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Differential Accumulation of Polyphenolics in Black Bean Genotypes Grown in Four Environments

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Environmental effects on polyphenolic composition of pigmented seed coat tissue were examined in four black bean genotypes, grown in four locations in Canada. Genotype was the most significant determinant in the phenotypic expression of flavonoid traits across four locations ($p < 0.0001$). The genotype × environment interaction was not significantly different for anthocyanin or extractable condensed tannin (syn. proanthocyanidin) but was significant for the bound anthocyanidin concentration ($p < 0.05$). One trace metabolite, (−)-epicatechin, was identified, but no flavonols were detected in the seed coats. Sequestration of anthocyanin in the seed coat was genotype-dependent and predominantly consisted of delphinidin with lesser amounts of petunidin and malvidin. Pigment sequestration in the two integument layers of the seed coat appeared to be mutually exclusive across all genotypes in terms of the pigment chemical character. Tissue-specific accumulation of extractable and bound anthocyanin in the outer integument was observed. The inner integument was devoid of anthocyanin, and the pigment consisted solely of condensed tannin inclusions. The occurrence of condensed tannin together with anthocyanin pigments, whether extractable or bound either by oxidation or by cross-linking, influenced the visual uniformity of seeds of bean cultivars. The co-occurrence of these compounds could have an effect on postharvest appearance during storage, on canning quality, and on the dietary effects of the putative functional food profile in the black bean market class.

KEYWORDS: Anthocyanin; black bean; condensed tannin; delphinidin; genotype; integument; *Phaseolus vulgaris*; proanthocyanidin; seed coat

INTRODUCTION

Polyphenolics, especially flavonoids such as catechins, anthocyanins, isoflavones, and flavonols, are considered important functional food characters connected with a good health and wellness profile in nutrition. Frequently, the antioxidant effects of polyphenols, together with the antiproliferative effects of flavonoids, are reported to contribute to wellness based on their documented influence on urinary tract infections, induced apoptosis for controlling cancer cell proliferation, and other similar physiological phenomena (*1*−*6*). These and other reports support the recommendation by national public health agencies of a diet with increased consumption of flavonoid-rich food such as fruit and the edible legumes (e.g. lentil, dry bean, field pea, soybean, and faba bean) (*4*, *7*).

Although black bean anthocyanins have been widely described (*8*−*11*), a comprehensive genotype by environment (G × E) assessment of the effect of growing location on the overall polyphenolic characteristics and concentration of individual classes of flavonoids has not been reported from the short season growing area. In past research with older varieties, selected Canadian black bean cultivars were assessed for anthocyanins in milled bean (*12*) and for canning quality (*13*). However, desirable, flavonoid-rich phenotypes grown in different locations have not yet been routinely screened to establish whether functional food attributes are consistently accumulated across genotypes and growing locations. In a cultivar development program, rapid, simple quantification of traits is necessary to evaluate large plant populations and make effective selections.

With this in mind, polyphenolics in seed coat tissue were quantified from four black bean genotypes grown at four locations, with the objective of assessing polyphenolic variation and G × E effects. Potential unfavorable quality attributes, in the form of high concentrations of condensed tannins (CT, syn. proanthocyanidins), were evaluated in decorticated seed coat tissue, in conjunction with the occurrence of other polyphenolics such as anthocyanins and trace flavonoid metabolites. A rapid, miniaturized extraction protocol was developed to facilitate screening for these compounds in a plant breeding program. These results are expected to provide plant breeders with an insight into the genotypic and environmental effects on dry bean phenotypes having these beneficial dietary traits. Accordingly, black bean cultivars could be developed to target known functional food traits and enhance desirable dietary profiles while avoiding traits that detract from dry bean quality.

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MATERIALS AND METHODS

Reagents. HPLC-grade chemicals were obtained from Sigma-Aldrich (Oakville, Ontario, Canada), unless otherwise noted. Reference compounds for authenticated standards (e.g., delphinidin, cyanidin, malvidin, (±)-catechin, (−)-epicatechin, kaempferol) were obtained from Fluka (Sigma-Aldrich, St. Louis, MO). Organic solvents were supplied by EMD Chemicals (Gibbstown, NJ) in OmniSolv grade. Acids and bases were of ACS quality. Filtered water was obtained from a Milli-Q Ultrapure water purification system (Millipore, Billerica, MA). Chemical reference compounds were weighed to 0.1 mg, and stock solutions were stored at −80 °C. Diluted working solutions were freshly prepared for each assay or HPLC analysis.

Plant Material. Black bean genotypes (UI 911 [UI] (original seed obtained from the University of Idaho), CDC Rio [Rio], AC Black Violet [BV], and CDC Jet [Jet]) were grown in a replicated randomized complete block design at four locations in Manitoba, Canada (Carman, [C], Portage la Prairie [P], Morden [M], and Winkler [W]) in 2006. Three biological replicates of each genotype were obtained from the Winkler location to test for variability across one environment. To assess the G × E effect, replicate subsamples of each genotype were derived from composite (bulked) samples taken from each location (C, P, M, W).

Seed Coat Preparation and Extraction. Whole dry bean seed of each genotype was moistened between wet filter papers at 4 °C to be decorticated by hand. The resulting seed coat tissue was freeze-dried and ground to pass through a 0.75 mm sieve of a ZM-200 Ultra centrifugal mill (Retsch, Haan, Germany). For isolation of metabolites in the inner and outer integument layers, the moistened seed coat was hand-separated under a dissecting microscope at 1.5× to 2× magnification. Dissected layers were photographed (3.2× magnification) mounted in water and after the addition of histochemicals (*n*-butanol−HCl, [BuOH−HCl] 70:30, v/v), as described previously for the microscopy of *Brassica* species seed coat tissue (*14*). Histochemical reactions distinguished anthocyanin, by an immediate change to a bright cherry-red appearance, from CT (slow brown-red appearance after 60 min, deepening to burgundy red after several hours) as depolymerization of the CT polymer occurred in the seed coat layers. The inner integument was a very fragile layer that tended to disintegrate as it was being removed from the outer layer. Inner layer remnants adhering to the outer integument were removed by gentle washes before extraction and HPLC separation.

Extractions and analyses were repeated at least three times with three technical subsamples for each biological or composite replicate. By use of a fast, miniaturized screening protocol, plant tissue was extracted in 2-mL microcentrifuge tubes (screw-capped polypropylene, with silicone O-ring seal). Unless otherwise stated, ground seed coat samples weighing 20−80 mg (±0.1 mg) were extracted in all the biochemical assays. To quantify extracts, supernatants were clarified in a fixed-angle Hereaus microcentrifuge (Thermo Fisher Scientific, Waltham, MA) for 2 min at 16060*g* and diluted as necessary. Results were converted to mg g^{-1} seed coat and reported as mean values of the repeated assays, using error bars to represent standard error of the means in graphs. Dissected integument layers for each genotype were collected in 2-mL microcentrifuge tubes and extracted sequentially with aqueous methanol (MeOH) and acetone as described for the seed coat extract assays, following. Liquid chromatographic (LC) separation and UV spectra, were obtained by a 1100 series HPLC (Agilent Technologies Canada Inc., Mississauga, ON) instrument equipped with an autosampler, quaternary pump, solvent degasser, UV photodiode array detection (UV-PDA), and a Zorbax XDB reversed-phase C_{18} column (3.0 mm × 150 mm, 5 μm particle size). Instrument separations and data analyses were controlled using ChemStation computer software for LC 3D systems (revision B.02.01-SR1[260]) with mobile phase modifications noted in each assay.

Biochemical Assays. (*1*) *Anthocyanin*. For rapid screening assays, tissue was incubated at room temperature in 1 mL of 80% aqueous MeOH modified with 0.05% trifluoroacetic acid (TFA). Samples were vortexed and then shaken on a rocking platform for 4 h to keep the tissue suspended. Both the 80% acidified MeOH and the 70% aqueous acetone fractions from the CT extraction were used to quantify total anthocyanin concentration in black bean seed coats. The two anthocyanin-containing fractions were quantified immediately after extraction to prevent loss of the unstable anthocyanin moieties. Delphinidin was used as a reference compound to quantify total concentration of all anthocyanin in the seed coat.

Anthocyanin identification was determined by comparison of relative retention time (RT), and UV spectral data from acid-hydrolyzed MeOH−HCl extractions (1% HCl, 10 min, 90 °C, partitioned against ethyl acetate to prepare the aqueous [lower] phase for UV spectral analysis) with those of anthocyanin reference standards (aglycones delphinidin, malvidin, cyanidin, pelargonidin), similarly separated and detected, as well as UV spectral properties (petunidin) from published reports (*8*, *10*, *15*). Initial experiments with acid-hydrolyzed seed coat extracts were spiked with trace amounts of two reference compounds (delphinidin, malvidin) to confirm their behavior in the sample matrix, and the HPLC mobile phase was modified with 0.05% or 0.5% TFA. Compounds in the unhydrolysed miniassay were separated using an aqueous MeOH HPLC solvent system (5% aqueous MeOH modified with 0.5% TFA as solvent A and 99.5% MeOH with 0.5% TFA as solvent B in a 25 min run time) and monitored at 530 nm.

(*2*) *Extractable CT*. The MeOH fraction (1) removed after centrifugation was replaced with 1 mL of 70% aqueous acetone for an overnight incubation (14−16 h) on the shaker. Polyphenols in each of the supernatants (filtered, 0.2 μm) were identified in HPLC separations by UV-PDA, using acetonitrile gradient elution as previously reported (*16*). A partially purified CT standard from *P. vulgaris*, prepared according to previous reports (*17*, *18*) and further processed to improve removal of bound protein, was used to quantify the CT component.

(*3*) *Bound Anthocyanidins*. The residual pellet from the MeOH−70%-acetone extractions was processed in the BuOH−HCl (70:30, v/v) assay to form monomeric anthocyanidins by cleavage of the CT polymer and quantified at the maximal absorbance nearest to 550 nm using a scanning spectrophotometer (*16*, *19*), modified by directly using the pellet in the 2-mL microfuge tube following the aqueous acetone extraction and omitting polyvinylpolypyrrolidone (PVPP). BuOH−HCl was added by weight (0.9080 g [~1 mL]) for more precision in the assay. Initial assays incorporated PVPP to bind CT and eliminate anthocyanin with MeOH washes; however, this procedure to eliminate anthocyanin from the residual pellet was ineffective and subsequently omitted. The final results are reported as "total bound anthocyanidins".

(*4*) *Trace Flavonoids*. The individual 80% MeOH and 70% acetone fractions were combined (separately for each black bean replicate), and this volume was reduced to about 200−300 μL under a stream of dried nitrogen. Aliquots in the 5−20 μL range were injected on the HPLC instrument under the same conditions for extractable CT and anthocyanin preparations. The RT and UV spectra of authentic reference compounds were used to identify other flavonoid metabolites in the seed coat.

Statistical Analysis. Data were analyzed using SAS PROC Mixed (SAS 9.2 for Windows V5.1.2600, SAS Institute, Cary, NC) to ascertain whether there were significant differences among genotypes and a significant replicate effect (W location only) and if there was an interaction with the location in which the plants were grown (G × E).

RESULTS

Rapid Screening Estimates of Flavonoid Traits in G × E Evaluation Trials. The miniaturized extraction protocol described here was tested for efficiency and found to be reproducible and sensitive down to the lowest mass extracted (14.0 mg). Preliminary experiments determined that the precision of the miniassay declined when tissue samples were greater than 80 mg or long sample cleanup methods were followed. The resulting anthocyanin showed no losses in this rapid miniassay, and high reproducibility was obtained when executed as described. Contrary to the stability of anthocyanin in the miniextracts over several days, acid-hydrolyzed extracts of anthocyanin (as aglycones) required prompt analysis. Delphinidin was more stable than the other pigments. In the unhydrolyzed acetone extract, CT was stable when the preparation was stored at −20 °C. At room temperature or 4 °C, the mixture darkened and CT appeared to oxidize to an unidentified product suspected to be a quinone. This is in keeping with observations reported for CT preparations of pinto bean (*16*).

The bound or otherwise cross-linked CT and anthocyanin were effectively measured by the aggressive BuOH−HCl assay with the

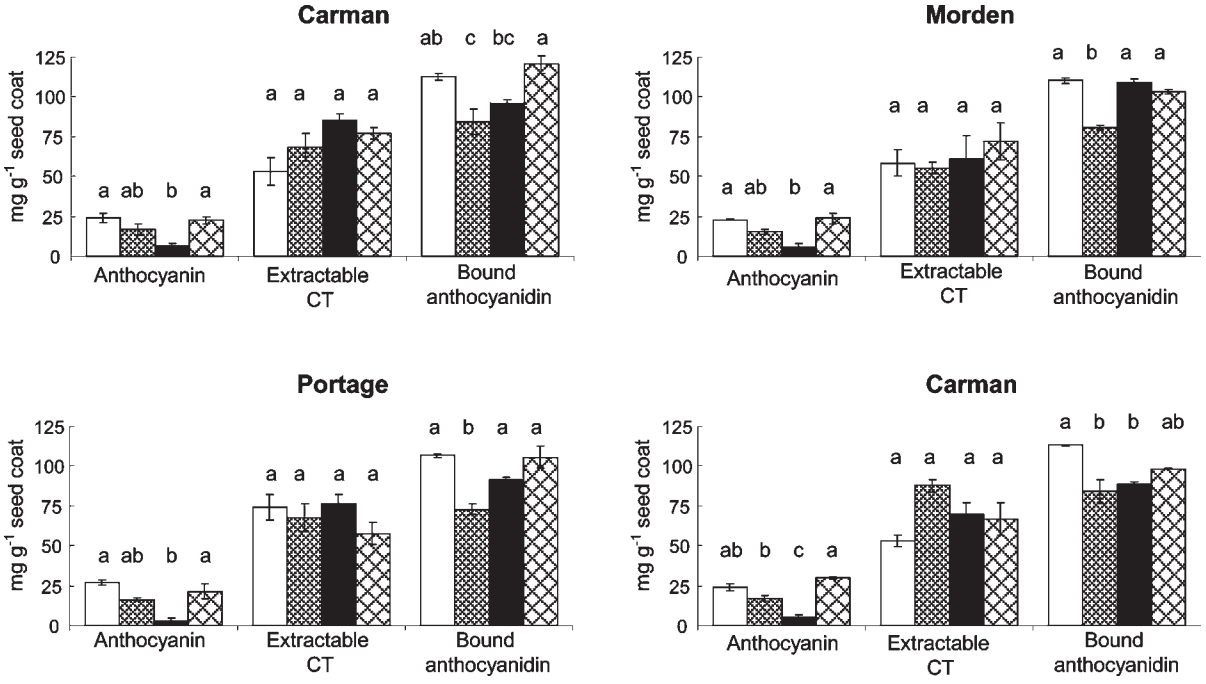

Figure 1. Concentrations of anthocyanin, extractable CT, and bound anthocyanidin found in seed coats of four black bean genotypes grown at four locations in Manitoba in 2006. Within each flavonoid class, mean values with the same letter are not significantly different at the indicated location. Genotypes are in the following order: Black Violet, CDC Jet, CDC Rio and UI 911.

residual pellet. Anthocyanin could not be removed completely by repeated washing with MeOH, although two concentrations of solvent (80% and 100%) and four extraction times (1, 7, 14, and 28 days) were tested. Even after a month of exposure to MeOH at either 80% or 100% concentrations, with and without an acidic modifier, the seed coat tissue remained heavily pigmented. For this reason, PVPP was omitted and the BuOH−HCl quantification was used to measure all bound flavonoids that converted to an extractable anthocyanidin molecule. In black bean, the wavelength maximum of the BuOH−HCl extract occurred at 550 nm, as opposed to the 538 nm in pinto bean, a reflection of the hydroxylation pattern on the B-ring of the flavylium ion (*20*).

Environmental Effects on Flavonoid Content in Black Bean Genotypes. There were no significant differences among biological replicates for any of the flavonoids tested at the one location (Winkler) where they were tested independently (all $p > 0.05$). The G × E interaction was not significant for anthocyanin ($p = 0.4548$) or for extractable CT ($p = 0.0708$) but was significant for the bound anthocyanidin trait ($p = 0.0050$) when samples were assessed from across the four growing environments (**Figure 1**). The G × E effect for bound anthocyanidin was noncrossover type, indicating that the ranking of genotypes was consistent across locations. UI 911 and AC Black Violet consistently had the highest concentrations of bound anthocyanidins, while CDC Jet had the lowest, across all four locations (**Figure 1**). The significant effect of genotype on the phenotypic expression of anthocyanin and bound anthocyanidin was evident across the four locations ($p < 0.05$, **Figure 1**). There were no significant differences in extractable CT at any location ($p > 0.05$).

Concentrations of extractable anthocyanin were lower than those of extractable CT or bound anthocyanidin. Accumulation of the different anthocyanin components followed the same pattern reported previously for UI 911 (*10*), with delphinidin as the predominant anthocyanin (**Figure 2**). Anthocyanin elution patterns were consistent among genotypes, and the anthocyanin wavelength maximum (530 nm) used to monitor the different compounds was reliable. Malvidin was the most likely to become

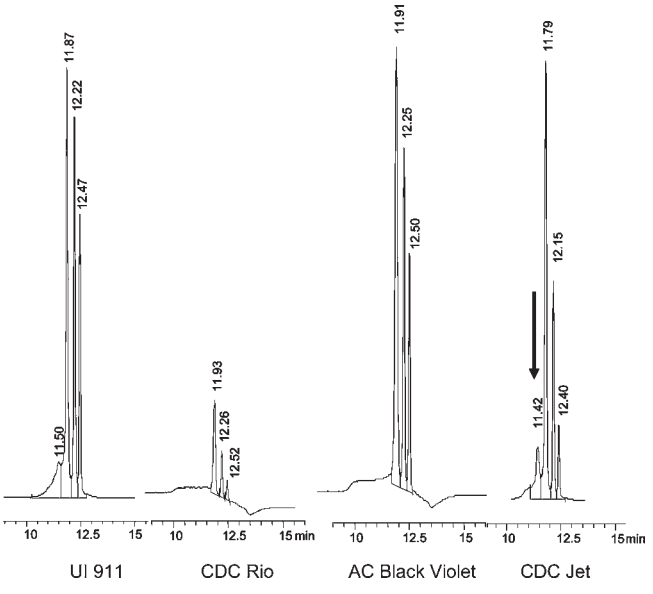

Figure 2. Anthocyanin elution profiles of seed coat extracts of black bean genotypes. The arrow at 11.42 min (CDC Jet) indicates an unknown trace anthocyanin. This was similar to the very minor peak detected in UI 911 extracts at 11.5 min Anthocyanin elution pattern was consistent over all extractions within each genotype in the order delphinidin, petunidin, and malvidin.

undetectable in the preparations because it was highly labile unless the extracts were analyzed promptly. Occurrence of a fourth anthocyanin moiety was evident in CDC Jet (**Figure 2**), similar to that reported in other accounts of black bean seed coat extracts (*9*, *10*). The cultivar CDC Rio consistently contained the lowest amount of extractable anthocyanin, and the seed coat color hue was variable in all the samples of CDC Rio. Some seeds displayed a brown-purple tone; other individual seeds showed patchy pigment patterning that was especially evident after

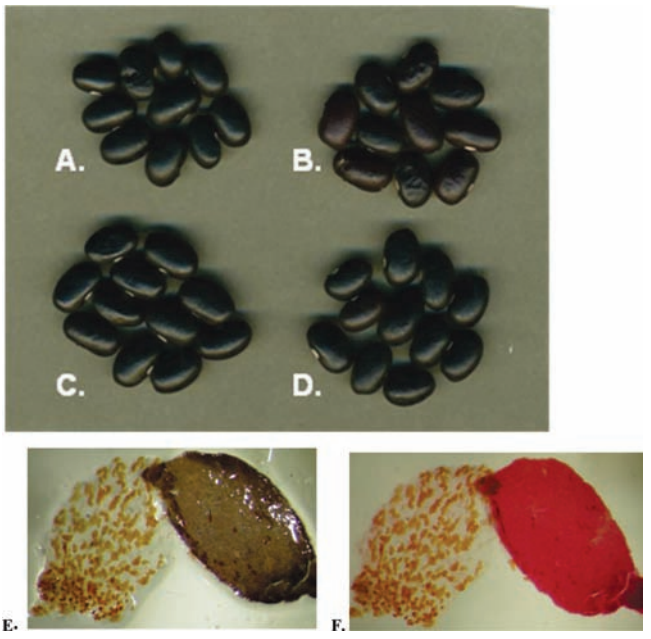

Figure 3. Whole seed and histochemically stained dissected integuments of black bean. Whole dry black bean seeds were grown at Winkler, Manitoba, in 2006: (**A**) UI 911, (**B**) CDC Rio, (**C**) AC Black Violet, and (**D**) CDC Jet. Inner (left) and outer (right) integument layers of CDC Jet before (**E**) and after (**F**) staining in BuOH−HCl (magnification, 3.2×).

moistening and viewing at 2× to 3× magnification (data not shown).

Trace Flavonoid Accumulation. Insoluble residues in the concentrated extracts interfered with an accurate quantification of the trace metabolites. Despite this result, it was evident that all the cultivars similarly accumulated (−)-epicatechin. Without exception, the flavonol class of polyphenolics (e.g., kaempferol) was not detected in the black bean seed coat extracts.

Differential Accumulation of Polyphenolic Products in Seed Coat Integument Layers. The seed coat of black bean genotypes showed considerable diversity with respect to the polyphenolic composition, although these traits are not visually evident in the mature, dry bean (**Figure 3A−D**). In the dissected, unstained black bean seed coat, the inner integument layer contained CT as isolated inclusions, and the outer layer contained only anthocyanin (**Figure 3E**,**F**). These tissue-specific chemical attributes were mutually exclusive, as monitored by both the histochemical reaction and the presence/absence of the distinctive UV spectra in the individual integument preparations analyzed by HPLC-separated UV spectra (LC data for dissected integuments not shown). The predominant anthocyanin in the outer integument was delphinidin (together with minor amounts of petunidin and malvidin).

DISCUSSION

Dry beans are primarily sold on the basis of their seed attributes such as size, shape, and color. Seed color is a complex phenotype affected by several genes in the flavonoid pathway as well as the environment in which the parent plants are grown. Black bean and other polyphenolic-rich crops are promoted as a wholesome addition to the diet (*7*), and it is desirable to select for those that contain high levels of these compounds that produce a good color and provide health benefits. Currently, black bean breeders rely on seed coat color at harvest to determine which genotypes stay in the program for further evaluation and which are dropped because of poor color development. Understanding the role of the various flavonoids in the development of this color could help breeders make more informed choices.

Evaluation of the Rapid Miniassay Protocol for Screening Black Bean Accessions. While selection based on seed coat color at maturity is reasonably effective, this is based on a comparison to standard varieties and the effect of environment can lead to retention of lines that would have been dropped had the true expression level of the polyphenolics been seen. There is a need for a high throughput, quantitative method that uses small amounts of tissue that can be used to make more effective selections.

The miniaturized screening assay described here can contribute a small part to the selection of black bean lines in breeding programs by efficiently quantifying pigmentation characteristics, the main factors in seed coat appearance. The rapid miniassay employed the novel approach of three extraction procedures for three forms of seed coat pigments from only one small tissue sample. Anthocyanin characterization was particularly prone to time-related problems and stability in processing large populations. By a change from multiple MeOH extractions with sample cleanup procedures (*8*−*11*) to a 4 h extraction on a rocking shaker and by use of 0.05% TFA in 80% aqueous MeOH, the large-scale genotype screening assays resulted in a precise and reproducible quantification of anthocyanin. Other choices to acidify the alcohol were more likely to degrade the anthocyanins, a finding also reported by Dao et al. (*21*). In a long extraction procedure that must remain consistent across a large number of samples, total anthocyanin concentration declined, which also defeated the purpose of a rapid screening assay using a small sample size. Of more concern, the malvidin-attributed peak in the LC separations (the most labile compound) declined rapidly during a lengthy extraction procedure even in a refrigerated cabinet. Non-interference of acetone and other organic solvent residues in CT quantification was established previously (*22*,*23*), and the reproducibility of our miniassay confirmed that there was no problem in using an acetone-extracted tissue pellet for the subsequent assay of bound CT and unextractable anthocyanin.

This "three-step-per rep" extraction is important in a cultivar development program where a rapid and efficient evaluation of traits is necessary when dealing with large plant populations. In Canadian-grown black bean genotypes, traits such as yield, seed appearance, days to maturity, drought tolerance, and disease resistance are the primary factors that contribute to selection. The miniaturized screening assay developed in this study may enable breeders to routinely quantify seed coat characteristics of dry bean.

Flavonoid Concentrations and Environmental Effects. Both anthocyanins and CT were observed in all genotypes examined. Unlike pinto bean seed coat, where no anthocyanin is produced, and the flavonol kaempferol accumulated as an aglycone, black bean seed coat appeared to bypass that product in favor of anthocyanin biosynthesis. Although the flavonoid content varied according to the growing conditions, the profile of anthocyanin accumulation was conserved in the seed coat and compared well to the benchmark established in UI 911 (*10*). The co-occurrence of these compounds could have an effect on postharvest appearance during storage, on canning quality, and on the dietary effects of the putative functional food profile in the black bean market class.

There was no effect of replicate within a location on the levels of any of the compounds studied ($p > 0.05$). This means it is possible to reduce the number of samples screened per location by pooling samples from across the test replicates. As a result, this approach was used to investigate the effect of location on the concentrations of these compounds. While there were significant differences in absolute values of the concentrations, there was no

difference in ranking across the different locations for any of the compounds studied (**Figure 1**). This suggests that within a breeding program, selections could be made on the basis of the ranking of lines using pooled seed samples from one location.

CDC Rio has a tendency to have a brownish appearance (**Figure 3B**). This is likely due to the presence of CT in the inner seed coat layer coupled with a reduced level of anthocyanins present relative to the other lines that have a blacker appearance. Since it was not the line with the highest level of CT, it would appear that it is the anthocyanins that cover up the brown appearance of the CT that lead to a more appealing seed coat color rather than a reduction in CT.

Significance of Pigment Sequestration on the Importance of the Phenotype in Black Bean. Tissue-specific expression of two major classes of flavonoids (CT and anthocyanin) is reflective of complex metabolic partitioning in black bean. The anatomy of readily separated integument layers accumulating characteristic bioproducts may be part of an intricate metabolic partitioning cascade uniquely associated with black beans. A distinct inner integument with tissue-specific flavonoids was absent in pinto bean seed coat (*16*) and the integument layers did not readily separate, compared to black bean.

For cultivar development, consideration of such tissue targeting by metabolites may become important should plant breeders want to address problems such as the ones evident in the pigmentation of CDC Rio. This genotype had a low concentration of anthocyanin compared to the other genotypes (**Figure 1**) and is known to produce red-brown off-colors in the mature seed. The lack of the desirable purple-black appearance and a distinct brown tone is reflective of the anthocyanin-poor and CT-rich phenotype that this cultivar revealed in the analyses. Fixing this problem should involve crosses with genotypes high in anthocyanin concentration. The tissue-specific nature of flavonoid accumulation may also help explain dry beans that are two-toned in color, e.g., the yellow and pink Flor de Junio types from Mexico.

Reports on black bean have confirmed that more than 2% of the weight in black bean is attributed to flavonoids or antioxidant phenolic acids based on radical scavenging assays and LC separations to quantify the polyphenolic concentration (*3*, *9*, *10*, *12*, *24*). These data, together with the results from the subset of Canadian-grown genotypes presented here, demonstrate that the seed coat is a remarkable tissue with untapped potential, and through selection, the healthy benefits of this whole food could be further enhanced.

ABBREVIATIONS

CT, condensed tannin; G × E, genotype by environment; PVPP, polyvinylpolypyrrolidone.

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