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Research article

Variations in chlorogenic acid levels in an expanded gene pool of blueberries

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Abstract: Blueberry (*Vaccinium* spp.) fruit is a rich source of chlorogenic acid (CA), among other polyphenolics, which have been associated with human health maintenance and chronic disease prevention. This study was designed to examine variation in CA over a wide range of blueberry genotypes (different species and genetic backgrounds) including commercial cultivars, breeding selections and breeding populations that contributed to current commercial cultivars. Fully ripe fruit was evaluated for CA concentration in two successive years, and concentrations ranged from 33–71 and 58–139 mg/100 g (frozen fruit) in southern highbush (4×) and rabbiteye (6×) blueberry commercial cultivars, respectively. In the breeding selections (4×), which comprised an expanded gene pool, the CA ranged from 33–107 mg/100 g. Further gene pool expansion associated with the establishment of the breeding populations (4×) resulted in a wide range of CA concentrations with significant increases (up to 156 mg/100 g) compared to current commercial cultivars (4×). Ploidy level had a strong correlation (r = 0.7) with CA accumulation in the blueberry genotypes investigated in this study, and a significant positive correlation between CA level and anthocyanin level in blueberry was also observed (r = 0.30, 0.51. and 0.49 for commercial cultivars, breeding selections, and breeding populations, respectively). The large variation in CA accumulation indicated that significant genetic variation for CA exists among blueberry species which can be successfully utilized in breeding programs to aid in developing phytochemically-enhanced blueberry varieties.

Keywords: *Vaccinium* spp.; phytochemicals; commercial varieties; breeding populations; polyploidy; HPLC-DAD

1. Introduction

One of the distinctive features of blueberry relative to many other commercially-available fruits is that it accumulates a particularly broad range of compounds demonstrated to have health-protective properties, including fibers, folate, ascorbate, carotenoids, and a diverse and highly-concentrated array of flavonoids (flavanols, flavanones, flavonols, flavones, anthocyanins) and non-flavonoids (phenolic acids, stilbenes) [1-4]. This complex, concentrated and diversified bioactive phytochemical profile is responsible for blueberry's selection as an intervention in clinical trials related to cognitive function, diabetes, cardiometabolic diseases, blood pressure and exercise performance [2,5-7].

Significant variations in levels of individual health-relevant flavonoid classes have been recorded for many popular highbush (*V. corymbosum*) and rabbiteye (*V. virgatum*) varieties [2,4], and recently the positive and negative influences of interspecific introgression on blueberry anthocyanin levels and acylation and glycosylation patterns were reported [8]. Chlorogenic acid (5-*O*-caffeoylquinic acid), one of the major phenolic acids in blueberry, has been linked to anti-obesity benefits, as well as a protective role against oxidative stress [9,10]. Elevated levels of chlorogenic acid have been associated with reduced incidence of Parkinson's and Alzheimer's diseases and diabetes [11,12]. Chlorogenic acid levels in blueberry are known to vary depending on genotype, but also stage of fruit maturity, year-to-year climatic differences, storage of fruit postharvest, and organic versus conventional cropping practices can all exert significant influence on levels of accumulation [2,13].

This study was designed to investigate the variations in chlorogenic acid (CA) content in a large number of blueberry commercial cultivars, breeding selections and breeding populations grown in North Carolina over two consecutive years. These blueberry genotypes are composed of different genetic backgrounds and ploidy levels (4×, 5×, 6×) and are currently part of the ongoing public NC State University blueberry breeding program.

2. Materials and methods

2.1. Plant material

A total of 16 commercial cultivars, 13 breeding selections (clones), and 23 breeding populations (F_1 cross progenies) of blueberry were evaluated for chlorogenic acid (CA) content in this study. The commercial cultivars included: Columbus, Ira, Montgomery, Onslow, Powderblue, Premier, Tifblue and Yadkin (rabbiteye blueberry, 6×), Arlen, Legacy, Lenoir, O'Neal, Ozarkblue, Pamlico, and Sampson (southern highbush blueberry (SHB), 4×), and Robeson (5×). The breeding selections were SHB, developed through NC State University's breeding program and included: NC 3961, NC 4263, NC 4365, NC 4385, NC 4398, NC 4900, SA-10:135 (NC 4399), SA-13:75 (NC 4807), SA-4:2 (NC 4563), SHF2B1-20:21 (NC 5018), SHF2B1-21:3 (NC 5021), SHF2B1-25:25 (NC 5042), and SHF2B1-25:41 (NC 5043). The breeding populations, also developed through the same breeding program, consisted of varying numbers of progeny plants generated from the following crosses: "Arlen" × "Georgiagem" (74 plants), CHID2-14:73 × open pollinated (OP) (28 plants), NC 1223 × "Columbus"

(90 plants), NC 2873 × OP (11 plants), NC 2898 × G-615 (37 plants), NC 3147 × "Legacy" (six plants), NC 3147 × NC 4562 (two plants), NC 3252 × OP (six plants), NC 3958 × OP (40 plants), NC 4165 × OP (six plants), NC 4295 × "Arlen" (34 plants), NC 4297 × "Ozarkblue" (69 plants), NC 4299 × "Ozarkblue" (164 plants), NC 4302 × "Georgiagem" (two plants), NC 4302 × "Sunshine Blue" (nine plants), NC 4562 × NC 3476 (31 plants), NC 4562 × NC 4179 (47 plants), NC 4562 × NC 4361 (50 plants), NC 4812 × OP (33 plants), NC 81-10-2 × "Columbus" (69 plants), NJ 89-158-24 × "Columbus" (37 plants), "Reveille" × NC 3476 (55 plants), and "Reveille" × NC 3920 (six plants). Detailed pedigree information (ploidy levels, origin, and % of parental species contribution) for the commercial cultivars, breeding selections and breeding populations were reported previously [8]. Blueberries had trickle irrigation, and no pesticides were applied because the genotypes were being screened to evaluate natural resistance to insects and fungi. Each genotype was replicated at minimum three times. Populations were composed of single plant progenies generated from crossing two heterozygous parents. Breeding selections and populations were created and selected for fruit firmness, yield, suitability for mechanical harvest and adaptation to the NC environment in the current blueberry breeding program at NC State University.

2.2. Fruit sample preparation

Blueberry field planting and establishment in the Piedmont Research Station, Salisbury, NC were detailed previously [8]. Fully ripe fruit were harvested at a uniform stage of maturity (when 75% of berries were fully ripe on any plant) and from comparable locations on each plant in the summer of 2010 and 2011. In both years, approximately 500 g of fruit were harvested all at once from each plant and packed on ice. Fruit were transported to the adjacent laboratory at the Plants for Human Health Institute (PHHI), NC Research Campus (NCRC), Kannapolis, NC. After transport, fruit were frozen in liquid nitrogen and stored at −80 °C, then lyophilized using a freeze dryer (VirTis 24Dx48; SP Scientific, Stone Ridge, NY) with a temperature controlled chamber for samples. Freeze-dried fruit were then stored at −80 °C until extraction. Weights of samples were taken before and after lyophilization to estimate dry matter content (DM percent) in the fruit.

2.3. Extraction and HPLC-DAD analysis

For each plant, lyophilized tissue (2.5–3.0 g/plant) was extracted with 30 mL of 0.3% acetic acid in MeOH:H_2O (70:30, v:v). Ground samples were transferred into 50-mL tubes, vortexed, and then centrifuged for 15 min at 3400 g_n. The supernatants were decanted into 100-mL volumetric flasks, extraction was repeated two more times and the final volume was brought to 100-mL. Extract was filtered into 2-mL amber vials using 0.2-µm polytetrafluoroethylene syringe filters (Fisher Scientific, Pittsburg, PA). A 10 µL aliquot was injected into a 1200 high-performance liquid chromatography (HPLC) system (Agilent Technologies Inc., Santa Clara, CA) equipped with an ultra violet-visible spectrophotometry (UV-VS) diode array detector (DAD), controlled-temperature autosampler (4 °C), and column compartment (30 °C). Chemstation software (Agilent Technologies Inc., Santa Clara, CA) was used as the system run controller and for data processing. Chlorogenic acid separation and quantification was performed using a reversed-phase column (250 mm × 4.6 mm × 5 µm (Supelcosil LC-18; Supelco, Bellefonte, PA)). The mobile phase consisted of 5% formic acid in H_2O (A) and 100% methanol (B). A step gradient of 10%, 15%, 20%, 25%, 30%, 60%, 60%, 10%, and 10% of solvent B

at 0, 5, 15, 20, 25, 35, 36, 37 and 40 min, respectively, at a constant flow rate of 1 mL/min applied for samples and CA commercial standard (Sigma Aldrich, St. Louis, MO). CA in samples was quantified using a standard curve with peak areas recorded at 325 nm associated with concentrations of 1.0, 0.5, 0.25, 0.125, 0.063, 0.031, 0.016 and 0.008 mg/mL in 100% MeOH. To facilitate comparison with published data, CA concentrations in this study were converted back to a frozen fruit basis and presented as mg/100 g frozen fruit.

2.4. Statistical analysis

Analysis of variance (ANOVA) was performed using the general linear model procedure (PROC GLM) with random effects using SAS software (version 9.4; SAS Institute, Cary, NC, 2012). Mean separations were performed using LSMEANS statement and Tukey's honest significant test. For the commercial cultivars or breeding selections (replicated clones) the statistical model used was $y_{ijkl} = \mu + G_i + Y_j + P_k + R(Y)_{(j)l} + GY_{ij} + \varepsilon_{(ijkl)}$; where y = response from the experimental unit, μ = overall mean, G = genotype (cultivar or clone), Y = year, P = plant, R(Y) = replication within year, GY = genotype × year interaction effect, ε = experimental random error. For the breeding populations (with single plant genotypes within a population) the model used was $y_{ijk} = \mu + G_i + Y_j + P(G)_{(i)k} + GY_{ij} + \varepsilon_{(ijk)}$; where y = response from the experimental unit, μ = overall mean, G = cross, Y = year, P(G) = plant within cross, GY = cross × year interaction effect, ε = random error. The PROC MEANS statement was used to compute genotype mean, standard deviation, and range within the 2 years (2010 and 2011) separately.

3. Results and Discussion

3.1. Chlorogenic acid concentration

In this study, chlorogenic acid (CA) was assayed as the major phenolic acid in blueberry fruit, where it was eluted at 13.3 minutes after injection of samples into the HPLC-DAD (Figure 1). This was in agreement with previous reports which indicated that CA was the major phenolic acid found in blueberry [1,2,14]. The CA concentrations observed in this study for the commercial cultivars were comparable and in agreement with previous reports [2,14,15]. Blueberry ploidy level, number of plants, and descriptive statistics for the chlorogenic acid concentrations in the cultivars, clones, and breeding populations are presented in Table 1. Consistent and comparable CA content was observed from year to year, with minor deviations in the case of the breeding populations. While 4× cultivars showed an average of 49 mg/100 g FW, the CA average for the 6× cultivars was 102 mg/100 g FW (Table 2). Yadkin, Powderblue and Ira contained the highest CA concentrations among the cultivars, at 139, 132, and 126 mg/100 g FW, respectively. These three cultivars differed significantly from the 4× cultivars and some of the 6× cultivars; namely Montgomery, Onslow, and Premier. The significant variation for CA among blueberry genotypes that we observed in this study was consistent with previous reports [16-20]. The clones and breeding populations, with a broader gene pool, contained a mix of significantly higher or much lower CA content depending on the genetic background. The CA content ranged from 33–107 mg/ 100 g in breeding selections (Table 2). The clones NC 4398, SA-4:2, and SHF2B1-20:21 contained the highest CA concentrations among breeding selections (i.e., 77, 107, and 107 mg/100 g FW, respectively). With the expansion of the genetic background to include a larger

number of species compared to the breeding selections, the CA content was significantly higher in some crosses, but ranged from 46–137 mg/100g FW in 4× crosses. For 6× crosses, the CA mean for certain crosses (i.e., NC 1223 × "Columbus" which contained 50% germplasm from the cultivated species *V. virgatum* and 50% from the wild *V. virgatum*) was significantly higher (156 mg/100 g), with some plants within this cross reaching 258 mg/100 g over two years of the evaluation. Detailed pedigree information for all plants evaluated in this study was previously reported [8]. However, when Columbus (6×) was crossed with two different breeding lines (NC 81-10-2 and NJ 89-158-24), no improvement in CA occurred.

Table 1. Descriptive statistics for the chlorogenic acid concentration (CA), standard deviation, and range in the blueberry commercial cultivars, breeding selections, and breeding populations evaluated in 2010 and 2011 at Salisbury, NC.

	Plants evaluated (no.)[a]	2010	2011
		CA (mean ± SD; mg/100 g)[b]	CA (mean ± SD; mg/100 g)
Commercial cultivar			
Southern highbush (4×)			
Arlen	5	55 ± 19	68 ± 32
Legacy	6	47 ± 12	57 ± 12
Lenoir	4	46 ± 9	32 ± 8
O'Neal	3	37 ± 3	38 ± 3
Ozarkblue	2	58 ± 1	69 ± 17
Pamlico	5	46 ± 9	45 ± 6
Sampson	5	36 ± 3	31 ± 10
Rabbiteye blueberry (6×)			
Columbus	3	112 ± 16	102 ± 12
Ira	6	132 ± 54	121 ± 58
Montgomery	5	84 ± 75	54 ± 28
Onslow	11	89 ± 22	55 ± 9
Powderblue	5	162 ± 39	103 ± 23
Premier	10	71 ± 21	46 ± 7
Tifblue	11	143 ± 28	76 ± 17
Yadkin	5	134 ± 62	144 ± 61
Interploid hybrid (5×)			
Robeson	3	48 ± 2	38 ± 7
Breeding selection (clone)			
Southern highbush (4×)			
NC 4263	5	60 ± 11	79 ± 41
NC 4365	3	57 ± 12	41 ± 6
NC 4385	3	64 ± 19	53 ± 15
NC 4398	3	57 ± 18	87 ± 6
NC 4900	3	58 ± 5	53 ± 10
SA-10:135	6	69 ± 22	61 ± 16

SA-4:2	3	88 ± 14	116 ± 16
SHF2B1-20:21	2	100 ± 1	110 ± 43
SHF2B1-21:3	4	68 ± 10	67 ± 9
SHF2B1-25:25	2	36 ± 6	48 ± 20
SHF2B1-25:41	5	33 ± 8	39 ± 10
Rabbiteye blueberry and derivatives (6×)			
NC 3961	3	38 ± 17	43 ± 10
SA-13:75	6	32 ± 12	30 ± 7
Breeding population (cross)			
Southern highbush (4×)			
Arlen × Georgiagem	74	55 ± 22	59 ± 25
NC 2873 × OP	11	45 ± 9	59 ± 10
NC 2898 × G-615	37	54 ± 25	57 ± 21
NC 3147 × Legacy	6	81 ± 32	78 ± 25
NC 3147 × NC 4562	2	135 ± 34	139 ± 15
NC 4295 × Arlen	34	44 ± 13	52 ± 14
NC 4297 × Ozarkblue	69	65 ± 23	72 ± 32
NC 4299 × Ozarkblue	164	58 ± 19	68 ± 23
NC 4302 × Georgiagem	2	39 ± 12	82 ± 10
NC 4302 × Sunshine Blue	9	55 ± 19	73 ± 26
NC 4562 × NC 3476	31	54 ± 20	70 ± 40
NC 4562 × NC 4179	47	61 ± 19	78 ± 29
NC 4562 × NC 4361	50	79 ± 25	88 ± 37
Reveille × NC 3476	55	43 ± 20	50 ± 20
Reveille × NC 3920	6	89 ± 17	131 ± 68
Rabbiteye blueberry and derivatives (6×)			
NC 1223 × Columbus	90	168 ± 59	143 ± 48
NC 81-10-2 × Columbus	69	53 ± 22	59 ± 23
NJ 89-158-24 × Columbus	37	65 ± 25	64 ± 19
5× open-pollinated			
CHID2-14:73 × OP	28	81 ± 32	90 ± 28
NC 3252 × OP	6	56 ± 22	90 ± 44
NC 3958 × OP	40	79 ± 36	94 ± 33
NC 4165 × OP	6	61 ± 22	63 ± 30
NC 4812 × OP	33	65 ± 33	77 ± 32

[a] Number of blueberry plants evaluated for chlorogenic acid (CA) in both years.

[b] Means of CA concentrations (milligrams per 100 g frozen fruit) ± standard deviations for each year.

Table 2. Mean concentration for chlorogenic acid (CA) in the blueberry commercial cultivars, breeding selections, and breeding populations evaluated in 2010 and 2011 at Salisbury, NC.

	CA (mg/100 g)		CA (mg/100 g)
Commercial cultivar		Breeding population (cross)	
Southern highbush (4×)		Southern highbush (4×)	
Arlen	62 de [a]	Arlen × Georgiagem	57 efg
Legacy	51 e	NC 2873 × OP	52 fg
Lenoir	38 e	NC 2898 × G-615	55 fg
O'Neal	41 e	NC 3147 × Legacy	80 cde
Ozarkblue	71 b-e	NC 3147 × NC 4562	137 ab
Pamlico	45 e	NC 4295 × Arlen	48 g
Sampson	33 e	NC 4297 × Ozarkblue	68 def
Average	49	NC 4299 × Ozarkblue	63 d-g
Rabbiteye blueberry (6×)		NC 4302 × Georgiagem	61 d-g
Columbus	109 a-d	NC 4302 × Sunshine Blue	64 fd-g
Ira	126 ab	NC 4562 × NC 3476	62 d-g
Montgomery	68 cde	NC 4562 × NC 4179	70 def
Onslow	72 b-e	NC 4562 × NC 4361	83 cd
Powderblue	132 ab	Reveille × NC 3476	46 g
Premier	58 e	Reveille × NC 3920	110 bc
Tifblue	109 abc	Average	70
Yadkin	139 a	Rabbiteye blueberry (6×)	
Average	102	NC 1223 × Columbus	156 a
Interploid hybrid (5×)		NC 81-10-2 × Columbus	56 fg
Robeson	43e	NJ 89-158-24 × Columbus	64 def
Breeding selection (clone)		Average	92
Southern highbush (4×)		5× open-pollinated	
NC 4263	70 b	CHID2-14:73 × OP	85 cd
NC 4365	55 bcd	NC 3252 × OP	73 def
NC 4385	64 b	NC 3958 × OP	87 cd
NC 4398	77 ab	NC 4165 × OP	62 d-g
NC 4900	61 bc	NC 4812 × OP	71 def
SA-10:135	65 b	Average	76
SA-4:2	107 a		
SHF2B1-20:21	107 a		
SHF2B1-21:3	66 b		
SHF2B1-25:25	44 bcd		
SHF2B1-25:41	33 cd		
Average	68		
Rabbiteye blueberry and derivatives (6×)			
NC 3961	46 bcd		
SA-13:75	31 d		
Average	39		

[a] Means with different letters are significantly different at $p < 0.05$.

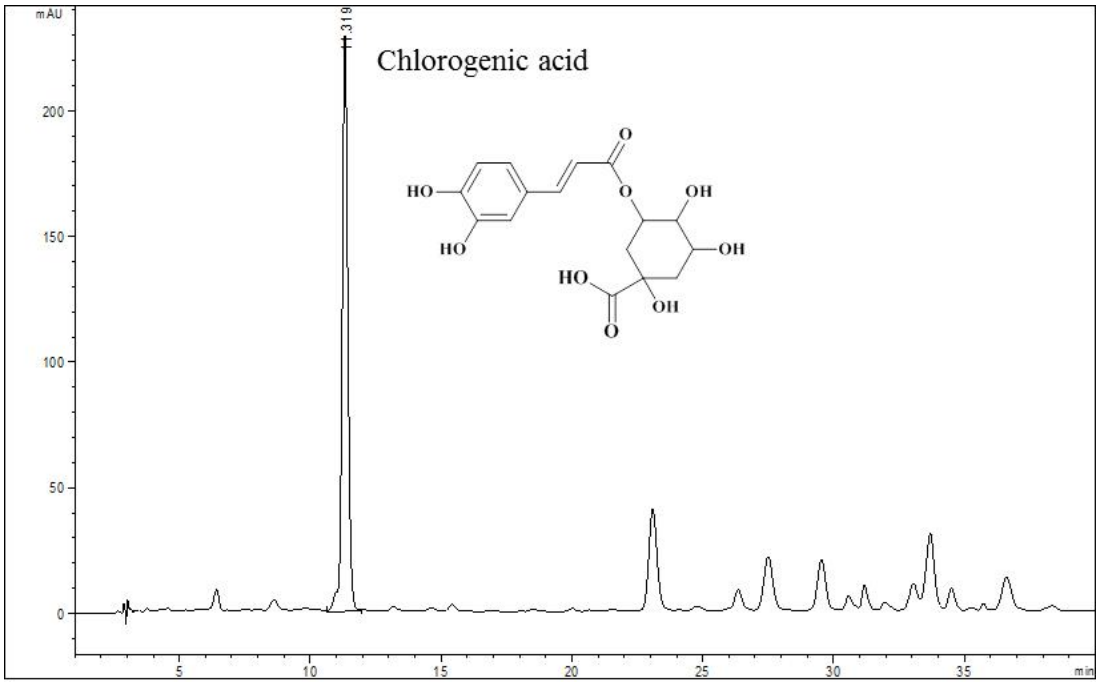

Figure 1. Representative HPLC chromatogram for chlorogenic acid detection in the commercial cultivar Arlen monitored at UV 325 nm (2010).

3.2. Analysis of variance (ANOVA)

ANOVA revealed that genotype was the main source of variation for CA in the commercial cultivars, breeding selections (clones), and breeding populations (Table 3). While there was a year effect on the CA concentration, it was small compared to the genotype effect. Other experimental factors including individual plants, replications, and genotype × year interactions did not show any significant effect on CA accumulation in the commercial cultivars. A similar trend was observed with the breeding selections genotypes (Table 3). The genotypic variation (calculated as a percentage of the MS associated with genotype over the total MS for the statistical model) constituted 64% and 50% of the total variation in CA in the cultivars and clones, respectively. In the breeding populations, since the F_1 heterozygous plants were segregating for all of the polyphenolic phytochemicals measured in this study including CA, significant variation was observed within populations, and in the genotype × year interaction. However, the genotype effect constituted 72% of the total variation observed over the two years of this study, which can have significant implications for blueberry breeding efforts.

3.3. CA correlations

Two types of correlations were examined with CA in this study; correlation between CA and ploidy level, and correlation between CA and anthocyanin (ANC) content. Anthocyanin ranges in these blueberry genotypes were previously reported [8]. A strong positive correlation was observed between ploidy level and CA content ($r = 0.75$) in the commercial cultivars. In the segregating populations, the correlation was not as evident.

A significant moderate correlation was observed between CA content in the blueberry materials

Table 3. Sources of variation (ANOVA) for chlorogenic acid (CA) in the blueberry commercial cultivars, breeding selections, and breeding populations evaluated in 2010 and 2011 at Salisbury, NC.

Commercial cultivars			Breeding selections		Breeding populations		
Source	df	MS[a]	df	MS	Source	df	MS
Genotype[b]	15	128.3**	12	35.0**	Genotype	22	721.7**
Year	1	49.4*	1	16.2*	Year	1	145.9**
Plant	10	8.3	5	2.9	Plant (genotype)	883	12.9**
Rep (Year)	2	6.9	2	9.6*	Genotype × Year	22	28.1**
Genotype × Year	15	18.8	12	4.0	Error	883	4.4
Error	134	8.6	63	2.4	R^2		0.88
R^2		0.70		0.76	CV		21
CV		27		20			

[a] MS (mean square) values for chlorogenic acid are divided by 100.

[b] Genotype refers to the commercial cultivar, breeding selection, or breeding population evaluated over two years.

* and ** are significant at $p < 0.05$ and $p < 0.01$, respectively.

Table 4. Pearson's correlation coefficients between chlorogenic acid (CA) and different classes of anthocyanins (ANC) in the fruit of blueberry cultivars, selections, and populations evaluated over two years (2010 and 2011) at Salisbury, NC.

ANC class [a]	Chlorogenic acid (CA)		
	Commercial cultivars	Breeding selections	Breeding populations
Aglycones			
Delphinidin-	0.06	0.45**	0.44**
Cyanidin-	0.51**	0.39**	0.50**
Peonidin-	0.51**	0.62**	0.39**
Peonidin-	0.40**	0.23*	0.31**
Malvidin-	0.09	0.43**	0.43**
Glycosides			
Galactose-	0.17*	0.21*	0.46**
Glucose-	0.41**	0.61**	0.24**
Arabinose-	0.09	0.30**	0.47**
Acylated-	−0.18*	−0.22*	−0.06
Total ANC	0.30**	0.51**	0.49**

* and ** are statistically significant at $p < 0.05$ and $p < 0.01$, respectively.

[a] Classes are aglycone- or glycoside-based anthocyanins [8].

used in this study, and their ANC content. This is particularly true with all ANC classes except for the acylated anthocyanins (Table 4). CA showed positive and significant correlation with total anthocyanins ($r = 0.5$) in the clones and breeding populations but was lower ($r = 0.3$) in the commercial cultivars. This positive correlation with ANC was expected, since CA and ANC share the same

biosynthetic pathway, as derivatives from L-phenylalanine (Figure 2). These correlation trends are of particular interest in that it may facilitate selection for improved blueberry phytochemistry, using CA as a phenotypic marker that is easy, fast, and inexpensive to measure using traditional HPLC methods. When selecting for higher CA, ANC may indirectly be elevated at the same time. This can play a significant role in blueberry breeding, since quantitatively assaying ANC with a complex profile is prohibitively expensive and time-intensive to perform on the large number of plants required for breeding programs. Data obtained in this study showed that significant genetic variability exists among blueberry breeding material established in NC State University's breeding program that can be used effectively to improve blueberry plants for CA and at the same time, indirectly improve ANC concentration. This is particularly important since breeding blueberry can be a long and demanding process that can take 10 to 20 years from the original cross to cultivar release [21]. Expanding the gene pool for blueberry resulted in enhanced CA content that may contribute to the blueberry phytochemical value and human health benefits.

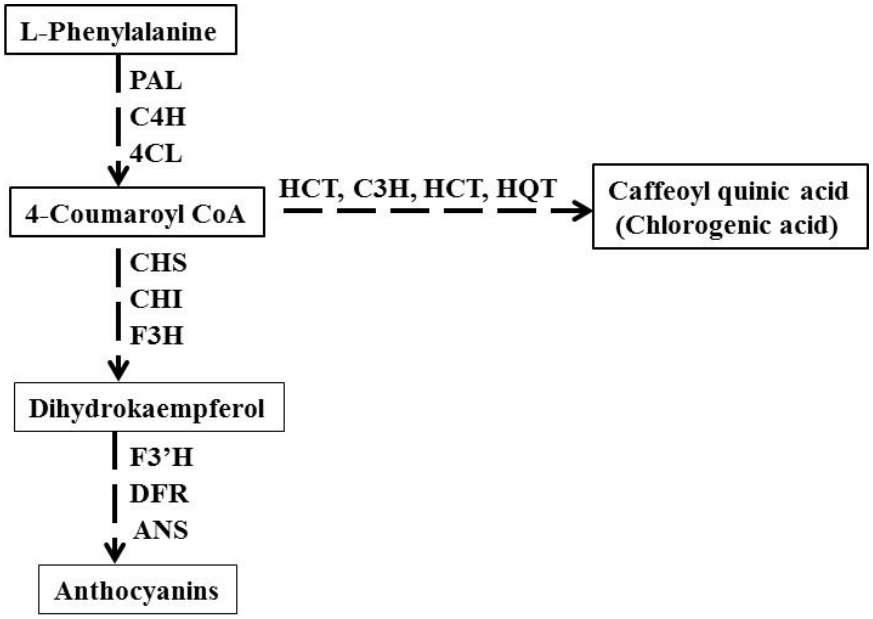

Figure 2. Simplified schematic of the phenylpropanoid pathway showing the biosynthesis of anthocyanins and chlorogenic acid in plants [23-25]. Dashed arrows represent multiple enzymatic steps. PAL = phenylalanine ammonia lyse, C4H = cinnamate-4-hydroxylase, 4CL = 4-coumaroyl-CoA ligase, HCT = p-hydroxycinnamoyl, C3H = coumaroyl ester3-hydroxylase, HQT = hydroxycinnamoyl CoA quinate transferase, CHS = chalcone synthase, CHI = chalcone isomerase, F3H = flavone 3-hydroxylase, F3'H = flavone 3'-hydoxylase, DFR = dihydroflavanol 4-reductase, ANS = anthocyanidin synthase.

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Conflict of interest

The authors declare no conflict of interest in this publication.

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