Seed Coat Shininess in *Phaseolus vulgaris*: Rescuing a Neglected Trait by Its Screening on Commercial Lines and Landraces

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Abstract

Seeds with shiny seed coat in common bean (*Phaseolus vulgaris* L.) are normally rejected by consumers due to their difficult cooking, however they can prevent insects and pathogens injuries. Seed coat shininess is known to be conditioned by the *Asp* gene with the dominant form and is also influenced by the *J* locus. The latter influences seed coat color and pattern and has been considered a precursor for proanthocyanidin only in the presence of the dominant allele. This work aimed at performing a series of morphological, biochemical and genetic analyses for screening seed coat shininess in common bean. We showed *Asp* and *J* can be easily distinguished. Colorimeter data (*L*a*b** system) can somehow provide a quantitative differentiation for seed coat shininess based on the *L** variable. Palisade epidermis length and seed coat roughness profiles obtained through electronic microscopy were good indicators for *Asp* phenotyping. Water uptake was affected by shininess but is probably influenced by more genes, even *J*, or due physiological responses. A vanillin-HCl test showed to be a suitable method for tracing condensed tannins. AFLP markers partially allowed the categorization of shininess phenotypic classes. These results could be helpful regarding selection purposes and breeding. Therefore, favorable selection to *Asp* would lead to better resistance to pathogens and insects. *J*, instead, could be selected for nutritional purposes, since proanthocyanidins are important anti-oxidant and anti-carcinogenic compounds.

Keywords: *Asper*, colorimeter, *Joker*, proanthocyanidins, segregation, water uptake

1. Introduction

Common bean (*Phaseolus vulgaris* L.) consumers identify their preferable commercial class, in part, considering color and its distribution pattern on seed coat. Therefore, breeders need to maintain genotype specific characteristics for each commercial cultivar. Knowledge about seed coat genetics is a fundamental issue to guide strategies for genotype selection (Bassett, 2007). Some studies have been focused in identifying genes associated to each color phenotype and determine their biochemical roles (Hosfield, 2000). Genes for seed coat traits are essentially involved in the phenylpropanoid pathway. The expression of each gene is reflected on the grains visual aspect and therefore on its acceptability to the market.

One trait observed by common bean consumers is seed coat shininess. In Brazil seeds with shiny seed coat are frequently rejected by consumers because cooking time is higher than with cultivars presenting dull or opaque seed coat. Despite this disadvantage, seed coat shininess is of great importance to the seed. The seed coat is the structure that provides protection to seeds from biotic (insects and pathogens) and abiotic (mechanical injuries, light, water or moisture) factors in natural conditions or even during grains storage. Seed coat shininess probably increases resistance to these stresses. Local farmers from Central America as well as Brazil, considering these potential benefits, also consume varieties with shiny seed coats. Diamant et al. (1989) showed that 18% among 600 people interviewed preferred shiny varieties in Guatemala.

Extensive phenotypic and genetic analyses led to the identification of genes that control different patterns (*T*, *Z*, *L*, *J*, *Bip* and *Ana*) and colors (*P*, *C*, *R*, *J*, *D*, *G*, *B*, *V* and *Rk*) of the seed coat. Nevertheless, many researchers diverged in their analyses due the variability and complexity of seed coat genes (Bassett & McClean, 2000; Hosfield, 2000). Furthermore, problems were extended to symbols attribution, being more than one symbol used for the same *locus* many times. Many of these genes exhibit epistatic interactions (McClean et al., 2002), turning

difficult their analysis separately.

Among all genes expressed on the seed coat, at least two have been associated to seed coat shininess: *Asper* (*Asp*) and *Joker* (*J*). Genotypes *asp asp* present dull seed coat and *Asp_* are shiny (Bassett, 1996). *Asp* is not related to seed coat color, controlling only the seed coat shininess (Bassett, 1996). This gene was ignored since its original publication (Lamprecht, 1940), because *J* was considered the gene for seed brilliance (Lamprecht, 1932, 1940). In this period, it was also used the symbol *Sh* (shiny) as being equivalent to *J* (Lamprecht, 1960).

Bassett (1996) has performed extensive studies with common bean seed coat, showing some very simple differences between *Asp* and *J*. The *asp* allele produces a paler seed coat than *j*, and *asp* is more frequent in cultivars than *j*. Additionally, the only visible effect of *asp* is the opaque seed coat, while *j* affects not only brilliance, but the color pattern of the seed coat. The main effect of *j* is to produce an immature and irregular color over the seed coat. The opposite should be expected with *J*. Moreover, in *jj* genotypes, the corona zone surrounding the hilum is usually pale or mostly uncolored and the seed coat exhibits a less shiny seed coat (Bassett, 1996).

The understanding of differences between these two genes improved based on their genetic mapping and biochemical analyses tracing their effect. *Asp* is mapped to linkage group B7 in common bean (Freyre et al., 1998; Miklas et al., 2000; Pérez-Vega et al., 2010). However, it is mapped only as a phenotypic marker. *J* is located on linkage group B10 (Freyre et al., 1998, Galeano et al., 2011). A RAPD marker tightly linked to *j* has been found and converted to a STS (Sequence Tag Site). However, this marker can only be used within the Mesoamerican gene pool due to the multiallelism of *J* (Bassett et al., 2002; McClean et al., 2002; Bassett, 2007). Recent advances with the molecular mapping of the phenylpropanoid pathway and the elucidation of proanthocyanidin synthesis in common bean may help do define a better marker for this and other genes (Caldas & Blair, 2009; Reinprecht et al., 2013; Yadegari et al., 2014).

Biochemical analyses have been provided more clearly for *J*. The dominant allele *J* indicates to be essential for the synthesis of proanthocyanidins or condensed tannins (Hosfield, 2000; Leakey, 1988). Proanthocyanidins are oligomers or polymers originated from condensation of units of flavan-3-ols, as catechins and epicatechins (Xie & Dixon, 2005; He et al., 2008; Hummer & Schreier, 2008; Zhao et al., 2010). In common bean, *J* is probably involved with the conversion of dihydroflavonol to leucocyanidin through Dihydroflavonol reductase (Hosfield, 2000), which further leads to proanthocyanidins synthesis. On the contrary, Beninger et al. (1998) and Beninger and Hosfield (1999) showed *jj* genotypes did not produce proanthocyanidins.

Asp causes a structural change on palisade epidermis and has no biochemical effect specified. Based on the structural effect, *asp* genotypes tend to present a thinner palisade epidermis layer than *Asp*. Besides, *asp* presents a rough seed coat surface while *Asp* exhibits an even surface (Beninger et al., 2000).

Considering the importance of the genes *Asp* and *J*, we performed a series of analyses in order to elucidate morphological, biochemical and genetic differences provided by these genes, with the following objectives: (i) provide a qualitative and quantitative method to differ shiny from dull seed coats; (ii) verify if variables such as palisade epidermis length, water uptake and tannin content could be associated to each gene and phenotype; (iii) verify if AFLP markers could differ these classes accordingly; (iv) analyze segregating populations to confirm genotyping.

2. Material and Methods

2.1 Plant Materials and Crosses

A set of testcross lines (Figure 1) varying for seed coat color, pattern and shininess from Bassett (1992, 1996, 1998a, 1998b, 2003) were used as control materials for morphological and biochemical analyses. Line 5-593 presents almost all loci for seed coat in the dominant homozygous form (*Stp T Z l⁺ Bip P [C r] D J G B V Rk Asp*) and has shiny seed coat (*J Asp*). Testcross line *j* BC₃ 5-593 (*j Asp*) has the recessive allele just for the *J* locus, being used as a control. Line *asp* BC₃ 5-593 (*J asp*) is recessive only for the *Asp* locus.

Morphological, biochemical and genetic analyses were carried out with the line Puebla-152 (P-152) and the cultivar Diamante Negro (DN). We also evaluated the landrace Serro Azul, which comprises the variants Shiny Serro Azul (SAB, from the Brazilian *Serro Azul Brilhante*) and Dull Serro Azul (SAF – *Serro Azul Fosco*) (Figure 1).

Puebla-152 (P-152) is a Mexican line from Mesoamerican group. This line has shiny black seed coat and was originally selected for high nitrogen fixation potential and resistance to root rot (Navarro et al., 2008; 2009). Diamante Negro was originated from the cross of CIAT lines XAN 87 x AS 367. Selection was held at CNPAF/EMBRAPA, Brazil, under artificial induction of common bacterial blight, being released as a cultivar in

1991. DN has high productivity and disease resistance (common bacterial blight and mosaic virus, and moderately resistant to anthracnose) (Empresa Brasileira de Pesquisa Agropecuária [EMBRAPA], 2010). Two parents, P-152 and DN-6, from the bean germplasm collection at CENA/USP were crossed, generating F_1 and being advanced to $F_{3:4}$ generation in 2010.

Figure 1. Profile of seed coat, hilum and corona zone for testers for the genes *Asper* (*Asp*) and *Joker* (*J*) (Bassett, 1996, 1998, 2003) e for breeding lines and landraces of common bean (*Phaseolus vulgaris* L.)

A. Profile of two genotypes contrasting for the *J* locus. Corona zone is represented on the right side, showing that *j* genotypes present it partially or totally uncolored. B. *Asp* alleles are distinguished by shiny (*Asp*) and dull (*asp*) seed coats. C. Reference line 5-593, with genotype *J Asp*, with intense shiny black seed coat. D. Tester *asp* BC_3 5-593, with dull seed coat. This tester means the only allele substitution was made on the *Asp* locus. E. Other tester with genotype *J Asp* but with brown color. F. Tester *j* BC_3 5-593, reference material for *J* study. G. Other tester with genotype *j Asp*, but with other color. H. and J. Landrace Serro Azul with variants Brilhante (Shiny Serro Azul) – SAB and Fosco (Dull Serro Azul) – SAF. I. Line P-152, with black shiny seed coat (*J Asp*). K. Cultivar Diamante Negro (CNPAF/EMBRAPA) with dull seed coat (*J asp*).

The landrace Serro Azul is traditionally cultivated by small farmers in Cunha county, Sao Paulo state, Brazil, under low technology conditions. This landrace is a local source for food and financial incomes. Serro Azul has been presenting low productivity and poor nodulation in soil fertility conditions of Cunha region. However, considerable improvement has been obtained when optimal fertilization conditions were adopted (Oliveira et al., 1998; Oliveira & Tsai, 2001). This landrace presents considerable morphological diversity. A source of 2 kg of seeds with dull seed coat was collected in Cunha and among all seeds we found only 10 with shiny seed coat. Dull seeds were called SAF and shiny seeds SAB. SAB plants were crossed to SAF and also the reciprocal (SAF x SAB, SAB x SAF). Crosses were advanced to $F_{3:4}$. Some results indicated SAB had better nodulation than SAF (Oliveira et al., 1998). An indirect survey for antrachnose resistance using detached leaves from grown plants showed SAB was resistant to some races of *Colletotrichum lindemuthianum* while SAF was highly susceptible (data not published). Also, some observations indicated high resistance to insects attack by SAB while SAF seeds were frequently injured in field and storage conditions (data not published).

2.2 Color and Shiniess Assessments

2.2.1 Visual Analysis

CIAT (1976) recommendations for seed coat shininess classification (shiny, intermediate and dull) were observed. However, morphological and biochemical aspects were the focus of this research. We evaluated 5-593 (Bassett, 1998) as the reference material for shiny seed coat (*Asp_*), *asp* BC_3 5-593 for dull seed coat and *j* BC_3 5-593 for the intermediate form. P-152, DN, SAB and SAF were classified accordingly. Progenies $F_{2:3}$ and $F_{3:4}$ of crosses P-152 x DN and SAF x SAB were evaluated based on parents and categorized according to color and shininess. Visual analyses were performed on Petri dishes filled with seeds over a white surface.

2.2.2 Spectrophotometric Method – Color System *L*a*b**

We tried to distinguish dull and shiny seeds by using the colorimeter Minolta Chroma Meter CR-300, obtaining values for three variables: *L** (brightness), *a** and *b** (color components). The Commission Internationale de L'Eclairage (CIE) recommended using the color measuring system *L*a*b** since it is similar to that human ganglion cells see: the amount of green or red as a*; the quantity of blue or yellow as b*; and the amount of brightness as L*. The *L** component is on the z axis, where 100 is the perfect white and 0 is black. Positive values of *a** turn red and negatives green. Positive *b** values turn yellow and negatives blue. We used *L** as a measure for brightness of the seed coat. Samples of about 30 to 40 seeds were placed in a small plate (13 cm^2) covering the entire bottom and then four measures were taken for each sample. Parents SAB, SAF, P-152, DN and crosses were all evaluated.

2.3 Microscopy Analyses of the Seed Coat

2.3.1 Scanning Electron Microscopy (SEM)

Plants of each line were grown in greenhouse and seeds were harvested, air-dried and stored in a cold chamber (8 ± 3 ºC) until analysis. We analyzed five seeds for each variety. Each seed was cut with a razor blade and fixed in a metal support (stub), sputter coated with gold and observed under a LEO 435 VP (Carl Zeiss, Jena, Germany), operating at 20 keV. SEM photos were taken from the opposite side to the hilum of each seed, in two fields of view, to highlight the palisade epidermis for length measurements. In total, 10 measurements were taken for each field of view. Measurements were performed using Image J software 1.44p and converted to metric system based on scales from the microscope.

2.3.2 Light Microscopy (LM)

Seeds of each genotype were sampled the same way as for SEM. Seeds were dehydrated with increasing concentrations of ethanol and then embedded with HistoResin Mounting Media kit (Leica Heldeberg) following manufacturer instructions. Images were observed under a Zeiss Axioskop 2 Microscope coupled with AxiosCamMR3 camera and a computer with software Image Pro-Plus, version 3.0.

2.4 Determination of Total Phenols, Total Tannins and Condensed Tannins From Seed Coats

Phenolics were determined using a sample of 50 seeds of each parent (P-152, DN, SAB and SAF) and two controls (5-593 with genotype *J* and *j* BC_3 5-593), all in triplicate. Samples were immerged in n-heptane for 96 hours and rehydrated. Seed coats were manually pelled and dried at 40 °C in Petri dishes. Phenolics were extracted with 200 mg fine powdered sample in 10 ml of acetone 70%. Samples were sonicated for 20 minutes and then centrifuged at 3000 g for 10 minutes, at 4 °C. Supernatant was removed and placed in other tube to proceed with determinations. The extraction procedure for total phenols and total tannins followed indications of Makkar et al. (1993). A standard curve was prepared with tannic acid, using Folin reagent and sodium carbonate

for reading. Condensed tannins determination followed procedures of Porter et al. (1986) with modifications. To the phenolic extracts was added butanol-HCl (95:5), followed by $FeNH_4S0_3$ 2% in HCl 2N and all samples were kept at 100 ºC for an hour, after that being cooled. Samples were read at 550 nm in a spectrofothometer and condensed tannins were expressed as leucocyanidin equivalents per kg of dry matter.

2.5 Histochemical Test With Vanillin-HCl

Three seeds of each parental were sectioned in 5-10 μm slices and colored with vanillin 10% solution in ethanol HCl (1:1), according to Broadhurst and Jones (1978). Materials were visualized with light microscope Zeiss Axiovert 35.

2.6 Water Uptake

Water uptake was determined for each genotype by adapting the procedures of Bushey et al. (2000), using three replicates. Samples were kept at 27 ± 1 ºC for eight hours. Every 30 min seeds were paper dried and weighted. Water uptake was expressed as the moisture content of seeds for each time point. The experiment was repeated three times.

2.7 Genotyping and Evaluation of Segregating Populations

Genotypes for each parents and for segregating populations derived (SAF x SAB and P-152 x DN) were all evaluated by visual analysis and also by colorimeter measures. Color and shininess groups were defined accordingly and genotypes based on loci *Asp* and *J* were defined.

2.8 Validation of Phenotypic Classes by AFLP Markers

A series of AFLP analyses was performed in order to validate phenotypic classes established on the basis of visual and colorimeter analyses. Plants of each material were grown in greenhouse and fresh new leaves were collected, frozen and grounded in liquid nitrogen. DNA was extracted according to Doyle and Doyle (1990), with modifications. Samples for the parents and bulks of progenies $F_{2:3}$ of the same phenotypic class were composed. The DNA of three progenies of each class was bulked and next prepared for fingerprinting. AFLP procedures were performed according to Vos et al. (1995), with modifications. Digestions were performed with 200 ng of DNA with *EcoRI* and *MseI*, and samples were ligated to specific adaptors. Pre-amplifications were performed with *EcoRI-A* and *MseI-A* primers and for selective amplifications 50 *EcoRI-N/MseI-N* (N = one, two or three selective bases) 50 primer combinations were used. Amplified samples were separated in polyacrylamide gels 6% under constant 40 W. Silver staining was performed according to Creste et al. (2001).

2.9 Statistical Analyses

Colorimeter data (*L*a*b** system measures) were analyzed through Shapiro-Wilk test (p < 0.05) in Statistix 8 (Analytical Software, Tallahassee) for normality test. Frequency distribution classes of the data were generated and *L*a*b** significances were evaluated through F-test and compared with Tukey (*p < 0.05*). Grouping of variables and association to phenotype (color and brightness) was also evaluated by principal component analysis (PCA), using CANOCO for Windows version 4.5 (Biometris, Plant Research International, The Netherlands). Interaction between brightness and colors was analyzed in a two-factor statistic design with F test (*p < 0.05*) and means were compared with Tukey (*p < 0.05*). Seed water uptake and cell length (SEM, LM) were compared with F-test followed by Tukey (*p < 0.05*) comparison. All analyses were performed with Statistix and PAST (Hammer et al., 2001). Segregation data were submitted to a chi-square test with the hypothesis that *Asp* controls seed coat shininess in the progenies derived from SAF x SAB and P-152 x DN. Genetic similarity analyses of AFLP profiles for the parents were performed with Bionumerics version 6.1, using Jaccard´s similarity index. Clustering analysis was performed based on UPGMA (Unweighted Pair-Group with Arithmetic Averages).

3. Results

3.1 Asp and J Genotyping

In first place, we visually compared our parent materials to the testers for seed coat genes, so we could define their genotypes. Comparisons with Bassett testers for *J* and *Asp* allowed the genotyping of the parents Puebla-152 (Mexican line with black and shiny seed coat), Diamante Negro (CNPAF/EMBRAPA, with black and dull seed coat) and the landrace Serro Azul (variants Serro Azul Brilhante and Serro Azul Fosco, respectively, with shiny and dull seed coat) for these loci. P-152 and SAB have shiny seed coat and regular color distribution with no change in corona zone, presenting *J Asp* genotype. DN and SAF have dull seed coats but a slight shiny aspect and therefore were classified as *J asp* (Figure 1).

3.2 Color and Shiness Assessments

After visual scoring, color and brightness measurements were taken to determine whether or nor there were differences between the genotypic classes. Seeds were categorized according to color and seed coat shininess, since color properties interfere with many variables studied such as $L*a*b*$ measures and tannin content. Genotypes P-152 and DN are black but differ for brightness. P-152 has shiny seed coat while DN is dull. SAB is brown and shiny while SAF is gray and dull. $F_{2:3}$ and $F_{3:4}$ progenies of each cross segregated for shininess and also colors, as shown later in segregation analysis. P-152 x DN cross generated only black progenies but segregating for shininess (SBL – Shiny Black and DBL – Dull Black). SAF x SAB differed both in colors and shininess, being grouped in shiny or dull brown (SB or DB) or shiny or dull gray (SG or DG), based on visual analysis (Figures 1 and 2).
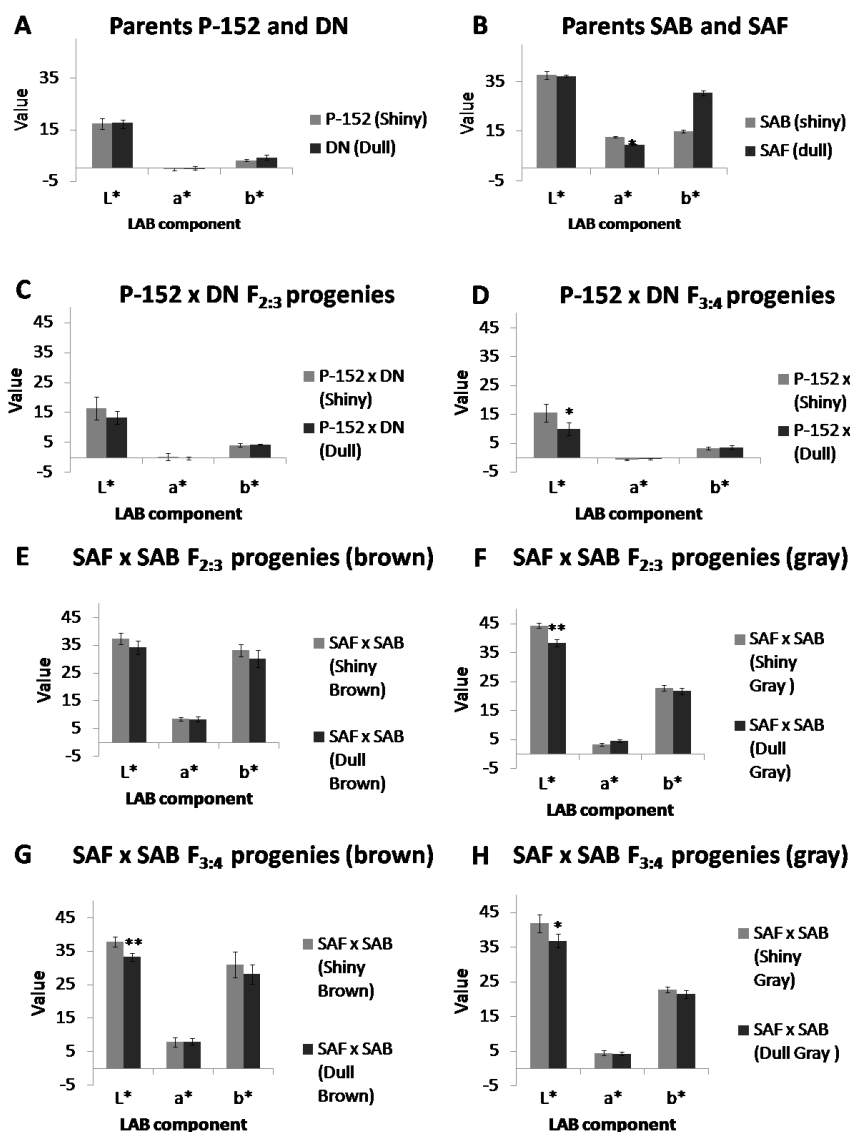

Figure 2. $L*a*b*$ measures obtained by colorimeter Minolta Chroma Meter CR-300 for common bean landrace Serro Azul, with variants Serro Azul Brilhante (SAB) and Serro Azul Fosco (SAF), the line Puebla-152 (P-152) and the cultivar Diamante Negro (DN)

Components $a*$ and $b*$ are for colors and the $L*$ component was attributed to shininess. $L*a*b*$ measures for the parents SAB and SAF and P-152 and DN (A and B) and for progenies $F_{2:3}$ and progenies $F_{3:4}$ of the cross P-152 x DN (C and D). Measures for progenies $F_{2:3}$ and $F_{3:4}$ of the cross SAF x SAB were separated by colors and shininess (E, F, G and H). We detected differences ($p < 0.05$) for the $L*$ component when $a*$ and $b*$ color components did not differ. *: indicates differences at $p < 0.05$. **: significance at $p < 0.01$.
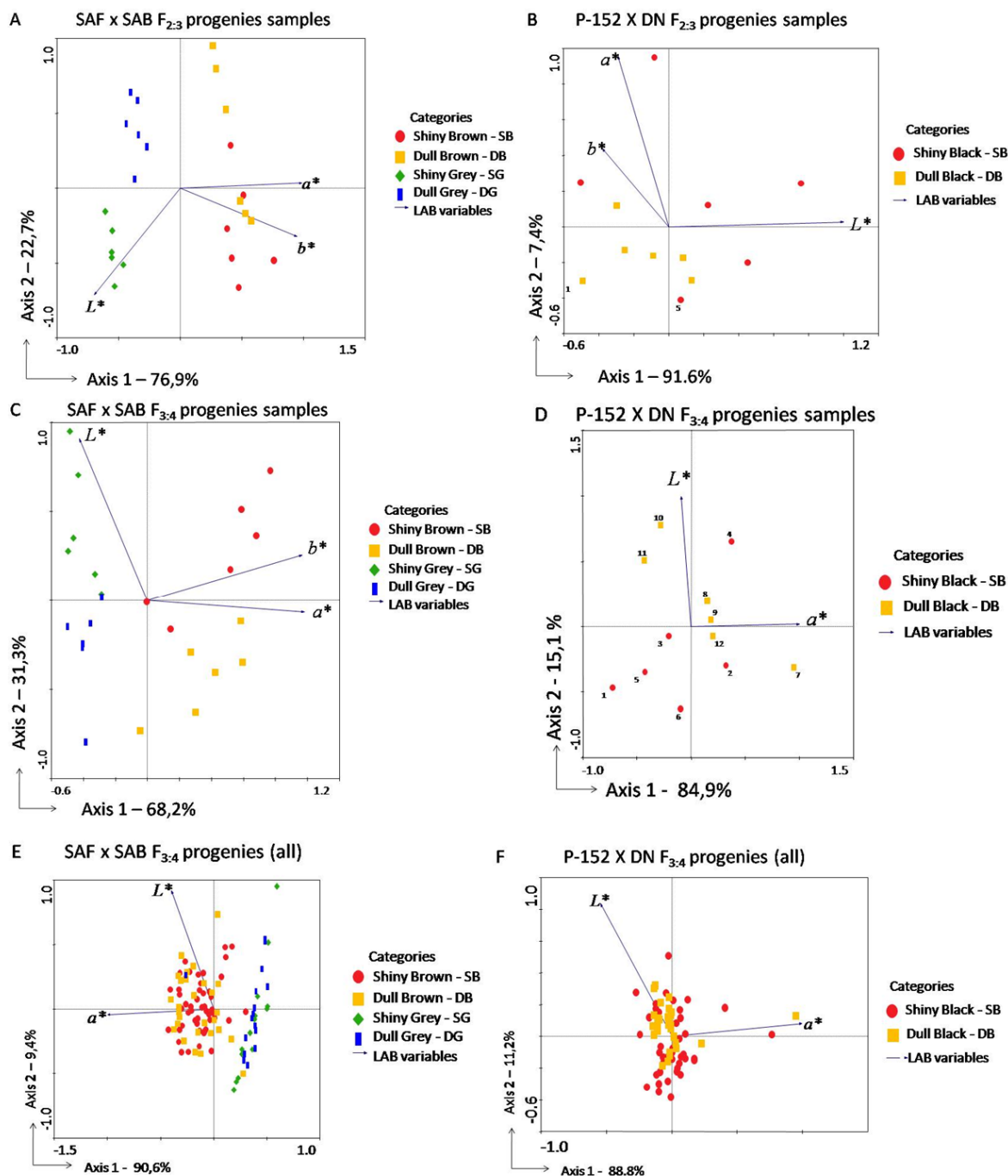
Figure 3. Principal Component Analysis (PCA) for $L*a*b*$ measurements of seed coats of progenies derived from crosses of common bean genotypes contrasting for seed coat shininess

Components $a*$ and $b*$ are for colors and the $L*$ component was attributed to shininess. A, B, C and D show $L*a*b*$ measures for progenies $F_{2:3}$ and $F_{3:4}$ of the crosses SAF x SAB and P-152 x DN using random samples of the progenies. E and F show the complete PCA profile, considering all $F_{3:4}$ progenies of these crosses.

Measures of the $L*a*b*$ system validated those classes (Figures 2 and 3). $L*$ and $a*$ fitted normal distribution but $b*$ did not in both crosses (SAF x SAB – $L*$: $p = 0.68$; $a*$: $p = 0.69$; $b*$: $p = 0.00$; DN x P-152 – $L*$: $p = 0.09$; $a* = p = 0.50$; $b*$: $p = 0.00$), according to Shapiro-Wilk test. We could not detect difference between SAB ($L* = 37.7$; $a* = 12.8$; $b* = 15.0$) and SAF ($L* = 37.4$; $a* = 9.7$; $b* = 30.6$) in the shininess $L*$ component, since $a*$ and $b*$ were significantly different and so $L*$ interacted with those. Also the parents P-152 and DN did not differ for $L*$ and $a*$, except for $b*$.

Nevertheless, among the progenies of both crosses (SAF x SAB and DN x P-152) we detected differences in the L^* component (Figure 2). Progenies $F_{2:3}$ and $F_{3:4}$ of P-152 x DN with dull seed coats presented lower L^* value than the shiny. The same was observed for the four classes (BS, BD, GS, GD) of the progenies of SAF x SAB. L^* values were lower in BD and GD. But comparisons for shininess could only be done when color component a^* and b^* did not interfere.

PCA analyses showed two components which explained at least 90% of the total variance (Figure 3). However, PCA only showed clear difference in brightness for the $F_{3:4}$ progenies. An interaction analysis (two-factor design) showed a significant interaction (F = 4.38, $p < 0.05$) between color and shininess, showing a^* and b^* interact with L^*. This makes difficult to distinguish shiny from dull seed coats and requires prior color differentiation. Therefore, only those materials with similar color (a^* and b^* not different between samples) can be distinguished based on L^*.

3.3 Microscopy Analyses

Previous studies have shown similar results as we are here, but this analysis was extended to more bean lines. As previously shown by Beninger et al. (2000), we verified that the line *asp* BC$_3$ 5-593 presents a rough textured seed coat when compared to line 5-593. Moreover, our work introduces the SEM profile of *j* BC$_3$-593 (Figure 4), not previously reported for this type of study. This tester shows just a slight roughness on the seed coat surface, almost imperceptible if compared to *asp* BC$_3$ 5-593. SAB and P-152 have an even surface, typical of shiny seed coats. SAF and DN are noticeable rough textured, as expected (Figure 4).

Palisade epidermis length measures were similar to Beninger et al. (2000), which have first measured it as a method to compare seed coats with differences in brilliance. The tester *j* BC$_3$ 5-593 presented no difference ($p < 0,11$) to 5-593 in length, but *asp* BC$_3$ 5-593 had lower length ($p < 0.02$). SAF presented lower value than SAB ($p < 0.02$). Non-significant difference was detected between P-152 and DN. They do not hold the same origin but were the parents of the study population (Table 1).

3.4 Water Uptake

Some reports have shown that seed coat shininess interferes with water uptake. Water uptake differed among the genotypes. However, the origin of each material had to be considered when evaluating this parameter. Different selection methods have taken genotypes to have divergent phenols or proteins concentrations, those who may interfere significantly with water imbibition. Overall, 5-593, *j* BC$_3$ 5-593 and *asp* BC$_3$ 5-593 showed a similar tendency of water uptake (Figure 5A). Even so, 5-593 had lower absorption rate than *asp* BC$_3$ 5-593. Interestingly, *asp* BC$_3$ 5-593 and *j* BC$_3$ 5-593 showed no difference (Figure 5A).

SAF had much higher water absorption than SAB (Figure 5A). Since these variants have the same origin, their comparison is appropriate. DN and P-152 had different results but P-152 absorbed water faster than DN, even the former being the shiny one. However, P-152 and DN have different origins, so comparisons are limited.

Table 1. Palisade epidermis length for genotypes contrasting for seed coat shininess (loci *Asp* and *J*)

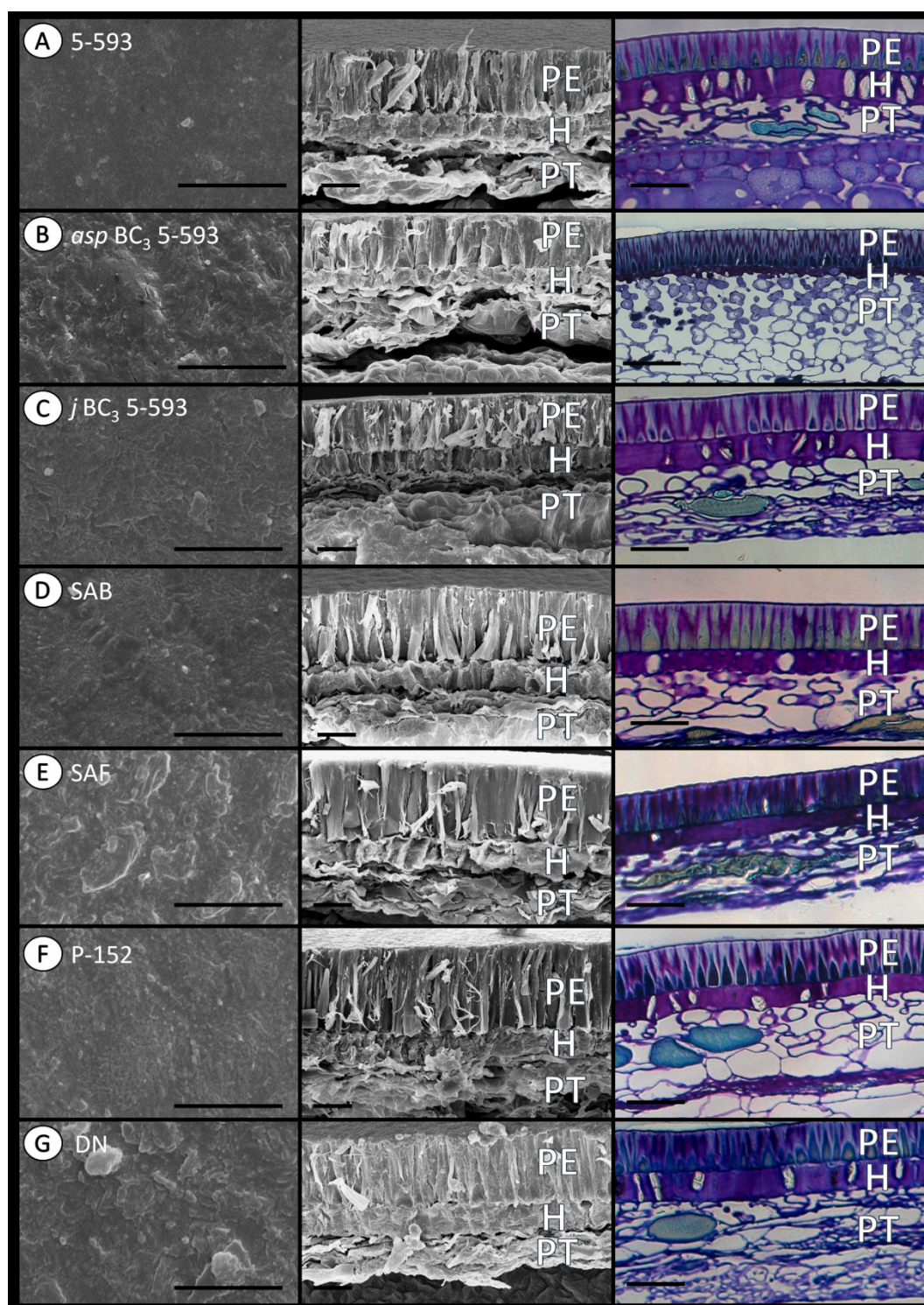| Cultivar	Genotype	Palisade epidermis length (μm)
5-593	*J Asp*	38,8 ±3,2 a
asp BC$_3$ 5-593	*J asp*	33,2 ±2.2 b
j BC$_3$ 5-593	*j Asp*	35,2 ±2,6 a
Serro Azul Brilhante	*J Asp*	40,8 ±2,0 a
Serro Azul Fosco	*J asp*	37,0 ±2,4 b
Puebla-152	*J Asp*	45,7 ±4,5 a
Diamante Negro	*J asp*	42,3 ±3,4 ab

Figure 4. Surface and cross section of the seed coat of different common bean genotypes contrasting for seed coat shininess

PE: palisade epidermis, H: hypodermis, PT: parenchymatous tissue. A. Line 5-593 (*J Asp*) shows a regular and even surface and palisade epidermis is longer than testers. B. Tester *asp* BC$_3$ 5-593 (*J asp*), with rough textured surface. C. Tester *j* BC$_3$ 5-593 (*j Asp*), somehow regular surface. D. Serro Azul Brilhante (SAB) (*J Asp*) and F. Puebla-152 (*J Asp*) also present regular and even surface while E. Serro Azul Fosco (SAF) and G. Diamante Negro (DN) have rough textured and irregular surfaces. Scale bars indicate length of 10μm on the first column. On the second column, bars representing 20 μm and on the third column 100 μm.
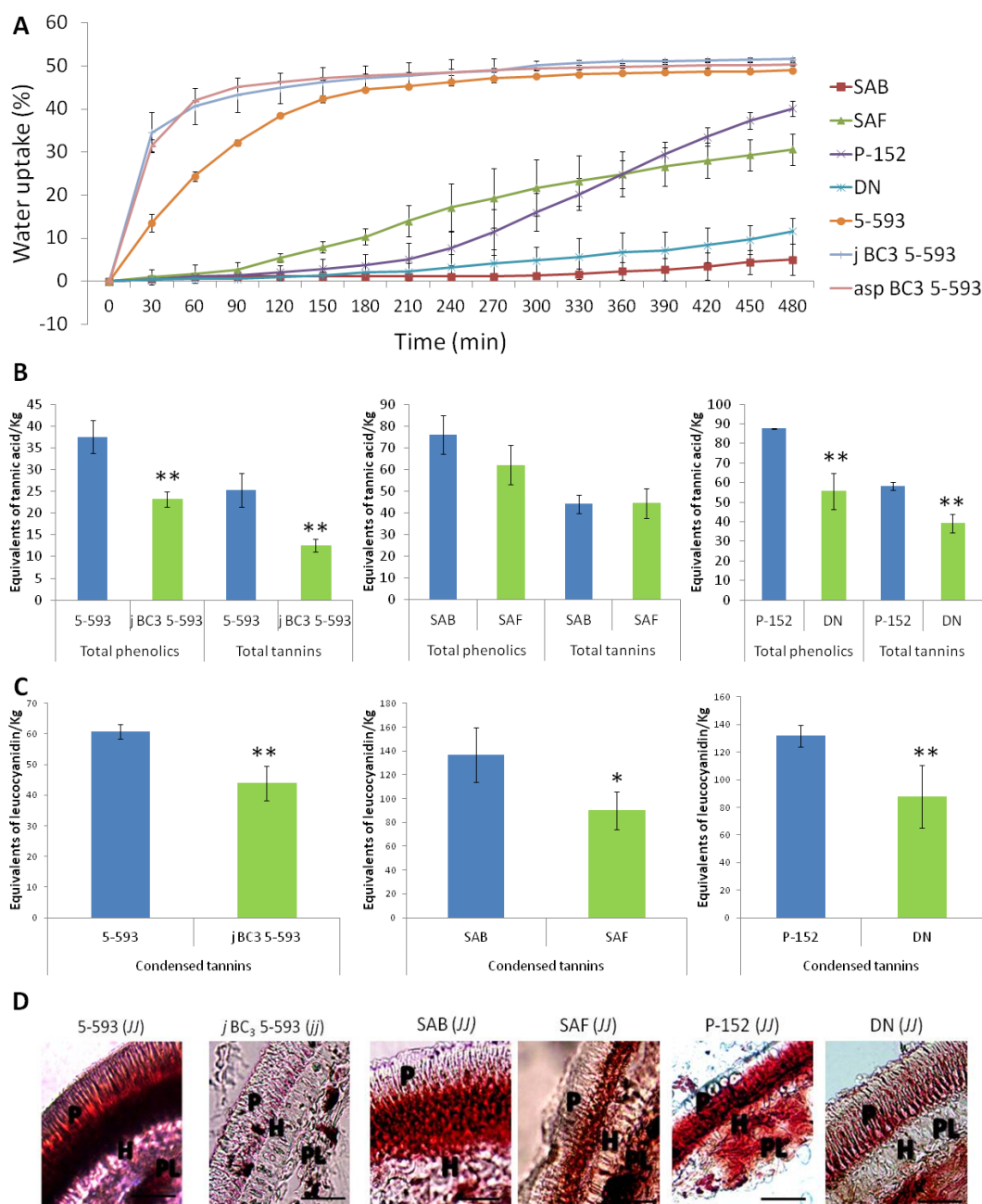
Figure 5. Water uptake profile, total phenols, total tannins, condensed tannins and vanillin-HCl test

A. Water uptake for genotypes 5-593 (*J Asp*), *j* BC₃ 5-593 (*j Asp*), *asp* BC₃ 5-593 (*J asp*), Serro Azul Brilhante (SAB), Serro Azul Fosco (SAF), Puebla-152 (P-152) and Diamante Negro (DN). B. Total phenolics and total tannins for genotypes. C. Condensed tannin amount for genotypes. D. Cross sections of seed coats colored with Vanillin-HCl for the genotypes contrasting for *J* locus. PE: palisade epidermis, H: hypodermis, PT: parenchymatous tissue. Bars represent 30 μm.

3.5 Phenols, Condensed Tannins and Histochemical Test

High levels of total phenols, tannins and condensed tannins were detected in SAB, SAF, P-152 and DN, compared to 5-593 and *j*BC₃ 5-593 (Figures 5B and C). We detected higher amounts of condensed tannins in the shiny 5-593 than in *j* BC₃ 5-593 (Figure 5C). The same was observed for the shiny parents SAB and P-152 compared to the dull SAF and DN.

Vanillin HCl test revealed that genotypes *J* presented strong red color along the seed coat, but weak staining was

observed for *j* BC₃ 5-593 on the palisade epidermis (Figure 5D).

3.6 AFLP Categorization

Genetic similarity was evaluated among all parents of the two crosses. SAB e SAF were close with 93.5% of similarity and DN and P-152 had 91% (Figure 6A). Bulk results for categories of colors and shininess (DN x P-152: BLS and BLD; SAB x SAB: BS, BD, GS and GD) showed the separation between crosses (Figure 6B and C). Overall, shiny bulks for DN x P-152 cross were somehow separated from the dull bulks. The bulks of SAF x SAB progenies were separated by color, but were mixed for the seed coat shininess trait. So, no specific bands to shiny seed coat were detected.
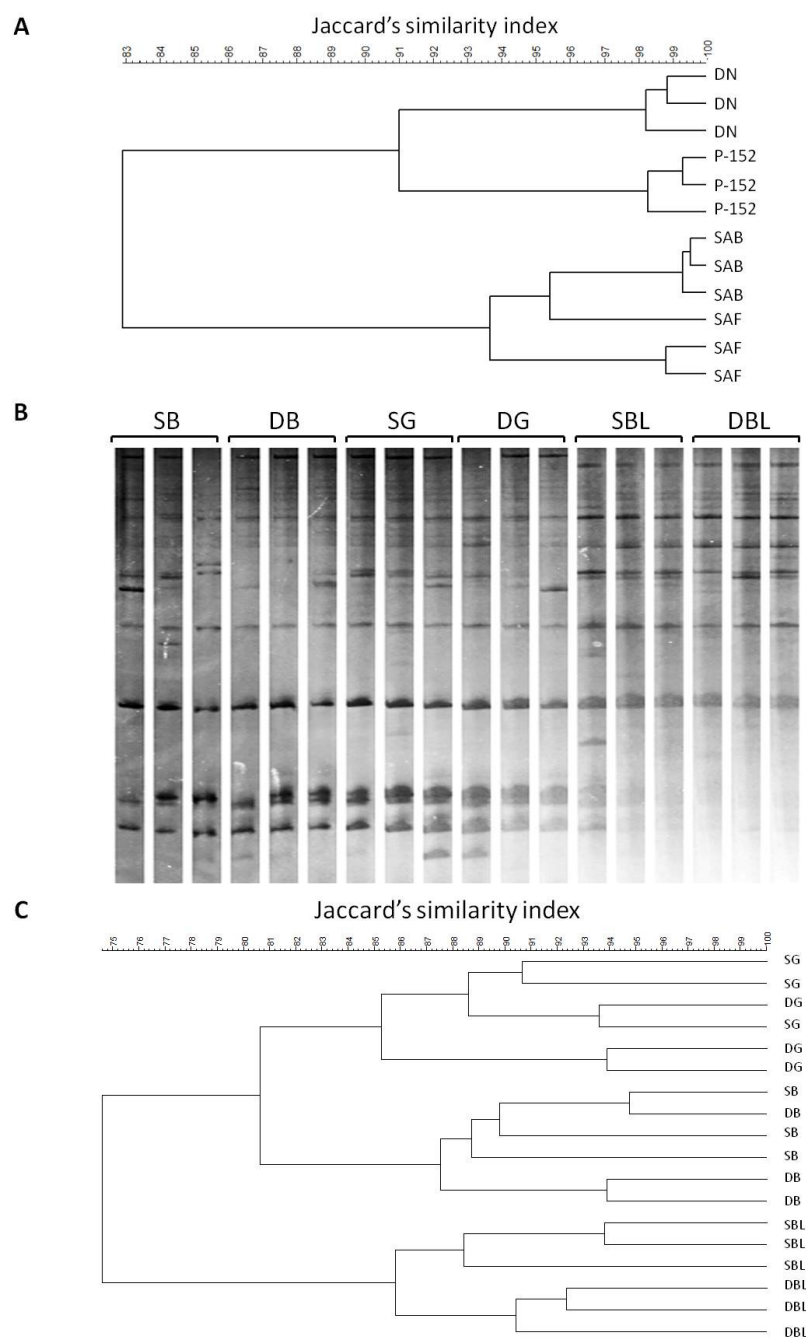

Figure 6. A. Similarity cluster of parents Serro Azul Brilhante (SAB), Serro Azul Fosco (SAF), Puebla-152 (P-152) and Diamante Negro (DN). B. and C. AFLP profile and clustering of progenies F₂:₃ from crosses SAF x SAB and P-152 x DN, showing different color and shininess classes. SB: Shiny Brown, DB: Dull Brown, SG: Shiny Gray, DG: Dull Gray, SBL: Shiny Black; DBL: Dull Black

3.7 Segregation Analyses

Since seed coat shininess is maternally inherited, when SAB was crossed to SAF, all F_1 seed presented dull seed coat. DN was crossed to P-152, so that all F_1 seeds were shiny. $F_{1:2}$ seeds of the two crosses were all shiny.

$F_{2:3}$ progenies segregated at the rate 3 shiny to 1 dull (Table 3). In $F_{3:4}$ generation, the segregation was of 5:3. Table 3 shows the observed and expected proportions of the two crosses between SAB and SAF (SAF x SAB; SAB x SAF) and P-152 x DN in generations $F_{2:3}$ and $F_{3:4}$. Results showed that a single gene with a dominant allele controls shininess in this varieties, which by all analyses performed (previous sections) showed to be *Asp*. *J* did not segregate, since all parents were *J_*.

Table 2. Segregation analysis for seed coat shininess in common bean crosses

Crosses and generations	Observed phenotype		Expected proportions	Expected phenotype		χ^2	*p*
	Asp (shiny)	*Asp* (dull)		*Asp* (shiny)	*Asp* (dull)		
Dull Serro Azul (SAF) x Shiny Serro Azul (SAB)							
SAF x SAB							
$F_{1:2}$	121	0	1:0	121.00	0.00	0.000	1.00
$F_{2:3}$	84	37	3:1	90.75	30.25	2.008	0.16
$F_{3:4}$	356	213	5:3	355.62	213.38	0.002	0.96
SAB x SAF							
$F_{1:2}$	163	0	1:0	163.00	0.00	0.00	1.00
$F_{2:3}$	122	41	3:1	122.25	40.75	0.001	0.97
$F_{3:4}$	479	263	5:3	463.75	278.25	1.337	0.25
Puebla-152 (P-152) x Diamante Negro (DN)							
$F_{1:2}$	75	0	1:0	75.00	0.00	0.00	1.00
$F_{2:3}$	54	21	3:1	56.25	18.75	0.360	0.55
$F_{3:4}$	258	160	5:3	261.25	156.75	0.108	0.74

4. Discussion

Here we showed a series of analyses aimed at the understanding of differences at the morphological, biochemical and molecular level for seed coat shininess in common bean. Our results were consistent with data already published, but also revealed new prospects on its evaluation. Furthermore, this was an initial step to resume a trait that has been neglected for breeding purposes, but may naturally be important for defense mechanisms of the seed or the plant itself. Besides, phenolic compounds accumulated on the seedcoat may represent important nutrition facts for human or animal consumption, so that this trait could have its roll to improve it.

4.1 The L* Variable to Show Differences in Shininess

Colorimeter data allowed the quantitative differentiation between the shiny and the dull seed coat, but were limited by color influence (components *a** and *b**) (Figures 2 and 3). Seeds must have similar *a*b** values so that these will not interact with *L** for shininess differentiation. Given the huge variety of seeds of common bean and considering a germplasm collection, it seems unviable. However, segregating populations can be somehow distinguished as we have shown by PCA analysis (Figure 3). Despite that, colorimeter measures show to be suitable for color distinguishing (PCA analysis).

4.2 Visual Scoring and Comparison to Testers to Show Differences Between Asp and J

J and *Asp loci* phenotyping can still be easily done based on characteristics previously shown by Bassett (1992, 1996, 1998a, 1998b, 2007). The phenotypes of the testers reveal clear differences among the alleles for *Asp* and *J* (Figure 1). Furthermore, we added some new characteristics that should be considered when genotyping seeds for those *loci* as presented on Table 2. *Asp* gives the real shiny aspect to the seed coat while *J* only intensifies it. On the contrary, *asp* gives the real dull aspect of the seed coat while *j* has no effect on brightness intensity.

Table 3. Main characteristics associated to *Asp* and *J* loci, involved in seed coat shininess in common bean

Genotype	Phenotype	Characteristics
J Asp	Shiny seed coat	Intense shininess but depends on the genotype, color and pattern.
		Even seed coat surface as showed by Scanning Electron Microscopy.
J asp	Dull seed coat	Dull seed coat due to *Asp* but with a slight shiny aspect, especially on hilum border, due to the dominant form of *J*.
		Rough textured seed coat surface due to *asp*.
j Asp	Intermediate shiny[†]	Seed coat shininess intensity is reduced by *j* effect. Seed coat color is irregular and corona presents partial coloring or totally uncolored.
		Regular and even seed coat surface.
j asp[‡]	Dull seed coat	Dull and rough textured seed coat is expected.

[†] It does not mean that intermediate shiny genotypes have *j Asp* genotype, because even *J Asp* genotypes present variations in shininess intensity.

[‡] That genotype was not studied, but was inferred.

4.3 Palisade Epidermis Length and Roughness of Seed Coat

It has been described that dull seed coats present a rough textured surface when compared to the shiny ones (Beninger et al., 2000, Lamprecht, 1940). It was clearly shown when cuticle was removed from the seed coat of the tester *asp* BC$_3$ 5-593 and the line 5-593 (Beninger et al., 2000). In this study, cuticle was not removed but similar pattern was observed (Figure 3). However, the reason for the structural change caused by *asp* is still unclear. In one attempt, seeds were treated with xylol for cuticle removal of the cultivar Shiny Crow (*Asp_*). When not treated with xylol, cuticle was regularly distributed over the seed coat. Treated seeds presented an uneven profile, similar to what is naturally observed for dull seed coats. Therefore, *Asp* could be involved in maintaining uniformity of seed coat surface (Bushey et al., 2002).

Palisade epidermis length was also clearly lower in dull seed coat genotypes than in shiny ones (Table 3, Figure 4). This may have a role in limiting the amount of anthocyanins accumulated on seed coat. Prior study has shown that then *asp* BC$_3$ was compared to 5-593, the former presented only 55% of delphinidin-3-glycoside, 58% of petunidin-3-glycoside and 55% of malvidine-glycoside, the three main kinds of anthocyanins found by HPLC in the seed coat (Beninger et al., 2000). Therefore, palisade epidermis length and seed coat roughness degree (rough or even) are good variables for *Asp* phenotyping. However, they require preparation of materials for microscopy.

4.4 Water Uptake

Water uptake assays are quicker to perform than microscopy analyses. With the method used here, we found Serro Azul Brilhante (shiny seed coat) absorbed low amount of water, the opposite of Serro Azul Fosco (dull seed coat) (Figure 5A). The presence of shininess on the seed coat has been associated to low water absorption. Using similar methods, Bushey et al. (2000) verified that among five cultivars, Raven (dull seed coat) presented the highest percentage of absorption (45%), while Shiny Harblack and Shiny Crow absorbed only around 1%. The same work also showed greater loss of anthocyanins in dull (Raven: 93%) than in shiny seeds (Shiny Harblack: 51%). Shiny seeds treated with xylol seem to uptake more water than untreated. Regular cuticle deposition on the seed coat influences water absorption potential (Bushey et al., 2002), and may be associated to *Asp*.

These results are consistent with SEM analyses. Higher palisade epidermis thickness and regular cuticle deposition on seed coat surface are limiting factors for water entry and distribution. Consequently, imbibition and germination are also delayed in shiny seeds, as observed for SAB (data not shown).

Nevertheless, Puebla-152 and Diamante Negro presented intermediate levels of water imbibitions in the five first hours and the values did not differ between those (Figure 5A). This highlights that is not just *asp* that is involved in water absorption, but other loci maybe conditioning it. Besides, SAB and SAF are all landraces with the same origin. P-152 and DN, otherwise, were originated from other sources and selected for different purposes. Then, it is possible that alleles of one or more genes had been selected, altering the pattern and potential for water absorption. It can be reinforced since the tester *j* BC$_3$ was not different from *asp* BC$_3$ 5-593 (Figure 5A), which means it is not just *asp* that may be involved in water uptake. Besides, water absorption rate could be due the

structural organization of the seed coat, not necessarily depending on a specific group of genes but on physiological processes. So water uptake seems not to rely only on *Asp* and cannot be used directly as a phenotypic indicator for this trait.

4.5 Condensed Tannins and Vanillin Test

All genotypes (SAB, SAF, P-152 and DN) were *JJ*, so detection of condensed tannins was expected in all samples (Figure 5C). However, in *j* BC$_3$ 5-593 condensed tannins were also detected even if in lower amount than in 5-593. This could be explained first by the lack of specificity of the method (Hummer & Schreier, 2008), since a lot of chemical compounds can interfere with their detection, as kaempherol (Beninger & Hosfield, 1999). Otherwise, in the bean cultivar Prim (*jj*), widely consumed in Chile, proanthocyanidins were not detected even with Thin Layer Cromatography (TLC). Samples applied in silica gel plates were colored with vanillin and were not stained in red as would be expected for the method (Beninger & Hosfield, 1999).

Then, here we applied vanillin test and further observation in optic microscope to verify if this method was more suitable. Interestingly, weak staining was observed on the palisade epidermis of *j* BC$_3$ 5-593 (Figure 5D), unlike for the others *J* (5-593, SAB, SAF, P-152, DN), which were all strongly red stained. These observations suggest histochemical tests and TLC are suitable for a qualitative evaluation of the presence of condensed tannins.

Despite the amount of condensed tannins was higher in shiny cultivars (5-593, SAB, P-152) (Figure 5C), as detected by acid butanol method, it is not possible to associate shininess to those compounds, given that *Asp* maybe involved in structural organization on the seed coat (Beninger et al., 2000). It is possible that other genes interacting to *J* are involved in condensed tannin accumulation on the seed coat. Recent works have suggested an oligogenic model for inheritance for such phenols (Caldas & Blair, 2009; Díaz et al., 2010). Moreover, epistatic and additive effects for colour genes have been shown (Yuste-Lisbona et al., 2014). However, these models still need to be better studied.

4.6 Segregation Analysis and AFLP Categorization

Segregation analyses showed that shininess is controlled by a single gene in the crosses evaluated (SAF x SAB; P-152 x DN) which is *Asp* (Table 3). *J* does not vary between parents and therefore in all progenies. Previous reports also showed the expected pattern regarding to *Asp* gene, as a F$_2$ population derived from BTS ('Black Turtle Soup') x Peru (Koenig & Gepts, 1989); a F$_5$ generation of the cross Dorado x Xan 176 (Miklas et al., 2000); and 104 RILs in F$_7$ of the cross Xana x Cornell 49242 (Pérez-Vega et al., 2010).

Since phenotypic classes were well defined, AFLP procedures were performed in bulks of progenies, to detect possible markers associated to seed coat shininess. But no specific fragment was detected even if categories for the traits shininess and color were considered (DN x P-152: BLS and BLD; SAB x SAB: BS, BD, GS and GD) (Figure 6B and C). UPGMA clustering showed separation among progenies of the cross P-152 x DN but not for SAF x SAB.

4.7 Implications of the Results

Our results showed different ways to phenotype and determine genotypic classes related to seed coat shininess in common bean. We discussed different properties conditioned by the genes *Asp* and *J* and how they could be distinguished by visual, spectrofotometric, microscopic, biochemical and molecular approaches. Moreover, these results have important implications on common bean selection and breeding.

These results suggest that seeds with dull seed coat are not necessarily absent from proanthocyanidins because the dominatn allele of *J* can be present even if the seed is dull (*asp asp*). This may be undesirable for commercial purposes, since proanthocyanidins are involved in seed hardening (Leakey, 1988) or seed coat darkening during storage (Junk-Knievell et al., 2008; Elsadr et al., 2011). Besides, some studies suggest that a high percentage of condensed tannins in some animal diets negatively affects digestibility of proteins and carbohydrates. This reduces weight gain and nutrient absorption efficiency (Li et al., 1996; Barahona et al., 1997; Barros et al., 2012; Lorenz et al., 2014). Legume tannins precipitate proteins and form complexes to iron in gastrointestinal lumen, reducing the availability of minerals from the grains (Mira et al., 2002). In common bean cultivars selection, if one desires to obtain varieties which produce seeds with small amounts or no condensed tannins, it is necessary to make selection on the *J* locus.

Nevertheless, recent evidences pointed monomers of proanthocyanidins as beneficial for human health as anti-carcinogenic, antioxidant and anti-inflammatory (Beninger & Hosfield, 2003; He et al., 2008; Zhao et al., 2010; Dzomba et al., 2013). In common bean, not only tannins, but another phenolic compound may even help in reducing inflamnation caused by certain diseases such as colitis (Zhang et al., 2014). Based on recent models of quantitative inheritance of condensed tannins (Caldas & Blair, 2009), selection could be directed towards low

levels of tannins to compensate for health and commercial benefits.

Another topic of huge concern is cooking time, which is often a reason for rejecting shiny cultivars, since they usually take longer to cook properly (Elia et al., 1997). Considering the shiny line P-152 of the present study, that absorbed water considerably fast, and the landrace SAB, that almost did not absorbed water, it is likely that selection of some other traits is also beneficial for cooking time. Then, not necessarily shiny cultivars are hard to cook. Some current examples can be mentioned with the Brazilian cultivars Ouro and Vermelho, which are shiny and easily cooked. These ones, then, would be similar to P-152. This highlights once more the importance of considering an oligogenic model to explain such variation, so that not only the shiny seed coat may be related to cooking time.

With all those explanation we could not forget the essentiality of the seed coat shininess since it can be a way for the seed or the plant to protect itself against insects and pathogens. Insects and pathogen resistance seem to be important traits associated to *Asp* but further work is necessary to explore the genetic and biochemical mechanisms involved.

Therefore, one selecting for pathogens and insects resistance should be looking at the *Asp* locus while other one, interested in digestive properties, could be dealing more with the *J* locus. Breeders should consider effects of both genes for taking decisions on what to select.

5. Conclusion

We showed several methods that can be used to phenotype and genotype for seed coat shininess in common bean. Visual scoring by comparison to testers previously developed for seed coat genes *Asp* and *J* can easily help to define the genotype of one cultivar. The L^* variable is able to distinguish shiny from dull seed coats, but no variation in color should be present. Palisade epidermis layer is a very good indicator of variability introduced by *Asp*, as other works had previously suggested. Water uptake is a fast method to such analyses, but other mechanisms beyond of genetics may explain its variation, which make difficult a direct association to seed coat shininess. Condensed tannins are more appropriate to phenotype and genotype for the *J locus*. The vanillin-HCl test showed to be a good method to qualitatively trace for the presence of condensed tannins. Such methods are suitable to phenotype and genotype populations of common bean and, therefore, select whether or not the shiny seed coat should be present.

Acknowledgements

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