y.O., 2004, "Inhibitory effects of *Caesalpinia sappan* on growth and invasion of methicillin-resistant *Staphylococcus aureus*," J ethnopharmacol, 91(1), pp. 81-87.

[18] Mar, W. and Lee, H.T., 2003, "A DNA strand-nicking principle of a higher plant, *Caesalpinia sappan*," Arch Pharm Res, 26(2), pp. 147-50.

[19] Mok, M.S., Jeon, S.D., Yang, K.M., So, D.S. and Moon, C.K., 1998, "Effects of Brazilin on induction of immunological tolerance by sheep red blood cells in C57BL/6 female mice," Arch Pharm Res, 21(6), pp. 769-773.

[20] Moon, C.K., Park, K.S., Kim, S.G., Won, H.S. and Chung, J.H., 1992, "Brazilian protects cultured rat hepatocytes from trichlorobromethane-induced toxicity," Drug chem toxicol., 15, pp. 81-91.

[21] Rabbani, G., Muchandi, I.S., Santosh, B.T. and Prashanth, P.M., 2010, "Effect of *Carissa crandas* L on saline modulated cardiac hypertrophy in rats," Journal of Pharmacy Research., 3(1), pp. 67-71.

[22] Rastogi, R.C., Vohra, M.M., Rastogi, R.P., Dhar, M.L., 1966, "Studies on *Carissa carandas* Linn. Part I. Isolation of the cardiac active principles," Indian J Chem, 4, pp.132.

[23] Reddy VL, Ravikanth V, Jansi-Lakshmi, V.V.N.S., Suryanarayan-Murthy,U. and Venkateswarlu, Y., 2003, "Inhibitory activity of homoisoflavonoids from *Caesalpinia sappan* against *Beauveria bassiana*," Fitoterapia, 74, pp. 600-602.

[24] Takamura, H. and Yamagami, A., 1994, "Antioxidative activity of mono-acylated anthocyanins isolated from Muscat Bailey A grape," J Agr Food Chem, 42, pp.1612-1615.

[25] Timberlake, C.F. and Henry, B.S., 1988, "Anthocyanins as natural food colorants," Progr Clin Biol Res, 280, pp. 107-121.

[26] Wang, H., Nair, M.G., Strasburg, G.M., Chang, Y.C., Booren, A.M., Gray, I.J. and Dewitt, D.L., 1999, "Antioxidant and anti-inflammatory activities of anthocyanins and their aglycone, cyanidin, from tart cherries," Journal of Natural Products. 62, pp. 294-296.

[27] Washiyama, M., Sasaki, Y., Hosokawa, T. and Nagumo, S., 2009, "Anti-inflammatory Constituents of Sappan Lignum," Biol. Pharm. Bull., 32(5), pp. 941 – 944.

[28] Wrolstad, R.E. and Heatherbell, D.A., 1968, "Anthocyanin pigments of Rhubarb, *Rheum rhaponiticum* Canada Red," J Food Sci, 33, pp. 592-594.

[29] Wrolstad, R.E., 1993, Color and pigment analyses in fruit products. *Oregon State University Agricultural Experimental Station,* Bulletin No.464 (reprinted).

[30] Wrolstad, R.E., Culbertson, J.D., Cornwell, C.J. and Mattick, L.R., 1982, "Detection of adulteration in blackberry juice concentrates and wines," J. Assoc. Off. Anal. Chem., 65, pp. 1417-1423.

[31] Wrolstad, R.E.; Hong, V.; Boyles, M.J.; Durst R.W. Use of anthocyanin pigment analysis for detecting adulteration in fruit juices. *In* Methods to Detect Adulteration in Fruit Juice and Beverages, Vol. I (S. Nagy and R.L. Wade, ed.). AgScience Inc., Auburndale, Fla. 1995.