CHARACTERIZATION OF POST HARVEST SEED COAT DARKENING AND CONDENSED TANNIN ACCUMULATION DURING SEED COAT DEVELOPMENT IN COMMON BEAN

(*Phaseolus vulgaris* L.)

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By

Hanny Elsadr

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ABSTRACT

Seed coat biochemistry and colour are highly variable in common bean (*Phaseolus vulgaris*) (syn. dry bean). Genetic studies of dry bean seed coat chemistry and colour have important implications in breeding efforts for improving nutrition and seed quality for consumer acceptance. The results of this thesis detail the phenotypic and genotypic characterization of seed coat post harvest darkening (PHD) in parents and progeny of crosses among them as well as the phenotypic characterization of seed coat condensed tannin (CT) accumulation in five genotypes of bean.

Seed coat PHD represents a problem for producers and consumers of several different market classes of dry bean. There are three post harvest darkening phenotypes: (i) non-darkening (ND), (ii) slow darkening (SD) and (iii) regular darkening (RD). The inheritance of PHD was determined by evaluating 28 populations derived from crosses between RD, SD and ND genotypes. Results suggest that at least two major, unlinked genes control the PHD trait in dry bean. Recessive epistasis with three phenotypic classes best explains the segregation ratios observed in populations from crosses between SD and ND parents. One gene, *J*, is responsible for whether a bean will darken and seeds of plants that are *jj* do not darken at all. Another gene, *SD*, influences the rate a seed coat will darken with seed from *sdsd* individuals darkening more slowly that those with the dominant *SD* allele.

Quantitative evaluation of seed coat PHD demonstrated that there was a wide range of darkening within any given PHD phenotype. Crosses made between the ND x ND cross class resulted in F_2 progeny that were all ND, however, a wide range of seed coat background colours was noted in the progeny. In several of the crosses made between ND x RD and SD x RD classes the resulting F_1 progeny were all RD; however, a wide range of RD phenotypes were observed in the F_2 progeny. These phenotypes are not likely due to quantitative trait loci (QTL) associated with the PHD trait, but rather a result of other chemical reactions occurring in the seed coat. Condensed tannins (CT; syn. proanthocyanidins), kaempferols, polyphenol oxidase (PPO) and possibly other compounds or enzymes may be interacting and causing this quantitative range within any given genotype as a function of environmental variability, genotype and their interaction. CT have been associated with PHD but are not responsible for the major difference

between RD and SD lines. They may, however be responsible for the quantitative nature of the phenotype.

Condensed tannins can be harmful or beneficial to human health and the environment depending on the amount present and where it is found in the plant. Manipulating the production, accumulation and form of CT in the seed coat of dry bean would be beneficial to bean producers, consumers and breeders. This experiment quantitatively and qualitatively evaluated differences in patterns of CT accumulation in the seed coats of five genotypes of dry bean which exhibited low, medium or high concentrations of CT in their seed coats at maturity. Condensed tannin content was assessed from seeds harvested every other day from 6 – 40 days after flowering (DAF) using a modified BuOH-HCl assay. Results illustrated that CT accumulated as early in low CT genotypes as in high CT genotypes. CT content stabilized after 14 DAF in low CT genotypes. By contrast, CT content peaked then leveled off 30 DAF in moderate and high CT genotypes. A reduction in CT content in the higher CT lines was observed in the final stages of seed development.

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CHAPTER 1: INTRODUCTION

Common bean (*Phaseolus vulgaris*) (syn. dry bean, snap bean) constitutes one of the "Three Sisters" that allowed the foundation of Native American agriculture (Dudman, 2005). Common bean is essential to the well being of more than 1 billion people worldwide; the seeds are a nutritional source of energy, carbohydrates, sugars, fat, protein, water, pantothenic acid, folate, calcium, iron, magnesium, zinc, vitamin A and C and dietary fiber (Dudman, 2005). Many developed countries, such as the United States of America (USA), depend on bean as a source of food, but moreover as a source of income. The USA is the primary producer and exporter of beans worldwide followed by Argentina (Galván et al., 2006).

Beans are eaten fresh (as a vegetable) or allowed to mature and dry for cooking purposes. Although dry beans, when cooked extensively, appear as a mass of undifferentiated paste; many people around the world take pleasure and care in selecting beans of a particular size, shape and colour. Common bean, therefore, retains importance in a diversity of cultures and traditions.

Common bean is cultivated in almost every country of the world. However, germplasm is believed to have evolved from two major gene pools, which represent the centres of diversity of bean; one originated in the Andes (Southern Peru, Bolivia and Northern Argentina) and the other in Mesoamerica (mainly Mexico) (Galván et al., 2006). These regions gave rise to beans with a diverse array of visual characteristics such as colour, shape and size, which form the foundation of the market class marketing system known today (Gepts and Debouck, 1991). Red, light red and white kidney, cranberry, black, navy, great northern and pinto are some of the more popular market classes of common bean grown in North America.

Canadian common bean production increased dramatically in the late 1980s and early 1990s, mainly due to the development of day neutral and early maturing varieties adapted to high latitude environments (Agriculture and Agri-Food Canada, 2010). Production continued to increase until recently; common bean production has decreased over the past few years and has now stabilized near 220 – 260 thousand tonnes per year (Agriculture and Agri-Food Canada, 2010).

Seed coat colour and biochemical components are highly variable in common bean. The nutrition profile and visual appeal of common bean represent the primary means by which consumers select beans in the marketplace. Development of reliable evaluation techniques for seed coat post harvest darkening (PHD) and condensed tannin (CT) concentration and content is

important for enhancing the efficiency of common bean breeding efforts for seed coat quality and nutritional traits. Identifying the genetic and biochemical controls of these traits will help lay a foundational background for future research and breeding efforts.

CHAPTER 2 : LITERATURE REVIEW

2.1 Common bean (*Phaseolus vulgaris* L.)

2.1.1Taxonomy

The taxonomic classification of beans is as follows: Kingdom Plantae, division *Magnoliophyta*, class *Magnoliopsida*, order *Fabales*, family *Fabaceae*, subfamily *Faboideae*. Within the subfamily *Faboideae* several genera commonly used as crops exist including *Phaseolus*, *Vigna*, *Cicer*, *Pisum*, *Lens*, *Lablab*, *Vicia*, *Glycine* and *Lupinus*. *Phaseolus* is the most commonly utilized of these genera worldwide.

Phaseolus can be divided into 4 cultivated species: *P. acutifolius* (tepary bean), *P. coccineus* (runner bean), *P. lunatus* (lima bean) and *P. vulgaris* (common bean) (Maynard et al., 1997). The thesis focuses on *P. vulgaris*.

2.1.2 Botany

Common bean is a self pollinated, dicotyledonous, frost sensitive, herbaceous, warm season annual legume (Biggs et al., 1997). It is a diploid species with 11 chromosome pairs (2n = 22). Beans have two main types of growth habits: bush and pole. Bush type beans grow to about 30 cm in height; pole type beans can grow several meters high and usually require trellising for support (Maynard et al., 1997). All varieties of common bean have alternate, green or purple leaves that are divided into three oval, smooth-edged leaflets (Wortmann, 2006). Each leaf is between 6 – 15 cm long and 3 – 11 cm wide. Flowers are white, pink, purple or a combination of these colours and are about 1 cm long. These flowers can produce green, yellow, black or purple pods that are about 8 – 20 cm long by 1 – 1.5 cm wide and contain between 4 – 6 seeds each. Most varieties of *P. vulgaris* produce smooth, plump, kidney-shaped beans that can be up to 1.5 cm long. Seeds of *P. vulgaris* range widely in colour and are often mottled in two or more colours (Wortmann, 2006).

2.1.3 Utilization and Importance

Common bean is a socially and economically important crop worldwide (Broughton et al., 2003). Many people, mainly in developing countries, depend on these beans as their primary

staple food. Many developed countries, such as the United States of America, depend on beans as a source of income.

Common bean is used in many different regions of the world for a variety of purposes. The seeds are consumed as a food and used for feed. Depending on the preferences of inhabitants in a region and the variety of bean cultivated, they can be directly eaten fresh (as a vegetable) or dried for cooking purposes. Many people around the world, particularly in Mexico, South America and Eastern and Southern Africa, use beans in traditional dishes (Broughton et al., 2003). Many bean producers also use the straw generated by bean plants as a source of feed and they themselves eat the green leaves as a leafy vegetable (Singh, 2000).

Beans are also able to fix atmospheric nitrogen into plant usable ammonium in agricultural systems. The ability for bean plants to fix nitrogen is particularly important in regions where industrial nitrogen is unaffordable or unavailable. In such circumstances farmers inter-crop or rotate beans with other crops, such as corn or grain crops, in order to reduce their dependence on industrial nitrogen. This technique is also an environmentally friendly practice because less inorganic nitrogen is required.

2.2 Seed Coat Post Harvest Darkening

2.2.1 Economic Importance of Seed Coat Post Harvest Darkening

Consumers take great pride in selecting beans of optimal colour, thus, seed coat colour plays an important role in price determination for both marketers and consumers. Bean seeds are graded using colour sorters in processing plants; discoloured beans are considered anomalies and represent waste or a price loss for sellers. The Canadian Grain Commission (CGC) uses colour as a primary means of classifying beans for quality control. The CGC's classification system has 6 grades with the highest grade being Extra No. 1 Canada and the lowest being No. 4 Canada. Beans with uniform size and good natural colour are classified as Extra No. 1 Canada while beans with off-colours are classified as No. 4 Canada. Economic value drops as quality decreases.

Seed coat post harvest darkening (PHD) is a phenomenon that causes a gradual change in the colour of the seed coat of some market classes of beans during storage. At least three PHD phenotypes exist: (i) non darkening (ND), (ii) slow darkening (SD) and (iii) regular darkening (RD).

RD beans tend to have lower standards of quality as they appear older compared to ND and SD beans. Therefore, as time passes in storage, ND and SD beans command a price premium above that of RD beans. This is particularly important for bean producers who store their seed for a period of time prior to sale. The longer the beans are stored the less profitable they are for the producer. Export producers can take advantage of this market by growing beans that are ND or SD and storing them until the RD beans locally grown in Mexico or in South America have darkened; the ND and SD bean producers can then enter these markets with lighter coloured ND and SD beans that are more profitable.

Nutritional quality, flavour and cooking duration of beans, however, should not be compromised for visual appeal. PHD is a means by which consumers can identify seed age. The advantage of RD beans is that older beans can be easily identified by consumers, thus, consumers can target their purchases to fresh beans that have better culinary quality and are more flavourful and fast cooking. By contrast, ND beans can remain on grocery store shelves or in producer or processor storage containers for years and consumers would not be able to tell them apart from freshly harvested beans by visual appearance alone. This represents a disadvantage to consumers who are paying a premium for a product that has unfavourable culinary characteristics.

Common bean is not the only crop that darkens as it matures and is stored. Several other legume crops also experience losses in value as a function of PHD of the seed coat. Faba bean (*Vicia faba* L.) cultivars with seed coats that contain CT darken significantly upon storage at room temperature for 6 months, while tannin-free cultivars have seed coats that do not darken at all (Crofts et al., 1980). Most market classes of lentil (*Lens culinaris* Medik.) have seed coats that naturally darken both with age and upon exposure to prolonged periods of high humidity (Kulkarni et al., 1989; Mills et al., 1999; Vaillancourt and Slinkard, 1985). As with common bean, aged lentils also have reduced seed viability, experience increased leakage of solutes, have

undesirable textures upon cooking and become unmarketable (Kulkarni et al., 1989; Mills et al., 1999). Reyes-Moreno et al. (2000) and Sefa-Dedeh et al. (1979) found that kabuli chickpea (*Cicer arietinum* L.) and cowpea (*Vigna unguiculata*) also have seed coats that naturally darken upon exposure to prolonged storage at high humidity.

2.2.2 Slow Darkening Beans

Seed coat PHD in certain market classes of common bean, namely pinto, cranberry, carioca or bayo beans, represents a problem for producers and consumers. Seed coat darkening is enhanced by prolonged storage under high light and moisture conditions and darker coloured pinto beans tend to be less desirable compared to lighter coloured beans (Junk et al., 2007). It is evident that SD beans are more desirable in the marketplace than RD beans and that breeding efforts should continue to pursue beans with seed coats that darken at a slow rate. This objective will benefit both producers, as they will get a premium for their beans, and consumers who will be more content with the product they purchase.

Currently, there are several means of controlling seed coat darkening in common bean; the most commonly used methods involve cultural practices, which are timely and costly. Understanding the genetic control of common bean PHD may offer solutions for a more practical and efficient means of limiting PHD. Furthermore, the effects of genetic improvement on PHD are permanent and far less costly in the long term.

Seed coat darkening may be reduced through the use of a variety of cultural practices including controlled atmosphere environments, radiation and harvest time. However, these practices tend to be impractical, expensive and sometimes ineffective. For instance, Sartori (1982) found that pinto beans stored in a controlled, nitrogen enriched atmosphere at 24 ºC and 75% relative humidity were far less subject to darkening after 6 months of storage compared to pinto beans stored for only two months in natural atmospheric conditions. However, the pinto beans exposed to both types of atmospheric conditions were hard, hard-to-cook (HTC) and had undesirable flavour.

Post harvest darkening in common bean is a function of temperature, humidity and light intensity and duration. Therefore, choosing the correct storage conditions may improve the shelf life of beans (Sartori, 1982). Storing beans in the dark at sub-zero temperatures with low

humidity may help reduce the effects of PHD. A freezer would suffice to meet all of these conditions, however, freezers are costly to purchase and operate.

Common beans stored at low moisture content darken more slowly than those stored at high moisture content (Park and Maga, 1999). One method of reducing seed moisture content is to rapidly heat beans at high temperatures followed by quick cooling (Aguilera and Steinsapir, 1985). Heating beans to 105 ºC in metal drums for three minutes followed by rapid cooling was less effective at reducing PHD when compared to heating beans at 70 ºC in an oven for one hour followed by rapid cooling (Aguilera and Steinaspir, 1985). However, it was noted that the beans heated to 105 ºC were softer when canned when compared to those beans heated at 70º C. An example of a practical use of this strategy can be found in Manitoba, where common bean producers use large bins to store their beans. Beans that are stored in bins earlier in the season tend to experience higher initial bin temperatures before the onset of the cold winter when compared to beans that are stored in the same bins later in the season (Mills et al., 1995). Therefore, delaying storage of beans in bins until the onset of the cooler days of late fall may help improve germination and cooking quality (Mills et al., 1995) and possibly reduce the rate of PHD.

Common bean seed coats darken in the field, prior to harvest, as a function of direct sunlight and heat penetrating through the pods. Gonzalez et al. (1982) found that harvesting beans at the semi-dry stage, when 50% of the pods on the plant are yellow, followed by 24 hours of exposure to temperatures of 27 ºC improved the colour, flavour and general acceptability of canned beans when compared to beans that were harvested at the dry stage (100% of the pods dry).

2.2.3 Evaluating Seed Coat Post Harvest Darkening

Junk et al. (2007) developed three protocols, namely the ultraviolet (UVC) light protocol, greenhouse protocol and cabinet protocol, for determining seed coat darkening in pinto beans. Although all three methods successfully distinguished RD from SD beans, the UVC light protocol was the quickest and most consistent and reliable.

In using the UVC light protocol only one side of each seed coat was exposed to UVC light, and thus, if the seed was to darken it would only darken on one side. The other side of the

seed remains, theoretically, unaffected. The UVC light protocol was most effective at maintaining this distinct separation of darkening between exposed and unexposed sides.

Junk et al. (2007) quantitatively phenotyped beans exposed to UVC light using a Hunter Lab colorimeter. This colorimeter was used to determine **L**, **a**, and **b** values for each seed coat surface. The **L** values (0 – 100) represented the degree of seed coat lightness; the greater the **L** value the lighter the seed coat. The **a** value represented the relative red-green hues of the seed coat; the larger the **a** value the redder the seed coat. The **b** values measured the relative yellow-blue hues; the larger the **b** value the yellower the seed coat.

Although quantitative phenotyping is accurate and effective, qualitative evaluation of PHD is more practical and economical. Post harvest darkening phenotypes can be easily distinguished from one another through qualitative evaluation in most populations, however, some populations are more variable and require quantitative phenotyping.

2.2.4 Seed Coat Post Harvest Darkening Chemistry

Seed coat PHD is believed to be controlled by changes in biochemistry associated with seed coat aging during storage. Phenols and phytates may be associated with PHD (Gesto and Vazquez, 1976). There is, however, a discrepancy as to whether total phenols increase or decrease with seed aging. Some studies demonstrated that an increase in phenols is associated with seed aging (Gesto and Vazquez, 1976); however, other studies have shown that total phenols decrease as seeds age (Hincks and Stanley, 1986; Martin-Cabrejas et al., 1997). Differences in extraction procedures were likely the cause of this discrepancy. Methanol soluble phenolic acid esters, free phenolic acids and cell wall bound phenolic acids are reported to increase over time as seeds age (Srisum et al., 1989). An increase in free phenolic acids was also reported over time in cotyledons; however, methanol soluble phenolic acid esters in the cotyledons decreased over time (Srisum et al., 1989). In another study, phenolic acids and phenols bound to cell walls or pectin in cotyledons were found to increase over time, while methanol soluble esters decreased over time (Garcia et al., 1998).

Results of a vanillin assay suggested that there was a significant difference in condensed tannin (CT) concentrations between SD and RD pinto beans both in aged and non-aged seed coats (Beninger et al., 2005). This study however, was conducted on parental genotypes and not

on populations segregating for PHD. A BuOH-HCl assay conducted on RD and SD recombinant inbred lines (RILs) from a segregating population from a cross between CDC Pintium x 1533-15 showed that there was no correlation between the concentration of CT and the degree of PHD (Marles et al., 2008a). They found that while CDC Pintium and 1533-15 had different levels of CT in some environments, CT was not associated with PHD in the segregating population. Although no direct correlation was found between CT and PHD, both Marles et al. (2008a) and Martin-Cabrejas et al. (1997) found that CT concentrations increased in the seeds of beans as they aged. By contrast, Stanley (1992) demonstrated that CT concentrations decreased in seeds as they aged. Sievwright and Shipe (1986) illustrated that over time and at high temperatures, CT concentrations increased during initial storage, plateaued and then decreased. This change is thought to be the result of a modification in the structural form of the seed coat rather than actual changes in seed coat CT concentrations. It is likely that the apparent increase in CT concentration during the initial stages of seed aging was due, in part, to small molecular weight non-tannin compounds developing into CT, whereas the apparent decrease in CT at later stages of seed aging is thought to result as a function of CT binding to macro molecules (Sievwright and Shipe 1986; Stanley, 1992).

In the study conducted by Beninger et al. (2005), kaempferols were found to decrease over time in the seed coats of CDC Pintium, a RD pinto bean. They also found that CDC Pintium had higher levels of seed coat kaempferol as compared to both aged and non-aged seed of 1533-15, a SD bean. These findings indicate that kaempferol concentrations may be contributing to the degree of PHD such that higher levels of kaempferol cause beans to darken more rapidly. SD RILs from a cross between CDC Pintium x 1533-15 had low kaempferol while RD RILs from this cross had high kaempferol (Marles et al., 2008a).

Peroxidase (POD), a phenol, and polyphenol oxidase (PPO), a phenol oxidizing enzyme may contribute to PHD. POD and PPO activity were found by Moura et al. (1999) to be elevated in RD beans when compared to SD beans both prior and after eight months of storage. However, in both RD and SD beans, overall POD and PPO activity decreased during this eight month storage period. Similarly, in a study conducted by De Oliveira Rios et al. (2002) elevated levels of phenols, PPO activity and POD activity in beans were noted after prolonged storage. Finally, Marles et al. (2008a) found PPO activity to be higher in RD than SD lines.

2.2.5 Genetic Improvement of Seed Coat Post Harvest Darkening

There is little published research on the improvement of PHD in common bean and the mechanisms underlying the genetic control of common bean PHD have yet to be determined. Previously, researchers believed that PHD was controlled by only one locus, the *J* locus, and that two alleles governed the inheritance of this trait (Basset, 1996). However, recent work at the U of S suggests that a one gene model with simple inheritance is unlikely in some populations or that several alleles are governing the inheritance of PHD in these populations.

2.2.5.1 The *J* Locus

According to the single *J* locus model, two alleles, *J* and *j*, control PHD in common bean; expression of the recessive allele *j* resulted in seed coats that are far less subject to PHD when compared to seed coats that have the dominant *J* allele (Basset, 1996; Prakken 1974; Prakken 1977 a and b). However, preliminary phenotypic evaluation of populations segregating for SD suggested that the existence of a second locus may be responsible for the degree of PHD.

J is a colour gene among many other colour genes that interact epistatically to form the various colour patterns observed on bean seed coats (McClean et al., 2002). The dominant *J* allele was found to be, at least in part, responsible for promoting the production of CT because CT were only produced in genotypes containing at least one dominant *J* allele (Feenstra, 1960; Beninger and Hosfield 1999a and b; Beninger et al., 1999 and 2000).

Two genotypes, namely PI 608686 and PI 608688, were classified as genetic tester lines for *jj* (USDA Genebank). PI 608686 and PI 608688 have black and purple seed coats, respectively; therefore, their darkening phenotypes cannot be determined. A second set of genotypes, namely KVxUI-1 and KVxUI-6 were developed to have a pinto pattern and are both putative *jj* tester lines developed by Dr. J. Meyers at Oregon State University (OSU), and therefore, are ND pinto beans and can be easily phenotyped for PHD. Utilization of these genotypes in crosses made for inheritance studies may be useful for identifying the genetic basis of the PHD trait.

2.2.5.2 Genotype, Environment and Their Interaction

The seed coats of some varieties of common bean tend to darken significantly within a few months after harvest while others tend to darken at a much slower rate (Junk et al., 2007). Although the genotype can influence the rate of darkening of the seed coat, storage conditions also influence the rate of darkening (Junk et al., 2007). Junk et al. (2007) tested genotype x environment (G x E) interactions as related to PHD across different outdoor (field) environments and between indoor (greenhouse) and outdoor (field) environments. There were significant G x E interactions associated with seed coat darkening of freshly harvested beans before being subjected to a darkening protocol. These differences could be due to variability across environments such as maturity date, harvest time and sunlight intensity. There was also a significant G x E interaction between beans grown in outdoor versus indoor environments. Beans grown in outdoor environments were lighter in seed coat colour than those grown indoors. These findings may be due to differences in harvest times relative to maturity as a function of variability in light, temperature and humidity (Junk et al., 2007). By contrast, once the seed coats were subject to the UVC protocol and were split into either SD or RD classes, there was no significant G x E interaction; SD beans were easily distinguished from RD beans upon darkening. Junk et al. (2007) concluded that PHD should not be classified based on freshly harvested seed that has not been subject to a darkening protocol. Rather, the pinto bean industry and breeders should darken the beans before making phenotypic judgments.

2.2.5.3 Simple Inheritance and Seed versus Seed Coat Generations

Junk et al. (2007) evaluated a series of populations made from both RD x SD and SD x SD parents. All populations of the SD and RD crosses segregated 3 RD: 1 SD in the F_2 and the $F_{2:5}$ RILs segregated 1RD:1SD with a distinct bimodal distribution of the L-value colour. This segregation pattern suggested that PHD followed simple inheritance and was controlled by a single gene with RD being dominant over SD. The phenotypes from the F_2s of populations made between 1533-15 (SD) x Saltillo (SD) crosses did not segregate for L-value colour. This suggested that both of the SD genotypes investigated were controlled by the same gene. However, Junk et al. (2007) noted that the frequency distribution was normally distributed within

the distinct SD PHD class, therefore, indicating that the SD trait may be influenced by modifying genes and environmental effects.

Assessing PHD in segregating populations can sometimes lead to confusion as seed coats are always one generation behind the generation of the embryo. Therefore, seeds evaluated for F_1, F_2, F_3, F_4 and F_5 seed coats actually represent the F_2, F_3, F_4, F_5 and F_6 embryo generations. This is because the DNA that codes for seed coat PHD of the harvested seed is not expressed until the seed is grown out in the following generation.

2.3 Seed Coat Biochemistry

2.3.1 Nutritional Benefits of Common Bean

Although plant breeders have exploited morphological trait variability in the past, there is a trend now towards biofortification as well. Biofortification involves the development of food crops rich in available micronutrients such as vitamin A, zinc, iron, calcium and folate. These compounds are beneficial to human health and there are growing concerns over human health in many regions of the world.

Biofortification can be feasibly conducted without compromising agronomic productivity of staple food crops (Nestel et al., 2006). Many micronutrients are essential to human health and are of growing importance in many regions of the world. For many vegetarians and inhabitants of Latin America and Africa, common bean is an important source of proteins and minerals (especially iron) (Moraghan et al., 2002). The distribution of iron between the seed coat and embryo of common bean is a genotypic trait (Moraghan, 2005). Moraghan et al. (2002) demonstrated that iron in the seed coat of common bean is more bioavailable than iron found in the embryo.

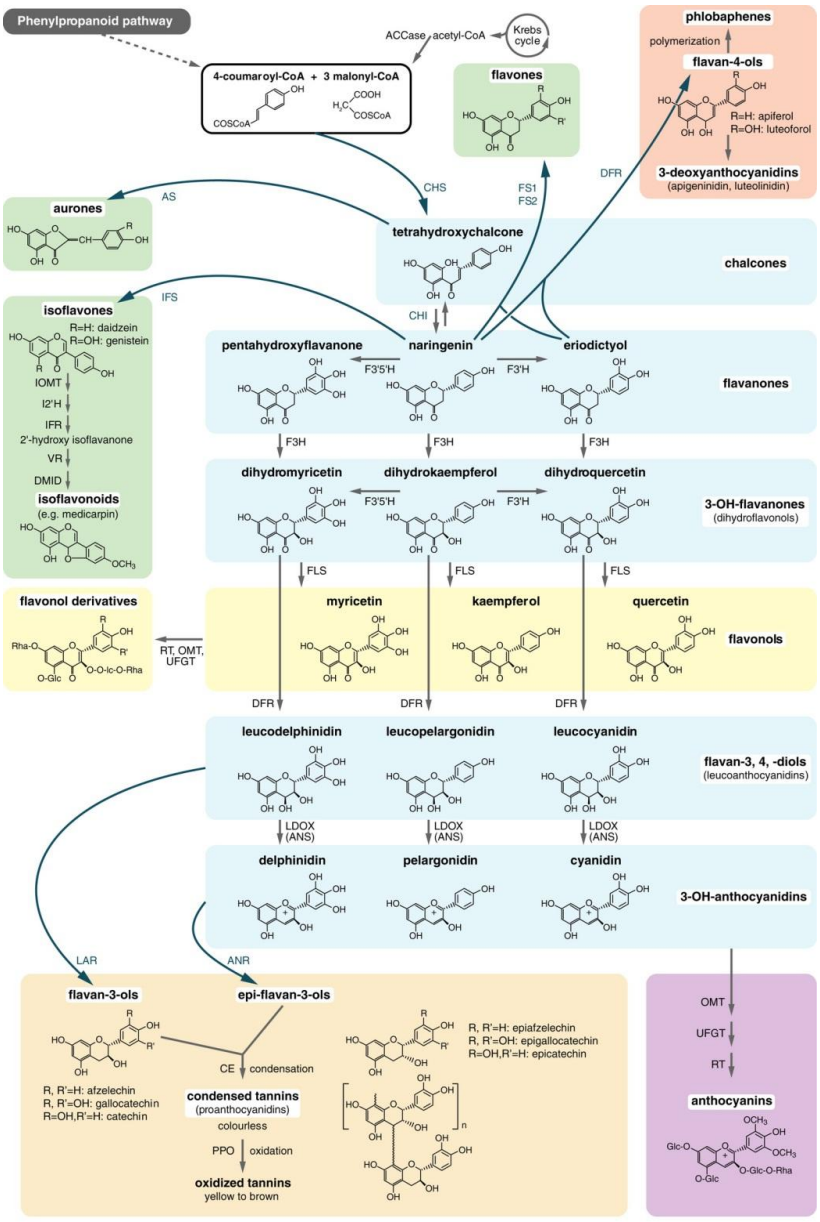
Researchers have recently become interested in understanding the genetic control of iron in legumes such as soybean and common bean (Moraghan, 2005). Moraghan (2005) illustrated that both environmental and genetic factors influence the degree of iron concentration in both the embryo (including cotyledons) and seed coats of common bean. Beans with white seed coats tended to have lower iron in their seed coats (approximately 5%) compared to beans with black seed coats (approximately 40%) (Moraghan, 2005); the bioavailability of iron in white compared to black beans is unknown.

2.3.2 Polyphenolics, Phenylpropanoids and Flavanols

Polyphenolic compounds (polyphenols) are a group of phytochemicals found in plants that are characterized by the presence of two or more aromatic rings, each bearing at least one aromatic hydroxyl and connected with a carbon bridge (Beecher, 2003). Polyphenolics are subdivided into hydrolysable tannins and phenylpropanoids such as lignin, flavanoids and CT (Dewick, 1995). Each of these subdivisions is derived from the secondary plant metabolic pathway known as the shikimate pathway (Dewick, 1995).

Phenylpropanoids are plant-derived organic compounds that are biosynthesized from phenylalanine. These organic compounds function as a defence against herbivores, microbial attack and other sources of injury. Phenylpropanoids protect plants from UV light, serve as structural components of cell walls, aid plants in molecule signalling and are components of pigment molecules (Hahlbrock and Scheel, 1989). Due to their diversity of functions, phenylpropanoids are vital compounds in most plants. The phenylpropanoid pathway consists of a series of steps that regulate the production of compounds such as CT (Hahlbrock and Scheel, 1989). Throughout this biochemical pathway there are a series of enzymes and metabolites produced, each of which may play a role in the regulation of CT in seeds and seed coats. One of the important biochemical pathways of CT development is the flavanoid pathway.

The flavanoid pathway is a branch of the phenylpropanoid pathway and is vital for the production of CT, anthocyanins and flavanols (Figure 2.1). Flavan-3-ols and epi-flavan-3-ols, which are both necessary for the production of CT, are biosynthesized from this pathway. Condensed tannin, anthocyanins and flavanol biosynthesis share common steps in the flavanoid pathway and the genetic and biochemical mechanisms of this pathway have been characterized in several plant species (Shirley et al., 1992; Holton and Cornish, 1995; Boss et al., 1996; Winkel-Shirley, 2001).



Lepiniec L, et al. 2006.
Annu. Rev. Plant Biol. 57:405–30

Figure 2.1 The Flavanoid Pathway. Adopted from Lepiniec, et al. 2006.

2.3.3 Condensed Tannins

Condensed tannins are polyphenols that contain hydroxyl (OH), carboxyl (COOH) and other suitable functional groups that form strong complexes with proteins, minerals and other macromolecules (Bate-Smith and Swain, 1962). CT are clear and colourless and result from the

condensation of flavan-3-ol units (Xie et al., 2003). Upon oxidation, CT turn brown resulting in a darkened background seed coat colour during storage (Debeaujon et al., 2001; Devic et al., 1999). CT polymers consist of 2 to 50 or more flavanoid units bound by carbon - carbon bonds (Hemingway and Karchesy, 1989). CT have molecular weights of 500 to 3000 U and can bind and precipitate proteins (Bate-Smith and Swain, 1962). CT are vital compounds in many plants because of their diversity of functions and can be present in leaves, stems, seeds, buds and roots and may help regulate tissue growth (Lees, 1993).

Most legumes, including bean, have seed coats that contain CT (Reed, 1995). CT content was found to be positively correlated with bean colour: red coloured beans contained the most CT while white coloured beans contain the least (Reed, 1995). This fact may suggest that CT play an important role in determining and regulating seed coat colour in common bean and other legume species.

It was once understood that CT have negative impacts on human health, however, it is now known that some CT are beneficial to human health while others have anti-nutritional values (Chung et al., 1998). Reddy et al. (1985) characterized the deleterious effects of tannins and grouped them as follows: depression of food/feed intake, formation of tannin complexes with dietary proteins and other food components, inhibition of digestive enzymes, increased excretion of endogenous proteins and effects on the digestive tract.

Foods rich in CT contain tannin chelated metal ions that are not bioavailable (Baynes and Batchwell 1990). Brune et al. (1989) suggested that the tendency of CT to form molecular complexes with iron reduces the bioavailability of iron in the gastrointestinal lumen of many mammals. Different forms of CT may cause varied effects on iron absorption in mammals (Brune et al., 1989). The wide range of forms associated with CT makes studying the effects of CT absorption of micronutrients in mammalian digestive systems difficult.

The seed coat of common bean can be highly variable in CT, ranging between 0.0 – 2.0%, depending on the species and cultivar of common bean as well as the colour of the seed coat (Reddy et al., 1985). In their review, Reddy et al. (1985) reported that bean varieties that were high in CT were of lower nutritional value than those with seed coats that were low in CT. The reduction in seed nutritional value is likely due to lowered protein digestibility as suggested by Hernandez et al. (1979) who fed human subjects black, red and white beans as their sole

source of protein. They found that humans fed the higher CT black and red beans had reduced protein digestibility compared to those fed white beans. In vitro and in vivo studies indicated that CT decreased protein digestibility (Reddy et al., 1985), which in turn, caused the inactivation of digestive enzymes making proteins insoluble. Therefore, the anti-nutritional value of beans high in CT is likely due to the ability of CT to bind certain proteins, both enzymatically and non-enzymatically, forming CT-protein complexes.

The anti-nutritional activity of CT can be reduced through soaking, de-hulling, cooking and germination of common bean (Reddy et al., 1985). However, some of these procedures can reduce other nutritional properties of beans through leeching of essential vitamins and minerals or by denaturing proteins (Reddy et al., 1985). Through classical breeding augmented with molecular tools such as marker assisted selection, the anti-nutritional properties of CT can be reduced by decreasing the relative proportion of CT in bean seeds by breeding for low CT bean varieties. However, the complete elimination of CT from bean seed coats may pose some difficulty to bean producers as CT serve many important functions in developing plants and seeds including protection against plant herbivores and disease, contribution to taste of many crops and they can act as a dietary antioxidant (Helsper et al., 1994; Gil et al., 2000). Condensed tannins play an important role as a natural fungicide against several disease organisms. Helsper et al. (1994) showed that CT-free near isogenic lines of faba bean (*Vicia faba* L.) were more susceptible to the foot rot pathogens, *Fusarium oxysporum* and *Fusarium solani*, as compared to lines containing CT.

2.3.4 Condensed Tannin Accumulation Studies

Currently there are no published studies on the accumulation of CT during seed coat development in common bean and little published work in other pulse crops. Grape (*Vitis vinifera*) is one species that has been studied for CT development, particularly during seed growth and development and fruit ripening. A reduction in the concentration and content of several polyphenols was noted during fruit ripening in the cultivar Cabernet Sauvignon and polyphenols were influenced by vine water status (Kennedy et al., 2000). Flavan-3-ol monomers, the basic building block of CT, were the most drastically affected polyphenol during berry ripening; this monomer de-cumulated rapidly as the berries ripened. The reduction of flavan-3-ol

is likely a result of the formation of CT through oxidative reactions, and therefore, although the monomers appear to have disappeared they have likely become part of CT polymers. These results illustrate that CT polymers accumulate while flavan-3-ol monomers de-cumulate during fruit ripening in grapes and that cultural practices such as watering regimes may be used to change the composition of CT and other polyphenols in grapes and other crops.

2.3.5 Condensed Tannin Genetic Studies

There is currently little published information on the genetic mechanism responsible for CT accumulation and binding in common bean and CT genetics in other pulse crops. The best understood system of CT genetics is in Arabidopsis (Nesi et al., 2001; Debeaujon et al., 2003; Bogs et al., 2007). Caldas and Blair (2009) conducted a quantitative trait locus (QTL) study that addressed the inheritance of seed coat CT and their relationship with seed coat colour and pattern genes in common bean. The study was conducted using three RIL populations from crosses between Andean and Mesoamerican genepools which were used to identify QTLs and for mapping STS markers associated with seed colour loci. In total 12 QTL on separate linkage groups in each population, which explained 10 – 64% of the phenotypic variation of CT, were identified. The Mendelian partly coloured pattern seed coat genes *Bip* and *Z* were found to be associated with loci on linkage groups B3 and B10. They also found that a QTL on linkage group B7 was associated with the *P* gene, the primary locus for colour expression in common bean. They concluded that inheritance of CT fits an oligogenic model and that QTL could be used to identify novel putative alleles at seed coat colour and pattern genes that control CT accumulation.

In soybean, four alleles at the *I* (inhibitor) locus control the absence, presence and spatial distribution of pigments in the seed coat (reviewed by Bemard and Weiss, 1973; Palmer and Kilen, 1987). Todd and Vodkin (1993) investigated the role of the *I* locus on development of CT in near isogenic lines of soybean. The lines they used had different allele combinations at this locus, and therefore, through the use of thin layer chromotography they were able to identify the allelic combinations that caused increased expression of CT. Todd and Vodkin (1993) found that yellow seeded soybean varieties, containing the dominant *I* allele, did not express CT in their seed coats. By contrast, black and imperfect black seeded soybean varieties, containing the

recessive *i* allele, had a significant amount of CT present in their seed coats. These findings indicate that the *I* locus may code for both seed coat colour and production of CT in soybean.

CHAPTER 3 : CHARACTERIZATION OF SEED COAT POST HARVEST DARKENING IN COMMON BEAN (*Phaseolus vulgaris* L.)

3.1 Introduction

Seed coat post harvest darkening (PHD) causes a gradual change in the colour of the seed coat of some market classes of bean during storage. For example, the seed coat of pinto or carioca beans often changes from a cream to a brown colour a few months after the seed has reached physiological maturity (Junk et al., 2007). Both genotypic and environmental factors can influence the rate and extent of PHD and darkening tends to occur more rapidly in environments prone to elevated temperatures, humidity and light duration and intensity (Park and Maga, 1999; Junk et al., 2007).

Junk et al. (2007) developed a protocol for determining the darkening phenotype of pinto beans using UVC light. This protocol allows for phenotyping to be carried out in several days rather than waiting several months for natural darkening to occur. There are at least three PHD phenotypes: (i) non darkening (ND), (ii) slow darkening (SD) and (iii) regular darkening (RD). Post harvest darkening of the seed coat in common bean is an undesirable characteristic that results in lost value to producers, exporters and vendors. Bean breeders have identified certain lines of common bean that darken slowly or not at all. There is an interest among North American bean growers to produce slow darkening beans, particularly for Central American markets. This objective will benefit both producers, as they may receive a premium for their beans, and consumers who will be more content with the product they purchase.

Studies have described genotypes associated with the PHD trait. According to Prakken (1974), the *J* locus is associated with PHD in common bean: the recessive *jj* produces seed coats that are far less subject to PHD compared to seed coats with the dominant *J* allele. The recessive genotype also, however, suffers from reduced levels of pigmentation in the colour of the seed coat pattern.

Initial observations of segregation among F_2 progeny of a cross between 1533-15, a SD pinto from the Crop Development Centre (CDC), and PI 608686 and PI 608688, both with the genotype *jj* (USDA Genebank), suggested a two-gene model rather than simply a new allele at *J*.

The hypothesis of this study was that a second locus (*sd*) controls the rate of seed coat PHD in common bean. This study was carried out with the objective of investigating how this second locus interacted with *J* using classical genetic techniques.

3.2 Materials and Methods

3.2.1 Parental Genotypes

A total of ten genotypes of common bean and the progeny of crosses derived from them were intercrossed to determine the genetic relationships among the different PHD phenotypes (Table 3.1; Figure 3.1).

Table 3.1 Parental genotypes, their sources of origin, market class and PHD phenotypes and putative genotypes.

Parental Line	Source of Origin	Market Class	PHD phenotype	Putative Genotypes
CDC Pintium	University of Saskatchewan	Pinto	RD	JJSDSD
Bayo Florida	Peru	Bayo	RD	JJSDSD
1533-15	University of Saskatchewan	Pinto	SD	JJsdsd
SDIP	University of Idaho	Pinto	SD	JJsdsd
Bayo Mochica	Peru	Bayo	SD	JJsdsd
Wit-rood	The Netherlands	Cranberry	ND	jjSDSD
KVxUI-1	Oregon State University	Pinto	ND	jjSDSD
KVxUI-6	Oregon State University	Pinto	ND	jjSDSD
PI 608686	Pullman USDA Genebank	Black	ND	jjSDSD
PI 608688	Pullman USDA Genebank	Dark Grey	ND	jjSDsd & jjSDSD

Figure 3.1 Parental PHD phenotypes (from left to right and down: CDC Pintium, Bayo Florida, 1533-15, SDIP, Bayo Mochica, KVxUI-1, KVxUI-6, Wit-rood, PI 608686 and PI 608688).

CDC Pintium is a RD, type 1 pinto bean developed and released in 1999 by the Crop Development Centre (CDC) in Saskatoon, Saskatchewan. CDC Pintium has a seed coat that changes from creamy white to dark brown colour after approximately six months of storage at room temperature under normal light conditions.

Bayo Florida is a RD bayo bean used at CIAT as a parent in a genetic mapping population. Bayo Florida has a seed coat that turns from a creamy white to a dark brown colour after approximately six months of storage at room temperature under normal light conditions (M. Blair, pers. comm.).

1533-15 is a SD, type 1 pinto bean developed and released in 2005 by the Crop Development Centre (CDC) in Saskatoon, Saskatchewan. 1533-15 has a seed coat that turns from creamy white to light brown colour, but does so at a much slower rate than CDC Pintium. After a year of storage at room temperature under normal light conditions, the 1533-15 seed coat remains very light in colour.

SDIP is a SD pinto bean developed by Dr. S.P. Singh at the University of Idaho-Kimberly Agricultural Research and Extension Centre (Singh et al., 2006). SDIP has a seed coat that is almost identical to 1533-15 and turns from a creamy white to a light brown colour. As with 1533-15, SDIP seed coats stored for a complete year at room temperature and under normal light condition remain very light in colour.

Bayo Mochica is a SD bayo bean used at CIAT as parent in a mapping population. Bayo Mochica has a seed coat that turns from a creamy white to a light brown colour, but does so at a much slower rate compared to Bayo Florida. After a year of storage at room temperature under normal light conditions, the Bayo Mochica seed coat remains very light in colour (M. Blair, pers. comm.).

KVxUI-1 and KVxUI-6 are ND pinto beans developed by Dr. J. Meyers at Oregon State University (OSU). Both of these lines have seed coats that are white with light brown spotting and retain their original colour at harvest after several years of storage at room temperature under normal light conditions. According to Dr J. Meyers, their genotype is *jj*.

Wit-rood boontje is a ND cranberry like bean from the Netherlands. Wit-rood has a seed coat that is white at harvest and remains white after several years of storage at room temperature under normal light conditions (K.P. Pauls, pers. comm.).

PI 608686 and PI 608688 are genetic testers for *jj* (Genetic Markers 39 and 41, respectively) obtained from the United States Department of Agriculture (USDA) Plant Introduction Station at Pullman, Washington.

3.2.2 Growing Locations

The plants used for this inheritance study were grown in (1) the phytotron, agriculture greenhouses, Preston field plots, Crop Development Centre (CDC) polyhouses on the U of S campus, (2) the Bovey greenhouses and phytotron on the U of G campus or (3) the quarantine greenhouses and Palmira/Cali field plots at CIAT.

3.2.3 The Crosses and Their Offspring

Crosses were made to generate a series of F_1 to F_5 seed coat generations for phenotypic and/or genotypic evaluation of PHD. The crosses were made by emasculating the flowers prior to them opening and then brushing the stigma with the donor parent pollen. The crosses included RD x SD, RD x ND, SD x SD, SD x ND and ND x ND and in many cases the reciprocals of these combinations (Table 3.2). The populations were generated either through single seed decent (SSD) or random selection of seed from each filial generation. The progeny from the crosses were checked for variability in seed size and seed coat patterning and/or darkening to verify that each cross successfully produced hybrids.

Table 3.2 Crosses evaluated in this PHD inheritance study, their location of development, parental genotypes and parental and F_1 phenotypes.

Cross #	Location Developed	Female Parent	Male Parent	Cross PHD phenotype	F_1 Phenotype
4119aS-1, 2, 4	U of S	KVxUI-1	P1608686	ND *x jj*	ND
4120S-1 to -3	U of S	KVxUI-1	P1608688	ND x*jj*	ND
4420bS-1	U of S	Witrood	PI 608688	ND x *jj*	ND
4119bS-2 to -4	U of S	P1608686	KVxUI-1	*jj* x ND	ND
4122S-1 to -4	U of S	P1608686	KVxUI-6	*jj* x ND	ND
4420aS-1 to -3	U of S	PI 608688	Witrood	*jj* x ND	ND
4123S-1 to -3	U of S	1533-15	P1608686	SD x *jj*	RD
4124aS-2, 3, 5	U of S	1533-15	P1608688	SD x *jj*	RD
4124bS-1 to -2	U of S	P1608688	1533-15	*jj* x SD	RD
3257S-1 to -8	U of S	PI 608688	1533-15	*jj* x SD	RD
4421S-1, 3	U of S	Pintium	PI 608686	RD x *jj*	RD
3254S-1 to -12	U of S	PI 608688	Pintium	*jj* x RD	RD
4422S-1 to -2	U of S	Witrood	KVxUI-1	ND x ND	ND
4423S-1 to -2	U of S	Witrood	KVxUI-6	ND x ND	ND
4086-1 to -2	U of S	1533-15	SDIP	SD x SD	SD
CIAT1	CIAT	Bayo Mochica	1533-15	SD x SD	SD
CIAT2	CIAT	1533-15	Bayo Mochica	SD x SD	SD
4084-1	U of S	1533-15	KvxUI-6	SD x ND	RD
4085-1 to -2	U of S	1533-15	KvxUI-1	SD x ND	RD
W18-1 to -33	U of G	Witrood	1533-15	ND x SD	RD
W19-1 to -70	U of G	Witrood	1533-15	ND x SD	RD
W52-1 to -18	U of G	Witrood	1533-15	ND x SD	RD
4121as-1	U of S	KVxUI-1	Pintium	ND x RD	RD
4121bs-1 to -3	U of S	Pintium	KVxUI-1	RD x ND	RD
CIAT22378-1	CIAT	Bayo Mochica	Bayo Florida	SD x RD	RD
CIAT22378-2	CIAT	Bayo Florida	Bayo Mochia	RD x SD	RD

Each cross served the purpose of either (i) verifying that two genotypes had a darkening phenotype that was controlled by the same gene(s), allele(s) and genetic mechanism(s) (SD x SD and ND x ND crosses), (ii) identifying the genetic control between RD and SD or ND phenotypes (RD x SD and RD x ND crosses), (iii) identifying the genetic control between SD and ND phenotypes (SD x ND crosses). In total, 28 crosses were generated for evaluation for their degree of PHD. Unfortunately, not all of these crosses successfully produced F_3 seed for F_2

seed coat evaluation and in some cases the seed coat could not be phenotyped due to colour distortions of underlying or overlying seed coat tissue.

3.2.4 Harvesting, Darkening, Scanning and Phenotyping Procedures

Plants grown in the field at Saskatoon were left to mature and dry out through September. Single plants were harvested in October. Single plants were placed into individual paper bags. All paper bags with single plants belonging to each line or family were separated in cloth bags tagged with the population number. The bags were then placed on non-heated dryers at the CDC laboratory for 2 – 4 days to ensure even drying. Dried plants were threshed and the seed was stored in paper envelopes at -20 ºC in the dark until phenotyping could commence. The same procedures were employed for plants grown in the greenhouse and in the phytotron throughout the year.

The UVC light protocol developed by Junk et al. (2007) was used to darken the seed coats of all seeds used in this experiment. Only the exposed half of the seed coats darkened, while the other half maintained its original colour. Visual comparisons were made between the exposed and unexposed sides of the seed as well as the exposed side of the seed with the seeds that had not been exposed to UVC light to facilitate more accurate phenotyping. Common bean seeds darkened using the UVC light protocol may darken further during storage; therefore, all seeds were scanned within two weeks of darkening. Six seeds of each parent and each progeny were scanned.

All seeds were visually phenotyped in 10 cm plastic weigh boats on a well lit table. The progeny from each cross and the two parents were assessed with each individual having half of their seeds with their exposed side facing up and half with their unexposed side facing up. A subset of checks, namely CDC Pintium (RD), 1533-15 (SD) and KVxUI-1 (ND) were used for comparison purposes. The progeny were then visually phenotyped for PHD by comparing the seed coat with the checks and parents; each individual was classified as ND, SD, RD or as bad seed (seed that was harvested pre-maturely or diseased and difficult to phenotype due to seed coat inconsistencies).

3.2.5 Statistical Analysis

A statistical analysis of observed versus expected segregation ratios for two and three class goodness of fit tests were compared using Fisher's exact chi square test and the Pearson chi square test. In all of the populations studied, the minimum sample size for a normal approximation to apply a goodness of fit test (Cochran, 1997; adopted from Bowley, 2008) using Fisher's exact test was met. Although the Fisher's exact test provides a conservative P-value estimate compared to the Pearson chi-square test, it was decided that the Pearson chi square test would be sufficient for the statistical analysis. This decision was made based on the fact that all of the statistical tests had P-values greater than the type 1 error rate decided on at the onset of the analysis using both the Pearson's chi-square and Fisher's exact tests. Therefore, there was no need to report both statistics in the study. Depending on the population and filial generation investigated a 1:0:0, 0:1:0, 0:0:1, 3:1:0, 0:3:1, or 9:3:4 (RD:SD:ND) expected segregation ratio was tested against observed segregations ratios.

The statistical analysis was performed using PROC FREQ of the SAS software (version 9.2, SAS Institute, Cary, NC). Pearson chi square P-values (type I error of 0.05) were used to determine the significance of observed versus expected segregation ratios. A test of heterogeneity was computed in situations where pooling individual populations or cross classes was possible. Tests of heterogeneity, H_0: the replicates are homogeneous, P-values (type 1 error of 0.05) were used to determine if individual populations could be pooled; pooling populations was desirable as it increased the sample size and the degrees of freedom (df) of the populations under investigation, therefore, strengthening the statistical test.

3.3 Results

Segregation ratios and chi-square values of several F_1-F_5 populations from a variety of crosses made between RD, SD and ND genotypes suggested a two-gene model for control of PHD in common bean. F_1 and F_2 population data are discussed below; F_3-F_5 population data can be found in Appendix A.

A total of eight F_1 and 289 F_2 individuals from six populations made from crosses between ND x ND or ND x *jj* tester lines were phenotyped. The F_1 progeny from these crosses were all ND (Table 3.2). Furthermore, all of the F_2 progeny from these crosses had ND seed coats. Therefore, genes controlling ND in the ND lines used in this inheritance study were allelic at the ND locus and Wit-rood, KVxUI-1 and KVxUI-6 were confirmed to be *jj* at the *J* locus.

A total of two F_1, 49 F_2, 43 F_3 and 104 F_4 individuals from four populations made from crosses between SD x SD (1533-15 x SDIP) were phenotyped. Two F_1 individuals from crosses between SD x SD (Bayo Mochica x 1533-15) were also phenotyped. The populations derived from the cross 1533-15 x SDIP did not segregate for PHD; the seed coats of progeny from these crosses were all SD (Table 3.2). Therefore, genes controlling SD in the SD lines used in this inheritance study were allelic at the SD locus.

A total of six F_1, 432 F_2 and 1241 F_3 individuals from 14 populations from crosses between ND x SD, SD x ND, SD x *jj* tester lines and *jj* tester lines x SD (1533-15, Wit-rood, KVxUI-1, KVxUI-6, PI 608686, PI 608688) were phenotyped. The F_1 individuals from these populations had RD seed coats (Table 3.2). F_2 progeny had segregation ratios that were not significantly different from 9RD:3SD:4ND (Table 3.3). F_3 families showed a wide range of segregation ranging from all RD, SD or ND as well as 3:1:0, 0:3:1, 3:0:1 and 9:3:4 (RD:SD:ND). In all cases the observed ratios did not significantly differ from the tested ratios. Crosses between SD and *jj* tester lines confirmed that the SD gene is not *J* and F_2 phenotypic ratios of 9RD:3SD:4ND suggest a two gene model for the PHD trait; segregation of F_3 families confirmed a two gene model. Furthermore, the fact that F_1 phenotypes were all RD indicates that RD is dominant to SD and ND.

Table 3.3 Observed and tested PHD segregation ratios and Chi-Squared, P-values and putative genotypes for the F_2 seed coats of ND x SD and SD x ND crosses.

Cross #	Female Parent	Male Parent	Observed F_2 Ratio (RD:SD:ND)	Tested F_2 Ratio (RD:SD:ND)	X^2	P-value	Putative F_1 Genotype
4123S-1	1533-15	P1608686	14:3:11	9:3:4	3.4444	0.1787	JjSDsd
4123S-2	1533-15	P1608686	19:7:6	9:3:4	0.7222	0.6969	JjSDsd
4123S-3	1533-15	P1608686	13:8:9	9:3:4	2.1926	0.3341	JjSDsd
4124aS-2	1533-15	P1608688	23:6:13	9:3:4	1.6582	0.5891	JjSDsd
4124aS-3	1533-15	P1608688	14:5:14	9:3:4	5.3569	0.0687	JjSDsd
4124bS-1	P1608688	1533-15	14:3:11	9:3:4	3.4444	0.1787	JjSDsd
4124bS-2	P1608688	1533-15	23:6:14	9:3:4	1.5685	0.4565	JjSDsd
4084-1	1533-15	KvxUI-6	16:4:8	9:3:4	0.4444	0.8007	JjSDsd
4085-1	1533-15	KvxUI-1	16:6:7	9:3:4	0.0728	0.9643	JjSDsd
4085-2	1533-15	KvxUI-1	11:3:2	9:3:4	1.4444	0.4857	JjSDsd
3257S	PI 608688	1533-15	5:1:2	9:3:4	0.2222	0.8948	JjSdsd
W18	Wit-rood	1533-15	17:1:10	9:3:4	4.8254	0.0896	JjSDsd
W19	Wit-rood	1533-15	44:9:16	9:3:4	1.9823	0.3712	JjSDsd
W52	Wit-rood	1533-15	9:5:1	9:3:4	3.7556	0.2457	JjSDsd

A total of seven F_1, 364 F_2 and 445 F_3 individuals from nine populations from crosses between ND x RD, RD x ND, RD x *jj* tester lines and *jj* tester lines x RD (CDC Pintium, Wit-rood, KVxUI-1, KVxUI-6, PI 608686 and PI 608688) were phenotyped. The F_1 individuals from these populations had RD seed coats (Table 3.2). All F_2 progeny had segregation ratios that were not significantly different from 3RD:1ND (Table 3.4) suggesting a single gene model with two alleles. F_3 individuals from the cross PI 608688 x CDC Pintium, however, showed segregation ranging from all RD, SD and ND as well as 3:1:0, 0:3:1, 3:0:1 and 9:3:4 (RD:SD:ND); in all cases the observed ratios did not significantly differ from the tested ratios (Table 3.5). F_3 segregation from crosses made between PI 608688 and CDC Pintium suggest a two gene model. The discrepancy between the F_2 and F_3 genotypes is believed to be a function of differences in the alleles present at a second locus in the different parental genotypes; one allele being dominant to the other. It is likely that seed from the PI 608688 genotype was heterogeneous, accounting for the wide range of segregation in the F_3. Furthermore, the fact that F_1 phenotypes were all RD indicated that RD is dominant to SD and ND.

Table 3.4 Observed and tested PHD segregation ratios and Chi-Squared, P-values and putative genotypes for the F_2 seed coats of ND x RD and RD x ND crosses.

Cross #	Female Parent	Male Parent	Observed F_2 Ratio (RD:ND)	Tested F_2 Ratio (RD:ND)	X^2	P-value	Putative F_1 Genotype
4121as-1	KVxUI-1	Pintium	71:21	3:1	0.2319	0.6301	JjSDSD
4121bs-1	Pintium	KVxUI-1	68:22	3:1	0.0148	0.9031	JjSDSD
4121bS-2	Pintium	KVxUI-1	69:18	3:1	0.8621	0.3532	JjSDSD
4121bS-3	Pintium	KVxUI-1	59:20	3:1	0.0042	0.9482	JjSDSD
3254S-1	PI 608688	Pintium	10:2	3:1	0.4444	0.5050	JjSDSD
4421S-1	Pintium	PI 608686	2:2	3:1	1.3333	0.2482	JjSDSD

Table 3.5 Observed and tested PHD segregation ratios and Chi-Squared, P-values and putative genotypes for the F_3 seed coat families of jj tester x RD cross PI 608688 x CDC Pintium.

Cross #	Female Parent	Male Parent	Observed F_3 Ratio (RD:SD:ND)	Tested F_3 Ratio (RD:SD:ND)	X^2	P-value	Putative F_1 Genotype*
3254S-1-1	PI 608688	Pintium	26:6:0	3:1:0	0.6667	0.4142	JJSDsd
3254S-1-2	PI 608688	Pintium	28:4:9	9:3:4	2.7561	0.2521	JjSDsd
3254S-1-3	PI 608688	Pintium	12:1:3	9:3:4	2.5833	0.2748	JjSDsd
3254S-1-4	PI 608688	Pintium	12:5:3	9:3:4	1.2667	0.5308	JjSDsd
3254S-1-5	PI 608688	Pintium	26:0:0	1:0:0	-	-	JJSDSD
3254S-1-6	PI 608688	Pintium	29:0:0	1:0:0	-	-	JJSDSD
3254S-1-7	PI 608688	Pintium	17:0:6	3:0:1	0.0145	0.9402	JjSDSD
3254S-1-8	PI 608688	Pintium	19:5:9	9:3:4	0.3064	0.858	JjSDsd
3254S-1-9	PI 608688	Pintium	29:0:0	1:0:0	-	-	JJSDSD
3254S-1-10	PI 608688	Pintium	25:0:6	3:0:1	0.5269	0.4679	JjSDSD
3254S-1-11	PI 608688	Pintium	0:0:7	0:0:1	-	-	jj__
3254S-1-12	PI 608688	Pintium	0:0:21	0:0:1	-	-	jj__
3254S-1-13	PI 608688	Pintium	0:0:10	0:0:1	-	-	jj__
3254S-1-14	PI 608688	Pintium	17:0:0	1:0:0	-	-	JJSDSD
3254S-1-15	PI 608688	Pintium	12:0:5	3:0:1	0.1765	0.6744	JjSDSD
3254S-1-16	PI 608688	Pintium	0:0:3	0:0:1	-	-	jj__
3254S-1-17	PI 608688	Pintium	8:0:2	3:0:1	0.1333	0.7150	JjSDSD
3254S-1-18	PI 608688	Pintium	7:0:1	3:0:1	0.6667	0.4142	JjSDSD
3254S-1-19	PI 608688	Pintium	5:0:1	3:0:1	0.2222	0.6374	JjSDSD
3254S-1-20	PI 608688	Pintium	8:0:4	3:0:1	0.4444	0.5050	JjSDSD
3254S-1-21	PI 608688	Pintium	9:0:4	3:0:1	0.2308	0.6310	JjSDSD
3254S-1-22	PI 608688	Pintium	8:1:0	3:1:0	0.9259	0.3359	JJSDsd
3254S-1-23	PI 608688	Pintium	6:0:0	1:0:0	-	-	JJSDSD
3254S-1-24	PI 608688	Pintium	9:0:3	3:0:1	-	-	JjSDSD
3254S-1-25	PI 608688	Pintium	7:0:0	1:0:0	-	-	JJSDSD
3254S-1-26	PI 608688	Pintium	6:0:1	3:0:1	0.4286	0.5127	JjSDSD

*PI 608688 may be jjSDSD or jjSDsd due to seed heterogeneity

A total of 495 F_5 individuals from reciprocal crosses between Bayo Florida (RD) and Bayo Mochica (SD) were phenotyped. There was no difference based on the direction of the cross and both populations had segregation ratios that were not significantly different than 3RD:1SD (Table 3.6), suggesting that PHD is controlled by two major genes in this population. Close examination of the phenotypes within each of the RD and SD classes suggested that there was a wider range of phenotypes within the classes and a quantitative trait loci (QTL) model may

be more appropriate for these populations. A different method of phenotyping, such as the use of a colorimeter, would be needed to classify the individuals to explore this further. Although a quantitative model may explain genetic differences, other factors such as variability in UVC light exposure or seed coat chemistry may influence PHD, affecting the results of phenotyping.

Table 3.6 Observed and tested PHD segregation ratios and Chi-Squared and P-values for the F_5 seed coats of crosses between Bayo Mochica and Bayo Florida.

Cross #	Female Parent	Male Parent	Observed F_5 Ratio (RD:SD:ND)	Tested F_5 Ratio (RD:SD)	X^2	P-value	Putative F_1 Genotype
CIAT22378-1	Bayo Mochica	Bayo Florida	164:62:0	3:1	0.7139	0.3982	JjSDsd
CIAT22378-2	Bayo Florida	Bayo Mochia	204:61:0	3:1	0.5547	0.4564	JjSDsd

A heterogeneity test (type I error rate of 5%) was performed in SAS after pooling all population segregation ratios from each of the classes of crosses, namely ND x ND, SD x SD, ND x SD, ND x RD and SD x RD. Tests for heterogeneity confirmed that each of the populations from each of the PHD classes could be pooled (P-values > 0.05) (Table 3.7).

Table 3.7 Pooled observed and tested PHD segregation ratios and chi-squared and P-values for all cross classes of the PHD trait.

Cross Class	Observed Ratio (RD:SD:ND)	Tested Ratio (RD:SD:ND)	X^2	P-value
ND x ND				
F1	0:0:8	0:0:1	-	-
F2	0:0:298	0:0:1	-	-
SD x SD				
F1	0:3:0	0:1:0	-	-
F2	0:49:0	0:1:0	-	-
NDx SD				
F1	6:0:0	1:0:0	-	-
F2	233:66:122	9:3:4	4.8475	0.0886
ND x RD				
F1	7:0:0	1:0:0	-	-
F2	279:0:85	3:0:1	0.5275	0.4677
SD x RD				
F5	368:123:0	3:1:0	0.0007	0.9792

3.4 Discussion

3.4.1 Qualitative Phenotyping Seed Coat Post Harvest Darkening

Common bean can be qualitatively phenotyped for PHD by visual evaluation of each sample independently or by comparative evaluation of all samples within a population or family. Visual evaluation can be conducted directly by analyzing the actual seeds, or scanned images of the seeds. Evaluation is best conducted by observing and comparing exposed and un-exposed sides of the seed coat. This method ensures that a relative comparison between the samples and true ND, SD and RD phenotypes is made. Hence, this allowed for a more accurate assessment of the relative degree of darkening that has occurred and ensuring that a relative comparison between the samples and true ND, SD and RD phenotypes was made. Qualitative evaluation of PHD is a quick and efficient method of phenotyping common bean for genetic studies and breeding.

3.4.2 Parental Genotypes

Parental genotypes were gathered from various locations across North and South America. The segregation ratios for the various PHD classes described previously were used to generate putative genotypes for each of the parents used in the inheritance analysis (Table 3.1). The results suggest that all parental RD and SD genotypes used in the inheritance analysis were homozygous dominant at the *J* locus. Parental RD genotypes were homozygous dominant and parental SD genotypes were homozygous recessive at the *SD* locus. Parental ND genotypes were always homozygous recessive at the *J* locus and in all instances, but one, parental ND genotypes were homozygous dominant at the *SD* locus. The seed used from the genotype PI 608688 is likely heterogeneous at the *SD* locus and homozygous recessive at the *J* locus. It is recommended that a subset of seeds from PI 608688 mixture be selfed for several generations to obtain both homozygous dominant and recessive genotypes at the *SD* locus for future genetic studies. Knowledge of the parental PHD genotypes identified in this inheritance study may prove to be an important tool for future inheritance studies and breeding work associated with seed coat genetics in common bean and other crops.

3.4.3 Genes and Alleles Controlling the PHD trait

Results from this inheritance study suggest that at least two unlinked genes control the PHD trait in common bean. One gene, *J*, is responsible for whether a bean will darken. The dominant allele *J* codes for darkening, while the recessive allele *j* is non-darkening. The second gene, *SD*, is responsible for how quickly a seed coat will darken and also has two alleles. The dominant allele *SD* codes for RD while the recessive allele *sd* codes for SD. Recessive epistasis with three phenotypic classes explains the segregation ratios of several populations. The gene *J* is epistatic to the gene *SD* resulting in the three phenotypic classes: ND, SD and RD, from nine possible genotypic combinations (Table 3.8). Any plant with the genotype *jj*, regardless of the genotype at *SD*, will be ND. Variability at the *SD* locus is only seen when *J* is dominant.

Table 3.8 Recessive epistasis of the PHD trait in common bean.

Allele combination	Expected phenotype
JJSDSD	RD
JJSDsd	RD
JJsdsd	SD
JjSDSD	RD
JjSDsd	RD
Jjsdsd	SD
jjSDSD	ND
jjSDsd	ND
jjsdsd	ND

3.4.4 Choosing Populations for Evaluating Seed Coat Post Harvest Darkening

The parents used in this inheritance study were of great value in identifying the underlying genetic control of PHD in common bean. However, some of the parents used in this study were more useful than others. The genotypes CDC Pintium, 1533-15, KVxUI-1, KVxUI-6 and Wit-rood had white or cream background colours prior to exposure to UVC light. The light background colour made these parental lines useful in genetic studies of PHD as it helped to reduce experimental error in phenotyping filial generations. Clearly genetic studies involving PHD should be conducted using genotypes with white or light cream background colour.

The genotypes Bayo Mochica and Bayo Florida had a darker cream background colour prior to exposure when compared to CDC Pintium, 1533-15, KVxUI-1, KVxUI-6 and Wit-rood. Although phenotypic evaluation of PHD in the two bayo beans was still possible, the darker background of these two parents made phenotypic evaluation more difficult and, therefore, less accurate. Future studies should avoid using genotypes such as Bayo Mochica and Bayo Florida, which have naturally darker seed coat background colours.

The degree of PHD variability observed in the Bayo Mochica x Bayo Florida population may be explained by several factors. One experimental factor that may affect phenotypic results is the light intensity distribution of the UVC light chamber, as discussed in the materials and methods section. UVC lights were alternated with fluorescent lights every 12 cm, thus, distributed 24 cm apart in the darkening apparatus. Beans placed directly under the UVC bulbs would be exposed to greater UVC light intensity then those positioned between UVC light bulbs. Thus, uneven distribution of light intensity may result in an equally uneven distribution in the degree of darkening, which would affect the precision of phenotyping, and thus, the results. Therefore, the quantitative range of phenotypes observed may be a function of experimental error. This implies that some phenotypes that would have normally been phenotyped as SD may have been phenotyped as RD, therefore, resulting in a larger number of RD phenotypes than was expected from a two gene model with epistatic interaction.

Another factor to consider is the possibility that the original pre-UVC exposed seed coat colour of the progeny from the CIAT22378-1 and CIAT22378-2 crosses demonstrated a high degree of variability. This variability may affect the apparent degree of darkening associated with the seed coats after exposure to UVC lights.

In order to correct for experimental errors associated with non-uniform exposure to UVC light and other seed coat chemistry effects, the compounds responsible for PHD must be identified so that the degree of darkening can be quantified. This method would eliminate dependence on the subjective human eye or instruments such as a colorimeter.

The *jj* tester genotypes PI 608686 and PI 608688 have purple and black seed coat colours, respectively. Therefore, phenotypic evaluation of PHD is not possible in either of these genotypes. Crosses had to be made between the PI lines and pinto lines with white or light creamy backgrounds in order to generate progeny with seed coat colours that could be

phenotypically evaluated for PHD. This method of analysis proved to be difficult sincemost of the crosses generated from the PI lines yielded F_1 progeny that had dark grey or purple seed coats, which could not be phenotyped for PHD. F_2 generations produced from crosses between the PI lines and pinto lines segregated for seed coat colour; some progeny were too dark to phenotype, others were difficult to phenotype due to their naturally darker seed coats, and yet other progeny had white or cream coloured seed coats, which were easily phenotyped. Future genetic studies involving the PHD trait in common bean or other crops do not use parental genotypes that are too dark to be phenotyped. Furthermore, as the number of progeny that cannot be phentoyped increases, the population size under evaluation decreases, and therefore, the strength of the statistical tests used for segregation analysis decreases. However, under circumstances where the genetic background of the PHD trait in a particular line is being investigated, but cannot be phenotyped for PHD, this method of inheritance analysis although difficult has proven to work successfully. PI 608686 and PI 608688 were also used to confirm that other pinto and cranberry beans were *jj*. This method of genetic identification was successful, however, we propose that PI 608686 and PI 608688 not be used as official *jj* tester lines in future PHD inheritance studies due to phenotyping difficulties associated with progeny derived from these lines. Instead, KVxUI-1 and KVxUI-6 may be used as jj tester lines as these lines have cream coloured backgrounds that can easily be phenotyped.

A wide range of seed coat colours were noted in all cross classes made in this inheritance study. Variability was apparent in the seed coats prior to UVC exposure. This background variability carried through after UVC light exposure causing a range of visible darkening phenotypes within the major ND, SD and RD phenotypes. This observation suggests that there is more dynamic seed coat chemistry at work, causing an underlying seed coat background and obscuring phenotypic evaluation from a visible perspective. This range makes phenotyping the PHD trait a quality judgment in distinguishing between ND and SD or SD and RD seeds in some cases. The potential for not correctly identifying a phenotype can result in distorted segregation ratios, however, the results obtained in this study showed statistically strong positive correlations between observed and expected phenotypic ratios when subjected to goodness of fit tests. The potential for genotypes to be misclassified is problematic for bean breeders despite the fact that background colours inevitably will contribute to the observed PHD colour of the commodity.

The background colour observed in common bean seed coats may be due to environmental effects or biochemical differences among various seed coat genotypes. Environmental and physiological conditions may make it possible that seeds maturing at different times on the same plant or on different plants may be subject to differing heat and light exposure. With respect to seed coat biochemistry, seeds from the same plant or from different plants may have varying degrees of biochemical interactions that are affecting background colour. In some cases, compound accumulation, oxidation and/or binding may occur more quickly in some seed coats when compared to others. This phenomenon could potentially be causing variability in the degree of seed coat background discolouration resulting in the variability observed in the final phenotypic evaluation of the PHD trait.

3.5 Conclusion

Clearly a more practical and efficient method of identifying PHD genotypes and phenotypes in order to improve selection practices for enhanced seed appearance in pinto bean is required. Several populations were generated between ND, SD and RD lines and F_1-F_5 generations were evaluated for PHD using the UVC light protocol. This project examined the phenotypic variability of PHD of several genotypes of common bean with the purpose of understanding the genetic control of the PHD trait.

The nature of segregation in several of the populations indicated that two unlinked genes control the PHD trait. Recessive epistasis with three phenotypic classes best explains the segregation ratios of several populations. The results from this inheritance study have led to a new understanding of the genetic control of the PHD trait. A new gene, which controls the level of PHD has been identified and was certainly different from the originally suggested gene, *J*, which was thought to be the only gene that controlled the level of PHD (Prakken, 1974). It is evident that there is a second locus that controls PHD darkening and *J* is epistatic to this locus. This new information warranted the need to name the new gene responsible for the degree of PHD. The accepted name for this new gene is *sd* after the slow darkening phenotype that results from the homozygous recessive form at this locus (BIC Genetics Committee, February 2011).

The segregation ratios for the various PHD classes described previously were used to suggest putative genotypes for each of the parents used in the inheritance analysis. The results

suggest that all parental RD and SD genotypes used in the inheritance analysis were homozygous dominant at the *J* locus. Parental RD genotypes were homozygous dominant and parental SD genotypes were homozygous recessive at the *sd* locus. Parental ND genotypes were homozygous recessive at the *J* locus and in all cases, but one, parental ND genotypes were homozygous dominant at the *sd* locus. Knowledge of the parental PHD genotypes identified in this inheritance study may prove to be an important tool for future inheritance studies and breeding work associated with seed coat genetics in common bean.

Further phenotyping needs to be completed to confirm the control of darkening by the two genes, but it is clear that the SD phenotype is not controlled by *J*. Markers will help to establish the genomic regions where these two genes are found and may eventually lead to the identification of the actual genes involved. The findings obtained from this research may be useful in understanding the seed coat darkening phenomenon in other bean species and other pulse, cereal and fruit, vegetable and beverage crops.

CHAPTER 4 : CONDENSED TANNIN ACCUMULATION DURING SEED COAT DEVELOPMENT IN COMMON BEAN (*Phaseolus vulgaris* L.)

4.1 Introduction

The majority of traits selected for in common bean breeding programs are related to growth habit, disease resistance, photoperiod sensitivity, maturity and final seed coat colour and patterning. More recently, several other aspects of bean seed characteristics, including micronutrient content, have become important to bean breeders. Biofortification involves improving the available nutrients such as vitamin A, zinc, iron, calcium and folate in food crops through breeding (Nestel et al., 2006). Many micronutrients are essential to human health and are of growing importance in many regions of the world. For many vegetarians and inhabitants of Latin America and Africa some varieties of common bean are an important source of protein and minerals (especially iron) (Moraghan et al., 2002). The distribution of iron between the seed coat and embryo of common bean is a genotypic trait (Moraghan et al., 2002).

Condensed tannins (CT; syn. proanthocyanidins) are an important group of chemical substances produced in several species of common bean. This substance can be harmful and/or beneficial to human health depending on the amount present and where it is found in the plant. Common bean seed coats are highly variable in CT and the nutritional value of beans with high CT concentrations in their seed coats is likely compromised (Reddy et al., 1985). The reduced nutritional value of high CT common bean may be a result of several factors associated with CT polymerization including depression of food/feed intake, formation of tannin complexes with dietary proteins and other food components, inhibition of digestive enzymes, increased excretion of endogenous proteins and effects on the digestive tract (Reddy et al., 1985). Manipulating the production, accumulation and form of CT in the seed coat of common bean may be beneficial to bean producers and consumers.

Therefore, common bean nutrition is particularly important to residents of many developing nations where beans are consumed as the main staple crop. The nutritional profile of some varieties of common bean, however, is sometimes deceiving as some of the nutrients found in the seeds and seed coats of this crop, although present in high concentrations, are not bioavailable due to compounds such as CT (Baynes and Batchwell, 1990). CT may be binding

some of these essential nutrients, hence, reducing the bioavailability of these nutrients. Therefore, consumption of tannin rich foods may lead to deficiency diseases such as anaemia (Matuscheck et al. 2001), which is common in many developing nations where iron is deficient in the diets of consumers.

Condensed tannin biosynthesis is controlled by the flavanoid pathway of the phenylpropanoid pathway, which is part of the larger shikimate pathway (Hahlbrock and Scheel, 1989). Throughout these biochemical pathways there are a series of enzymes and metabolites produced, each of which may play a role in the regulation of CT production in seeds and seed coats. Understanding the genetic mechanism by which CT are biosynthesized has been difficult due to the complex nature of the phenylpropanoid pathway.

Based on preliminary work on a limited number of genotypes, the accumulation of CT in common bean seed coats appears to begin very early on in seed coat development (Marles et al., 2008a). Accumulation was hypothesized to be either delayed or occur slower in genotypes which contained lower concentrations of extractable CT at maturity. It was also hypothesized that CT content would likely reach a peak in the seed coat of developing seeds and then begin to decline as the CT became bound and un-extractable. Genotypes with higher concentrations of extractable CT at maturity are likely to have higher rates of accumulation throughout seed coat development.

The objective of this study was to qualitatively identify when CT begin to accumulate in common bean seed coats during seed development and quantify the concentration and content of extractable CT throughout seed coat development in five genotypes of common bean that were known to have differing concentrations of CT in their seed coats at maturity. By analyzing the production and accumulation of CT and their key forming factors, plant breeders may be able to better understand the genetic controls that are responsible for up or down regulation of CT in common bean seeds. This knowledge may help reduce the anti-nutritional and anti-environmental properties associated with CT in common bean.

4.2 Materials and Methods

4.2.1 Growing Locations

The black screen houses at CIAT are located at recta Cali-Palmira km 17 (legal location 3 º 30´ N, 76 º 21´ W). A section of the screen house that was 24 m x 11 m was used to grow bean

plants; the screen house was oriented in a north/south direction along its length. Screen house temperatures were maintained between 22 – 35 ºC and 60 – 100% relative humidity. Plants were grown in 5 gallon pots using a clay soil placed on a gravel floor. In addition to the starter nutrient charge, the plants were irrigated once every two weeks with a 20-20-20 all purpose fertilizer at a concentration of 5 ml/L water; the fertilizer contained micronutrients. Soil was maintained between permanent wilting point and saturation using overhead irrigation and weeds were controlled manually. Pots were placed 30 cm apart in all directions. White flies were controlled as necessary using sticky traps.

A section of the quarantine greenhouse at CIAT that was 4 m x 8 m was also used to grow plants from Canadian-supplied seed. The greenhouse was maintained between 24 – 32 ºC during the day and between 20 – 22 ºC at night and 60 – 100% relative humidity. Plant growth and maintenance practices were the same as in the screen house.

4.2.2 Genotype Selection and Block Design

The objective of this experiment was to evaluate the difference in the pattern of CT accumulation in the seed coats of five different bean genotypes. Two separate experiments were conducted to address this objective. Genotypes from each of the experiments were evaluated for their accumulation of seed coat CT every other day starting from 6 days after flowering (DAF) to 40 DAF.

In the first experiment three genotypes, DOR364, RIL89 and RIL58, were assessed for CT accumulation in their seed coats. These genotypes were obtained from a RIL population from a cross between DOR364 x G19833. The DOR364 x G19833 population consisted of 88 individuals and was highly variable for total CT in the seed coat (M. Blair, pers. comm.). This same population has also been extensively mapped at CIAT for phytochemical composition and seed coat colour (Caldas and Blair, 2009). DOR364 is a Mesoamerican bean line that has a seed coat with an intermediate CT concentration at maturity (Blair et al., 2003). DOR364 is a small-grained, semi-determinate bush type, red seeded Mesoamerican advanced bean line released by CIAT. DOR364 has been extensively studied and is a parent of a well characterized bean genetic mapping population **(**DOR364 x G19833**)** developed at CIAT (Ariza et al., 2007). The average mature mass of a single seed of DOR364 was 264 mg. At maturity, the seed coat of DOR364

contained 17.8 mg g^{-1} of CT, which is an intermediate level relative to the RILs in the DOR364 x G19833 population (Blair, 2009). The male parent, G19833 is an Andean bean line, which has a seed coat with a similar CT content at maturity as DOR364. It was not used in this experiment as it did not grow well and there were insufficient flowers produced to conduct the experiment. RIL89 is a small-grained, indeterminate pole type, yellow seeded RIL from the cross DOR364 x G19833. The average mature mass of a single seed of RIL89 was 277 mg. At maturity, the seed coat of RIL89 contained 27.9 mg g^{-1} of CT, making it the highest CT line in the DOR364 x G19833 population (Blair, 2009). RIL58 is a medium-grained indeterminate pole type, brown seeded, RIL from the same RIL population. The average mature mass of a single seed of RIL58 was 375 mg. At maturity, the seed coat of RIL58 contained 10.3 mg g^{-1} of CT, making it the second lowest CT line in the population.

For the DOR364, RIL89 and RIL58 experiment, plants of each of the three genotypes were grown in a black screen house at CIAT under a 12-hour photoperiod; given that these genotypes are daylight sensitive, flowering only if day length is 12 hours or less. A total of 18 time points/genotype/replication were evaluated. Of the 28 pots per genotype, 18 were designated for the 18 harvest time points. The other 10 pots were used as extras to collect pods from any time points that were lacking in seed coat material due to insufficient harvest. Each pot contained three plants to ensure enough flowers were available for tagging. This practice maximized the number of seed coats available per time point/genotype/replication, thus ensuring enough seed coat tissue was available for determining CT accumulation over all time points. All pots within each genotype were randomized to reduce variability associated with screen house environments. This experimental design was repeated for each of the 3 genotypes; each replication being randomized and planted two weeks apart.

The second experiment was grown in the quarantine greenhouse and consisted of two parents of a RD x SD RIL population from the University of Saskatchewan: CDC Pintium and 1533-15. CDC Pintium has relatively higher CT concentrations when compared to 1533-15 (Beninger et al. 2005; Marles et al., 2008a). CDC Pintium is a medium-grained pinto bean that has been extensively studied for the PHD trait at the U of S. This early maturing, plump seeded, RD type I bush pinto bean was developed and released in 1999 by the Crop Development Centre (CDC), University of Saskatchewan. CDC Pintium had a seed coat that changes from creamy

white to dark brown after approximately six months of storage at room temperature under normal light conditions. The average mature mass of a single seed of CDC Pintium was 397 mg. Marles et al. (2008a) found that the seed coat of CDC Pintium was highly variable in CT content at maturity, ranging from 9.5 – 17.8 mg g^{-1} CT depending on the environmental conditions where the plants were grown. Despite this range, CDC Pintium was still considered a relatively low CT line, on average, when compared to DOR364, RIL89 and RIL58. The parent, 1533-15, is a medium-grained pinto bean that has also been extensively studied for the PHD trait at the U of S. This early maturing, plump seeded, SD, type 1 pinto bean was developed by the CDC and registered as CDC WM-1 in 2005. 1533-15 has a seed coat that still turns from creamy white to light brown colour, but does so at a much slower rate compared to CDC Pintium. After a year of storage at room temperature under normal light condition, 1533-15 seed coats remain relatively light in colour. The average mature mass of a single seed of 1533-15 was 397 mg. Like CDC Pintium, 1533-15 was highly variable in CT content at maturity ranging from 8.3 – 11.2 mg g^{-1} CT depending on the environmental conditions under which the plants were grown (Marles et al. 2008a). In some environments 1533-15 had a significantly lower CT concentration compared to CDC Pintium, however, in other environments no significant difference was noted between the CT concentration between these two lines. However, 1533-15 did not ever have higher CT concentrations than CDC Pintium when grown in the same environment (Marles et al. 2008a).

The block design of the CDC Pintium and 1533-15 trial was the same as the other lines; however, the plants had to be grown in a quarantine greenhouse due to Colombian government regulations regarding imported seed.

4.2.3 Experimental Design: From Planting to CT Assessment

4.2.3.1 Environment

All three replications for all five genotypes were planted in 5 gallon black plastic pots. The seeds were scarified by chipping a small section of the seed coat using a blade. Six seeds were planted per pot to ensure adequate germination. Once the seeds had germinated the seedlings were thinned to 3 plants per pot. Upon flowering, each plant was tagged daily until enough seed coat tissue was collected for the experiments or when the plants stopped flowering.

The planting, germination, thinning, flower initiation and flower termination dates for all five genotypes are presented in Table 4.1.

Table 4.1 Julian days for planting, germination, thinning, flower initiation and flower completion of DOR364, RIL89, RIL58, CDC Pintium and 1533-15 for each biological replication.

Genotype	Replication	Planting Date	Germination Date	Thinning Date	Flowering Date	Date flowering finished
DOR364	1	65	69	72	99	110
	2	79	82	89	114	139
	3	93	97	107	128	145
RIL89	1	65	69	72	100	122
	2	79	82	89	118	139
	3	93	97	107	128	152
RIL58	1	65	69	72	100	122
	2	79	82	89	118	161
	3	93	97	107	130	167
CDC Pintum	1	65	69	72	110	111
	2	79	82	89	124	126
	3	93	97	107	137	139
1533-15	1	65	69	72	110	111
	2	79	82	89	124	126
	3	93	97	107	137	139

4.2.3.2 Tagging, Harvesting and Seed Coat Handling Procedures

All flowers were tagged with a small jewelry tag on the morning that they opened. The jewelry tag was labeled with the Julian day and wrapped around the pedicels of the opening flowers using a thin string. Flowers and seeds from all dimensions of the plants were used for the CT assessment. The day of tagging (DOT), number of flowers tagged, pot number, number of pods harvested, day of harvest (DOH) and seed/pod age were recorded for all of the flowers tagged for each pot/genotype/biological replication. This information was used to identify the plants that needed to be harvested and to keep track of the amount of seed coat obtained or still required for the analyses. Duration of flowering was very short (2 – 3 days) for CDC Pintium and 1533-15 due to their determinate growth habit (Table 4.1). By contrast, RIL58 and RIL89 had a very long duration of flowering (20 – 30 days) due to their indeterminate pole type growth habit. DOR364 had an intermediate flowering and tagging duration due to its semi-indeterminate bush type growth habit.

Harvest occurred in mid to late morning (9:00 am – 11:00 am). Pods requiring harvest on plants in each pot were identified using a spreadsheet where all tagging data were recorded. Pods were removed from their pedicels with jewelry tags attached, placed in airtight Ziploc bags and placed on ice in an ice bath to reduce any further biochemical changes. All aborted flowers or pods that did not grow well were discarded. Working on ice, pods were split open using a scalpel and the seeds were removed. The pods were then placed in plastic tubes with lids and frozen at -20 ºC. Seeds from each pod/genotype/DAF/biological replication were packaged separately in the plastic tubes and the number and mass of the seeds from each pod was recorded. Following separation, the samples were frozen at -80 ºC.

A representative pod and seed sample from each genotype/DAF was scanned next to a Canadian penny. Scanning allowed for a visual illustration of the progression in pod, seed, and seed coat development.

Seeds that were harvested between 10 – 40 DAF were removed from the freezer, one set at a time, and their seed coats were separated from their cotyledons. The seed coats for all pods of each genotype/DAF/biological replication were combined and placed in separate 15 ml microfuge tubes plastic tubes; cotyledons were placed in a second set of tubes. Each set of tubes was labeled with the genotype, DAF, biological replication and the type of tissue present in the tube. The seed coat and cotyledon samples were then weighed on a fresh weight basis. The samples were then frozen at -80 ºC. A similar procedure was conducted for seeds harvested at 6 and 8 DAF; however, due to the difficult nature of removing seed coats from cotyledons and the fact that the majority of the seed mass was composed of seed coat at these younger DAF, entire seeds were used for the CT analysis.

Once the seeds were frozen to -80 ºC, they were removed from the freezer while in their plastic tubes and the caps of the vials were detached. A plastic mesh was placed on top of each set of vials and the vials with seed coats were placed in a freeze drier at -50 ºC at a pressure of 53 mBars for 48 hours. Once freeze dried, tissue was transferred into a plate shaker for grinding to a fine powder for five minutes. The ground tissue was then weighed and re-frozen at -80 ºC.

Samples were shipped to the U of S where they were placed in a -80 ºC freezer for 2 weeks and then shipped to the U of G where they were immediately re-frozen at -80 ºC. Mailing

time from CIAT to U of S and from U of S to U of G took 4 and 1 day respectively. The samples were not frozen during shipping.

4.2.3.3 Modified Butanol-Hydrochloric Acid Assay

Ground seed coat tissue was then treated to a modified butanol – hydrochloric acid (BuOH-HCl) assay. This protocol was modified from the original protocol described by Lees et al. (1993). The assay was conducted as follows: 20 – 40 mg of seed coat tissue was weighed into air tight screw on test tubes and 1 ml of 70:30 BuOH-HCl was added and mixed with a spatula. The solutions were vortexed for 10 seconds, incubated at 80 ºC for 30 minutes, vortexed again for 10 seconds and re-incubated at 80 ºC for 30 minutes. The heated tubes were then placed in an ice bath for 5 minutes, centrifuged at 12,000 – 14,000 rpm for 2 minutes, rotated 180 degrees and centrifuged again at 12,000 – 14,000 rpm for another 2 minutes. New sets of 2 ml tubes were labelled and 980 uL of BuOH-Cl and 20 uL of each of the extract solutions were added to each tube (dilution of 1:50). The contents of each set of diluted solutions were aliquoted into acid/solvent resistant spectrophotometer cylinder vials and the absorbance for each sample was read on a spectrophotometer at 538 nm. A wavelength of 538 nm was chosen to conduct the quantification because it is the wavelength that CT is absorbed maximally in pinto beans (M.A.S. Marles, pers. comm.). A control consisting of ground, freeze dried mature DOR364 seed coat tissue, was also assessed using the same BuOH-HCl assay described above. The control was assessed every time a subset of the experimental samples was subject to the BuOH-HCl assay to monitor the consistency between different sets of sample runs.

4.2.3.4 Standard Curves

Two standard curves were generated. The first standard curve was obtained from the control DOR364 tissue and the second standard curve was generated from a semi-purified CT prep obtained from pinto bean seed coat tissue obtained from Dr. Susan Marles at the U of S, which was previously used in the study conducted by Marles et al. (2008b).

The DOR364 standard curve was generated by measuring out 10, 20, 30, 40, 50, 60 and 70 mg of ground freeze dried mature DOR364 tissue into separate test tubes and subjecting these samples to the mini-BuOH-HCl assay described above. The samples were then read at 538 nm using an Ultrospec 2100 Pro UV visible spectrophotometer at 538 nm to generate a standard

curve. The absorbance values of each sample were then used to calculate DOR364 equivalents using the linear regression equation of the standard curve (Figure 4.1). This conversion made it possible to compare each of the samples, not only to each other, but also to the control. The data obtained from this standard could not be used for comparison with other CT experiments because different sets of mature DOR364 seed coat tissue are likely to be highly variable in CT content due to environmental differences each time a set of this genotype is grown.

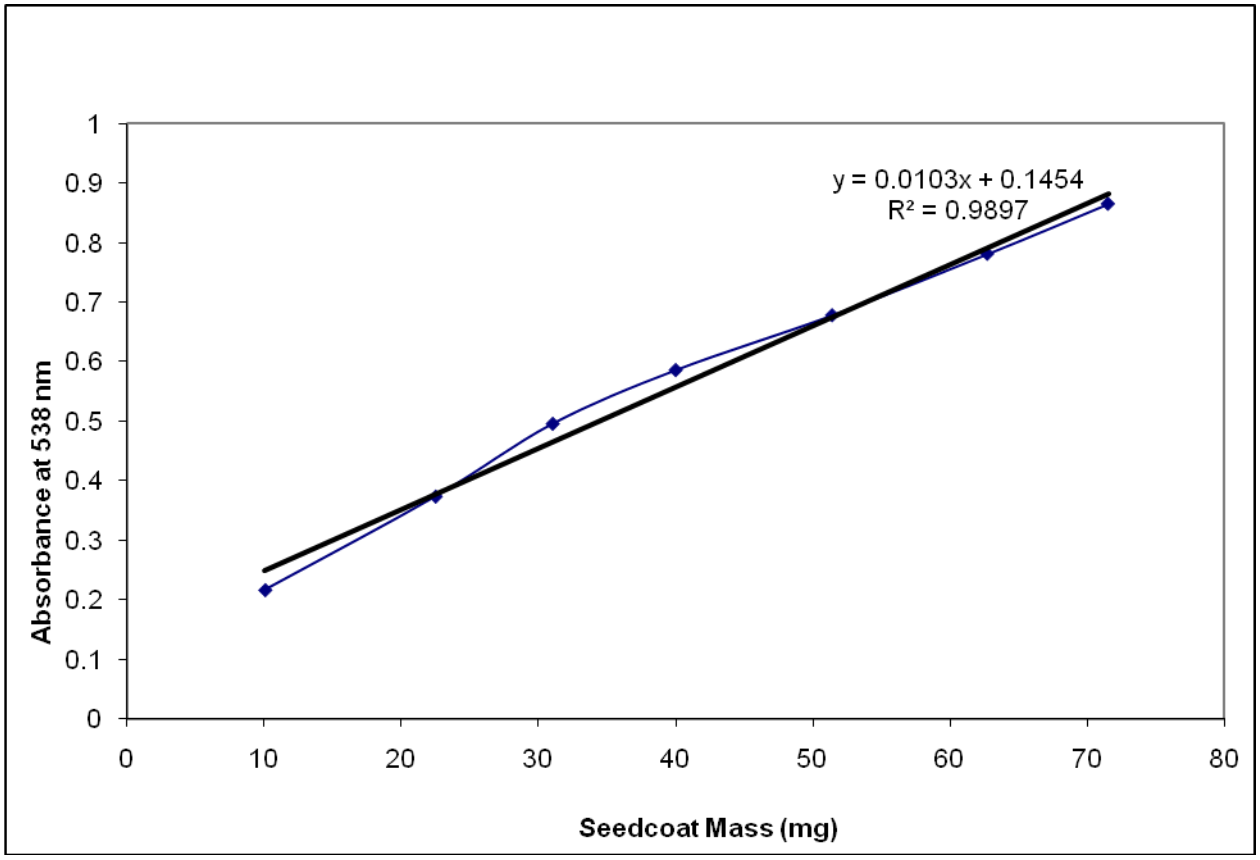


Figure 4.1 CT standard curve generated from mature ground freeze dried DOR364 seed coat tissue.

The semi-purified standard curve was generated by measuring out 4.492 mg of semi-purified CT prep into a test tube and subjecting this sample to the mini-BuOH-HCl assay described above. The sample was then separated into a subset of 5, 10, 15, 20, 25, 30, 35 and 40 uL solutions and 1 ml of BuOH-HCl was added to each subset; this resulted in a final dilution of 200, 100, 66, 50, 40, 33, 29 and 25x respectively. Absorbance values were then determined on the spectrophotometer at 538 nm to generate a standard curve (Figure 4.2). The absorbance

values of each sample were then used to quantify the approximate amount of CT present in each experimental sample using the linear regression equation of the standard curve. This standard could then be used to compare this set of experiments with other sets of experiments measured previously and in future provided a similar standard was used in other experiments.

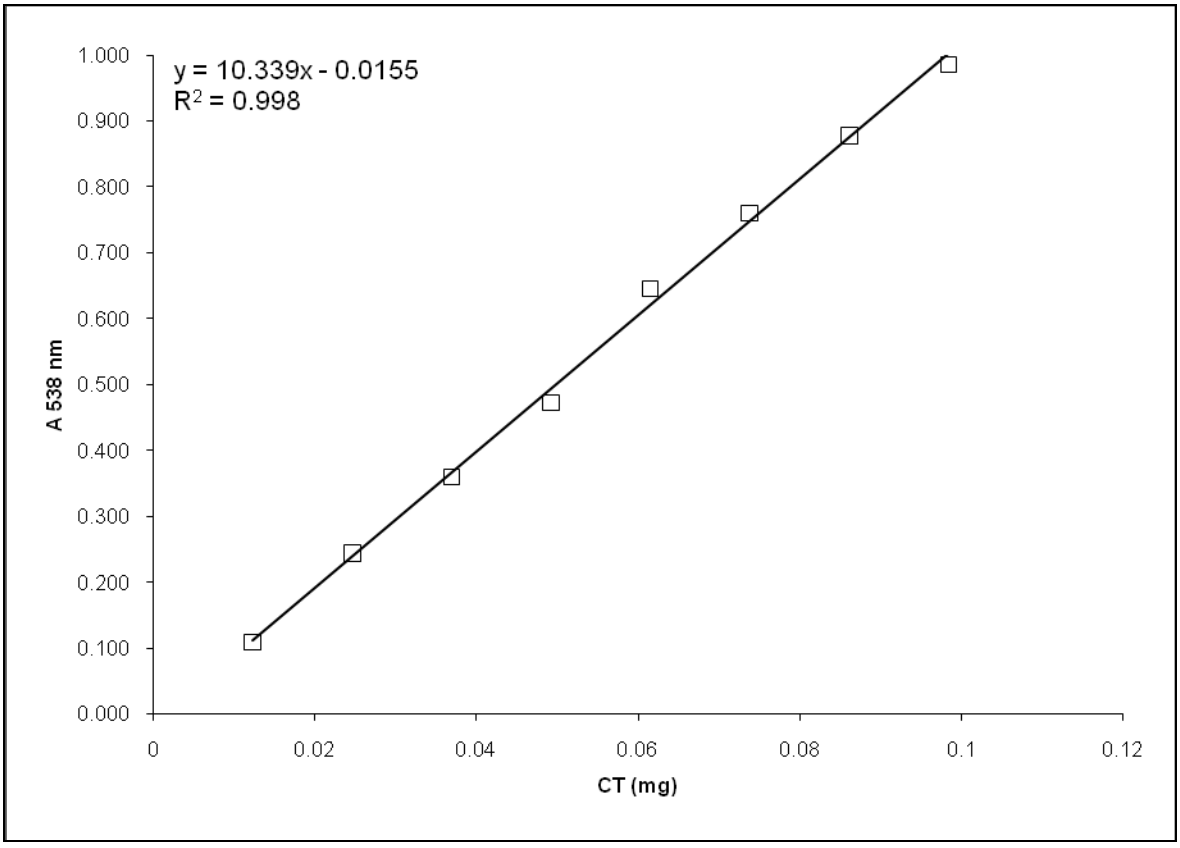


Figure 4.2 CT standard curve generated from semi-purified CT prep made from pinto bean seed coat extract.

4.2.3.5 Qualitative Assay

CT concentrations were also qualitatively assessed for each of the genotypes for each DAF using scanned images of the sample solutions in 96 well plates. Each scanned image contained (1) a check – pure BuOH-HCl, (2) solutions of mature DOR364 seed coat tissue at 5, 30, 60, 90 and 120 mg and (3) solutions of developing seed coats from 6 – 40 DAF for a given genotype (Figure 4.3). This procedure allowed for a visual demonstration of the quantification

results. The samples used for the qualitative assay were all standardized using 20 mg of each sample seed coat tissue and a dilution of 1:50 of the final CT solution. Equal volumes of solution from each sample were placed in every well of the 96-well plate, thereby ensuring that the scans were not variable due to differences in seed mass, dilution ratios or solution volume.

	A	B	C	D	E	F	G	H
1	BuOH-HCl					Developing seed coat (18DAF)		
2	Mature DOR364 5 mg					Developing seed coat (20DAF)		
3	Mature DOR364 30 mg					Developing seed coat (22DAF)		
4	Mature DOR364 60 mg					Developing seed coat (24DAF)		
5	Mature DOR364 90 mg					Developing seed coat (26DAF)		
6	Mature DOR364 120mg					Developing seed coat (28DAF)		
7	Developing seed coat (6DAF)					Developing seed coat (30DAF)		
8	Developing seed coat (8DAF)					Developing seed coat (32DAF)		
9	Developing seed coat (10DAF)					Developing seed coat (34DAF)		
10	Developing seed coat (12DAF)					Developing seed coat (36DAF)		
11	Developing seed coat (14DAF)					Developing seed coat (38DAF)		
12	Developing seed coat (16DAF)					Developing seed coat (40DAF)		

Figure 4.3 96-well plate layout for qualitative CT concentrations assessed in Figures 4.10 – 4.14.

4.2.4 Statistical Analysis

A statistical analysis of the DOR364 standard and the semi-purified CT standard was performed using Proc GLM of the SAS software (version 9.2, SAS Institute, Cary, NC). A regression of absorbance at 538 nm as the dependant variable and seed coat mass as the independent variable was conducted to obtain a linear regression equation for each standard. The R^2 value and linear equation for the DOR364 standard was 0.998 and ($Y = 10.339x + 0.0155$) respectively. The R^2 value and linear equation for the semi-purified standard was 0.9821 and ($Y = 0.0103x + 0.1454$) respectively. A histogram was generated for both data sets and in both cases the data was normally distributed.

The first experiment (DOR364, RIL89 and RIL58) was analyzed separately from the second experiment (CDC Pintium and 1533-15) due to differences in environmental growing conditions between the screen house and greenhouse.

A statistical analysis of technical replications was performed using Proc Mixed of the SAS software (version 9.2, SAS Institute, Cary, NC). There were no significant differences ($p >$

0.05) between the technical replications within each biological replication, therefore, technical replications were averaged and this average value was used in further analyses.

Statistical analyses of the variables DAF, genotype and biological replication was performed using a Proc Mixed repeated measures analysis (version 9.2, SAS Institute, Cary, NC). DAF, genotype and their interaction were classified as fixed effects. Biological replications and the interactions of biological replications with DAF were classified as random effects.

The DAF variable and DAF x genotype interaction was further partitioned into linear, quadratic and lack of fit regressions. An F-test (type 1 error of 0.05) was used to determine the significance of these effects. Proc Mixed was also used to compute least square means (lsmeans), standard errors (se) and to test for group effects (variation within groups of interest) by genotypes at each DAF through the use of variation slicing. Proc Univariate was used to test residuals for normality.

The initial analysis assumed (1) that the model was linear and additive and (2) the error terms summed to 0, were independent, random, homogeneous (common variance) and normally distributed. Plots of residuals by predicted, by treatment (DAF), by genotype and by block (replications) were used to test model and error assumptions. The data were found to be heterogeneous and a single model for all five genotypes combined was not possible due to the large degree of variability. For this reason a heterogeneous autoregressive order 1 (arh1) was used as the correlation structure in the repeated measures analysis with DAF as the repeated variable. Studentized residuals were compared to the critical values for Lund's test of outliers with a type 1 error of 0.05 (Bowley 2008 – adapted from Lund, R.W. 1975) and no outliers were found. Proc Nlin was used to compute coefficients of the regression equation and to develop a regression model that best described the response for each of the genotypes.

4.3 Results and Discussion

4.3.1 Seed Coat Mass

Seed and seed coat mass for all 18 time points were generated; both seed and seed coat mass across all 18 times points were highly correlated. The R^2 comparing seed coat and seed mass for DOR364, RIL89 and RIL58 taken collectively was 0.81 and that for CDC Pintium and

1533-15 taken collectively was 0.84. Therefore, only an analysis of seed coat mass throughout seed development was included in the discussion.

The seed coat masses of DOR364 and RIL89 were not significantly different from one another between 6 – 40 DAF ($P > 0.05$) (Figure 4.4). Seed coat masses of DOR364, RIL89 and RIL58 were not significantly different at 6, 8 and 10 DAF ($P > 0.05$). The seed coat mass of RIL89 was significantly lower than the seed coat mass of RIL58 between 12 – 40 DAF ($P < 0.05$). The seed coat mass of DOR364 was significantly lower than the seed coat mass of RIL58 between14 – 40 DAF ($P < 0.05$). Three asymptotes were observed for each of the three genotypes. The first asymptote appeared to be a linear increase in seed coat mass and was observed for DOR364, RIL89 and RIL58 between 6 – 20, 6 – 20 and 6 – 22 DAF, respectively. Following this increase, seed coat mass stabilized from 22 – 28, 22 – 32 and 24 – 30 DAF for the genotypes DOR364, RIL89 and RIL58, respectively and then a decrease in seed coat mass was observed from approximately 30 – 40 DAF for all three genotypes.

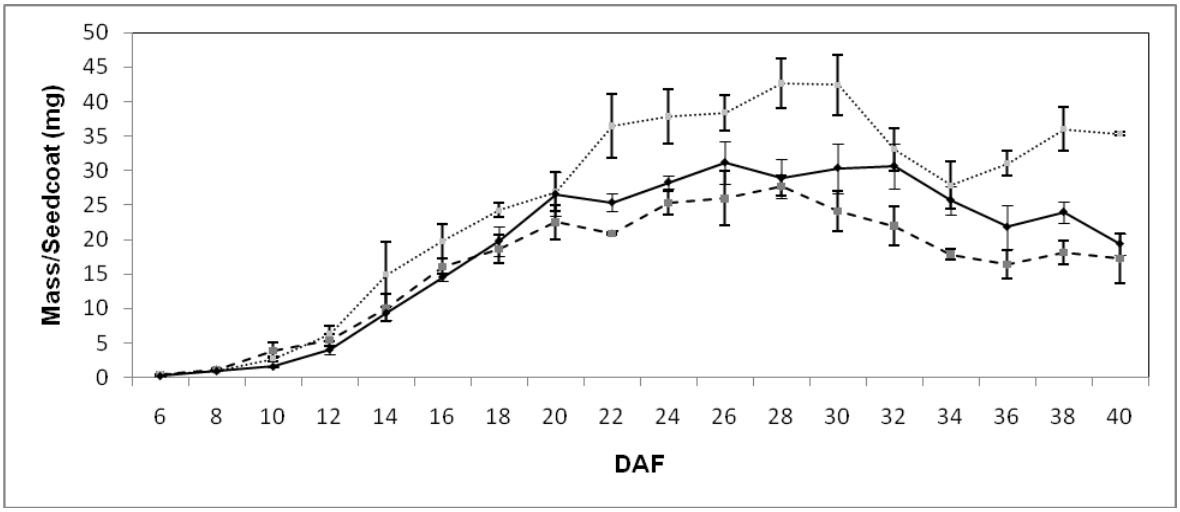


Figure 4.4 Dry developing seed coat mass for DOR364, RIL89 and RIL58 (averaged over three biological replications).

The seed coat masses of CDC Pintium and 1533-15 were not significantly different throughout development ($P > 0.05$) (Figure 4.5). Two asymptotes were observed: the first appeared to be a linear increase in seed coat mass and was observed between 6 – 24 DAF. Following this increase, a linear reduction in seed coat mass occurred from 26 – 40 DAF.

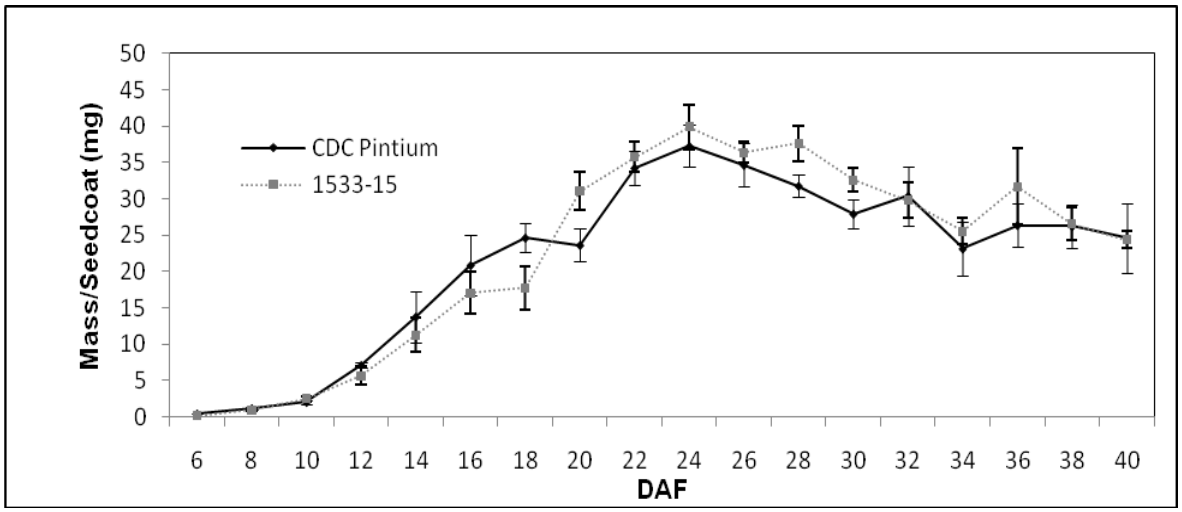


Figure 4.5 Dry developing seed coat mass for CDC Pintium and 1533-15 (averaged over three biological replications).

The development of seed coat mass for all five genotypes followed a quadratic polynomial trend. The initial increase in biochemical and structural components of the seed coat has likely caused the initial increase in seed coat mass. The stabilization phase in the genotypes DOR364, RIL89 and RIL58 and the turn over phase (peak of the quadratic regression) in the genotypes CDC Pintium and 1533-15 were likely due to the translocation of seed coat components within the seed coat or because the seed stopped growing and started to mature. The final reduction in seed coat mass was likely the result of the translocation of seed coat components into the cotyledons.

4.3.2 Seed Coat Mass and Condensed Tannin Content: Measuring Accumulation

Condensed tannin concentration explains the amount in a given mass of seed coat tissue as measured by the modified BuOH-HCl assay. CT content is obtained by multiplying the CT concentration by the average mass of the seed coat at a particular DAF. Therefore, CT content explains the amount of CT present in the entire seed coat. Knowing the amount of CT present in the entire seed coat is essential to evaluating accumulation trends since CT concentration may be diluted by sudden increases in seed coat mass. The observed differences between CT content and concentration are a function of changes in seed coat mass throughout seed development. To

demonstrate these differences a comparison between CT concentration vs. seed coat mass and CT content vs. seed coat mass in CDC Pintium was used (Figures 4.6 and 4.7). Because CT concentration is highly influenced by seed growth, a sudden increase in seed coat mass dilutes the CT in the seed coat making CT content appear to drop (Figure 6; 22 – 28 DAF) when in fact it is still accumulating (Figure 7; 22 – 28 DAF). CT content trends mirror seed coat mass trends (Figure 4.7). By contrast, CT concentration appears in some cases to be inversely related to seed coat mass (Figure 4.6). These differences should be taken into consideration when deciding which type of evaluation is best for the experimental objectives and hypotheses under investigation. It is clear that only CT content data should be used when describing the accumulation over time because CT content includes CT in the entire seed coat, whereas CT concentration only focuses on CT on a unit mass basis. CT concentration is best suited for comparing the same time point across genotypes and is particularly suited for relative comparisons of CT in mature tissue, but should not be used to monitor accumulation trends over time.

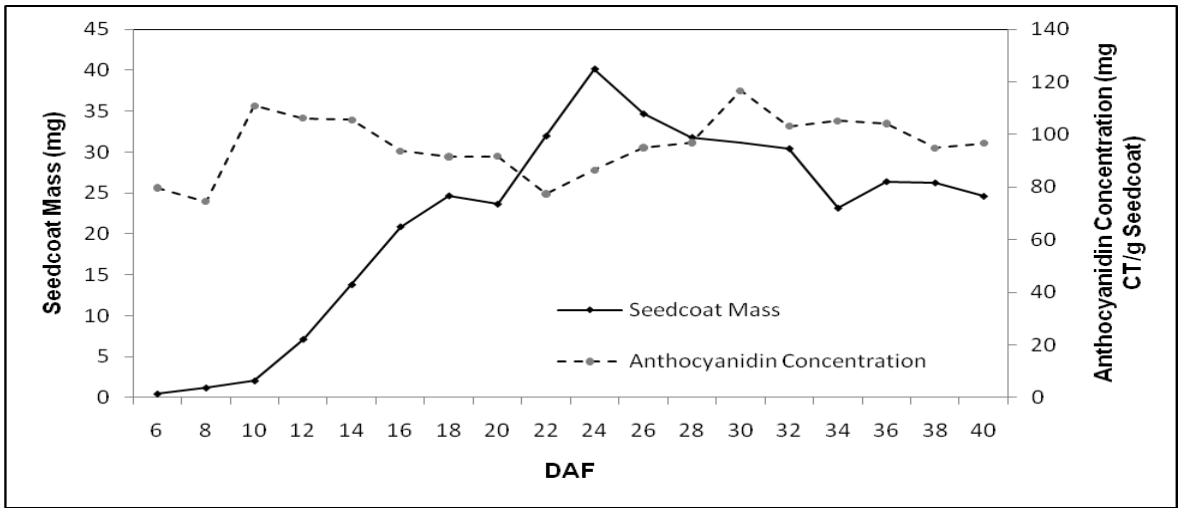


Figure 4.6 Condensed tannin concentration vs seed coat mass for CDC Pintium (averaged over three biological replications).

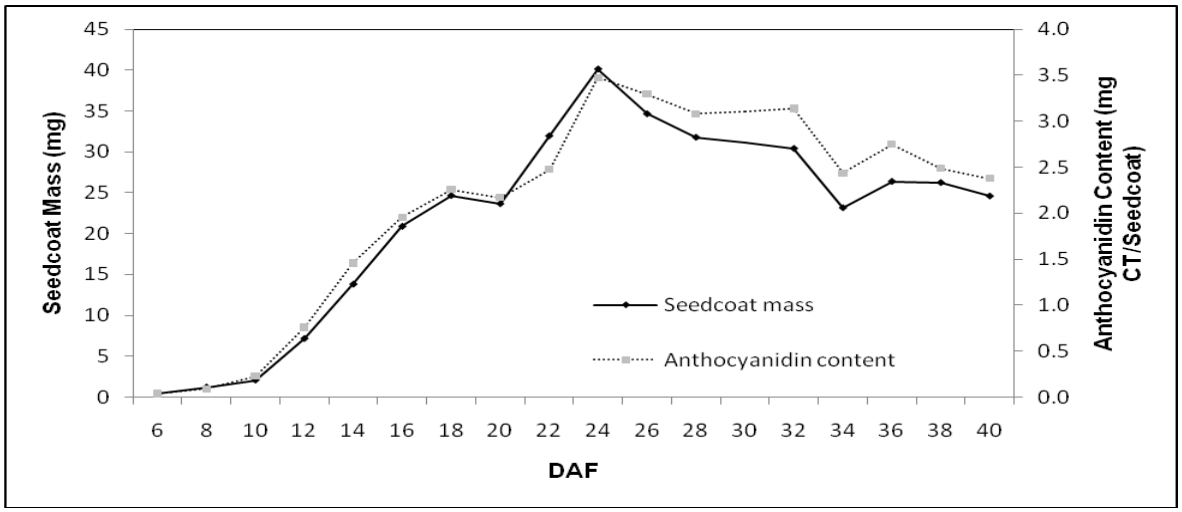


Figure 4.7 Condensed tannin content vs seed coat mass for CDC Pintium (averaged over three biological replications).

4.3.3 Qualitative Assessment of Condensed Tannin Concentration

Prior to the quantitative assessment of CT concentration and content each genotype was qualitatively assessed for CT concentration on a 96 well plate (Figures 4.8). These scanned solutions illustrated how CT concentration changed over time as was indicated by the colour of the solution. As the colour darkens, the amount of CT present in solution increases. The concentration trends that are quantitatively described below are apparent from these scans; differences in CT concentration can be seen both within each genotype as seed coat development progresses and between genotypes when comparing any given DAF.

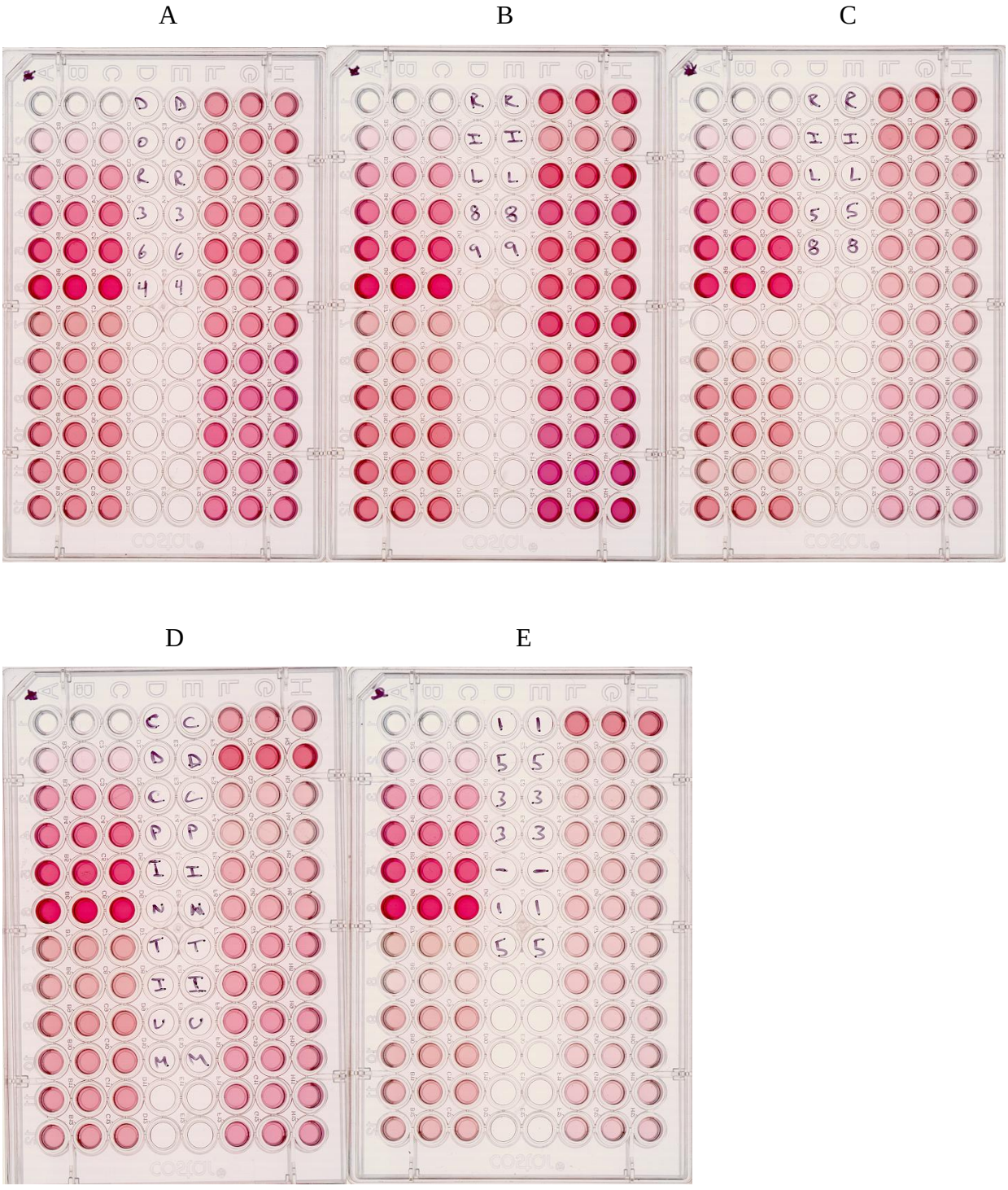


Figure 4.8 Qualitative assessment of CT concentration for DOR364 (A), RIL89 (B), RIL58 (C), CDC Pintium (D) and 1533-15 (E).

4.3.4 Condensed Tannin Trends During Seed Coat Development

CT content and concentration were calculated for both DOR364 and semi-purified CT equivalents and the results for both equivalents were highly correlated for each of the genotypes. The R^2 for sample values calculated with DOR364 equivalents compared to semi-purified CT for CT concentration and content in DOR364, RIL89 and RIL58 taken collectively were 0.99 and 0.98 respectively; R^2 for this same set of conditions in the CDC Pintium and 1533-15 experiment taken collectively were 0.99 and 0.94. Therefore, only the data generated using the semi-purified CT analysis was included in the discussion.

These correlations indicate that semi-purified preparations, which are technically difficult to make, are not necessary in CT evaluation if the objective is to only assess trends on a relative level. However, if CT quantification is required, semi-purified or purified CT would be necessary. Significant differences were found for the various genotypes, DAF, replications and their interactions for CT content and concentration ($P < 0.0001$) and each will be discussed separately below.

4.3.5 Condensed Tannin Content

In order to evaluate CT content, dry seed coat mass for all 18 time points/ genotype/ replication were recorded (Figures 4.4 and 4.5). This evaluation was made in order to determine if CT was accumulating throughout seed coat development or if what appeared to be an increase or decrease in CT was actually a function of seed coat growth and development. CT began to accumulate very early since it was already present at 6 DAF in all five genotypes. CT accumulated rapidly from 6 – 14 DAF for all five genotypes (Figure 4.9 and 4.10).

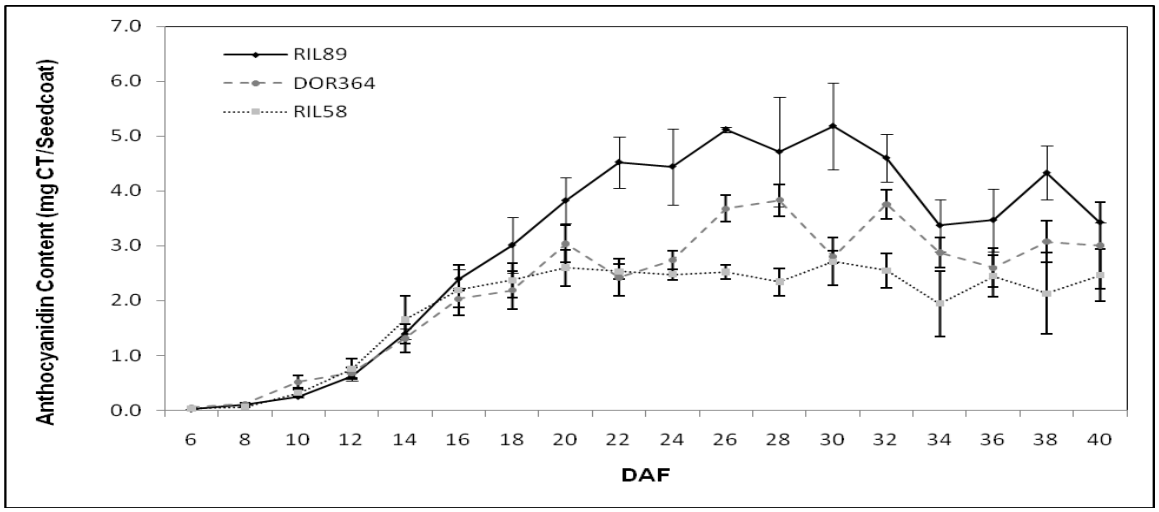


Figure 4.9 Condensed tannin content for DOR364, RIL89 and RIL58 (averaged over three biological replications).

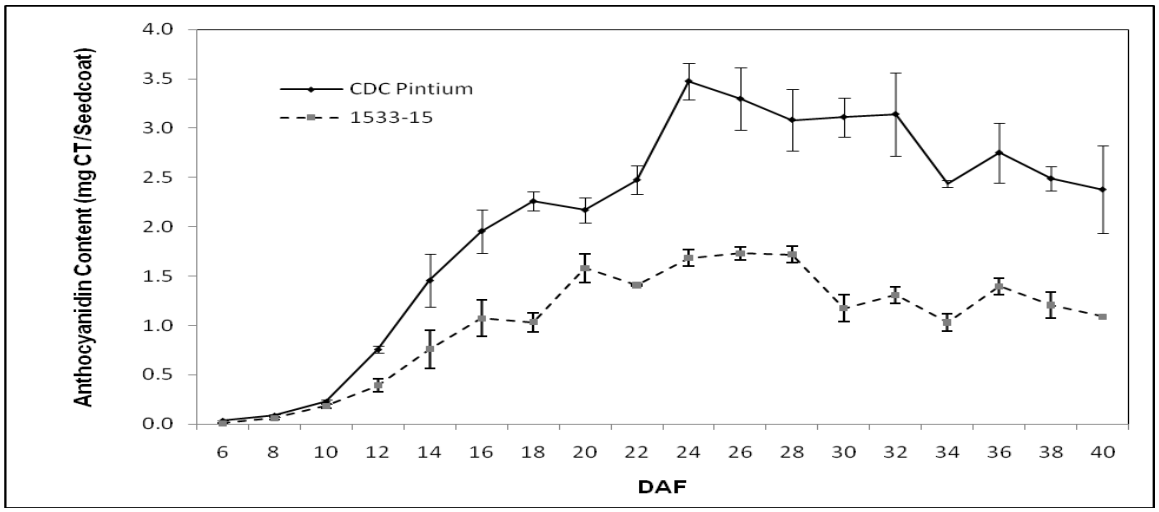


Figure 4.10 Condensed tannin content for CDC Pintium and 1533-15 (averaged over three biological replications).

The condensed tannin content of DOR364, RIL89 and RIL58 was not significantly different from 6 – 14 DAF ($P > 0.05$) (Figure 10); however, significant differences between these genotypes appeared 14 DAF where the CT content began to diverge. The CT content of RIL89 was significantly higher than that of DOR364 and RIL58 from 16 – 40 DAF ($P < 0.05$) and DOR364 had a higher CT content than RIL58 from 16 – 40 DAF. Three asymptotes were

observed for the CT content trend in RIL89. The first asymptote was linear and was observed between 6 – 22 DAF where CT content increased from 0.1 – 5.0 mg CT seed coat^{-1}. Following this increase, CT content stabilized from 22 – 30 DAF at approximately 5.0 mg CT seed coat^{-1} and then decreased from approximately 30 – 40 DAF where CT content dropped from 5.0 – 3.5 mg CT seed coat^{-1}. Only two asymptotes were observed for the CT content of DOR364 and RIL58. The first asymptote was a linear accumulation of CT content and was observed between 6 – 20 DAF for both genotypes. During this phase of accumulation CT content increased from approximately 0.0 – 3.0 mg CT seed coat^{-1}. This initial accumulation was followed by stabilization of CT content between 20 – 40 DAF in both genotypes at approximately 2.0 – 3.0 mg CT seed coat^{-1}.

CDC Pintium and 1533-15 did not differ in CT content from 6 – 10 DAF ($p > 0.05$) (Figure 11). Significant differences were noted between 6 – 14 DAF ($p < 0.0001$) even though the seed mass was not different between the two genotypes. CT accumulated rapidly from 6 – 24 DAF (0.0 – 3.5 mg CT seed coat^{-1}) and 6 – 20 DAF (0.0 – 1.50 mg CT seed coat^{-1}) for CDC Pintium and 1533-15, respectively. Following this initial accumulation, CT content stabilized between 20 – 30 DAF and 20 – 40 DAF for CDC Pintium and 1533-15, respectively. The last 10 DAF was marked by a reduction in CT for CDC Pintium where CT content dropped from 3.0 – 2.0 mg CT seed coat^{-1}.

Genotypes that had relatively high CT concentrations at maturity tended to have a high CT content throughout seed coat development when compared to moderate and low CT lines. CT began to accumulate early on in seed coat development in all five genotypes. Following this rapid increase one of three observations were recorded: (1) A slower increase, stabilization and then gradual decrease of CT content for those genotypes that were found to have relatively high CT at maturity, (2) a slower increase followed by stabilization for the moderate CT line or (3) a short increase followed by stabilization at a lower level for those genotypes that were found to have relatively low CT at maturity.

The initial accumulation between 6 – 14 DAF in all five genotypes was likely due to a higher ratio of CT production to CT binding in seed coats. Stabilization of CT content in the moderate and low CT lines was likely due to equilibrium between CT production and binding. The CT stabilization stage coincides with maximum seed coat size where seed coat mass also

stabilizes; this is the point where the seed has switched from growth to maturation. Finally, the reduction of CT content at 30 DAF in RIL89 and CDC Pintium was likely due to a higher ratio of CT binding to CT production and coincides with the drying down of the seed and seed coat. These trends imply that the rate of CT accumulation coincides with the rate of change of seed and seed coat mass.

4.3.6 Condensed Tannin Concentration

No significant differences in CT concentration were observed between RIL58 and DOR364 from 6 – 16 DAF ($P > 0.05$) (Figure 4.11); however, from 18 – 40 DAF, DOR364 had consistently higher CT concentrations than RIL58 ($P < 0.05$). By contrast, RIL89 had significantly higher CT concentrations than DOR364 from 6 – 16 DAF ($P < 0.05$) and no significant differences in CT concentrations were observed between these two genotypes from 18 – 40 DAF ($P > 0.05$). RIL89 and RIL58 had significantly different CT concentrations throughout the entirety of seed coat development ($P < 0.05$), except at 6 DAF where no differences were observed ($P > 0.05$).

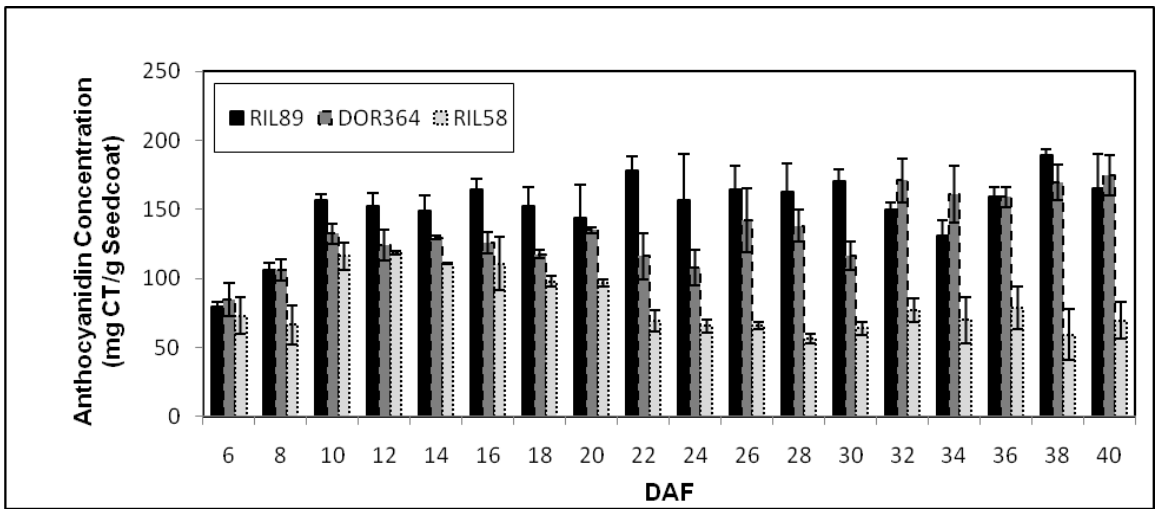


Figure 4.11 Condensed tannin concentration for DOR364, RIL89 and RIL 58 (averaged over three biological replications).

CDC Pintium maintained higher CT concentrations than 1533-15 over all time points ($p < 0.0001$) (Figure 4.12).

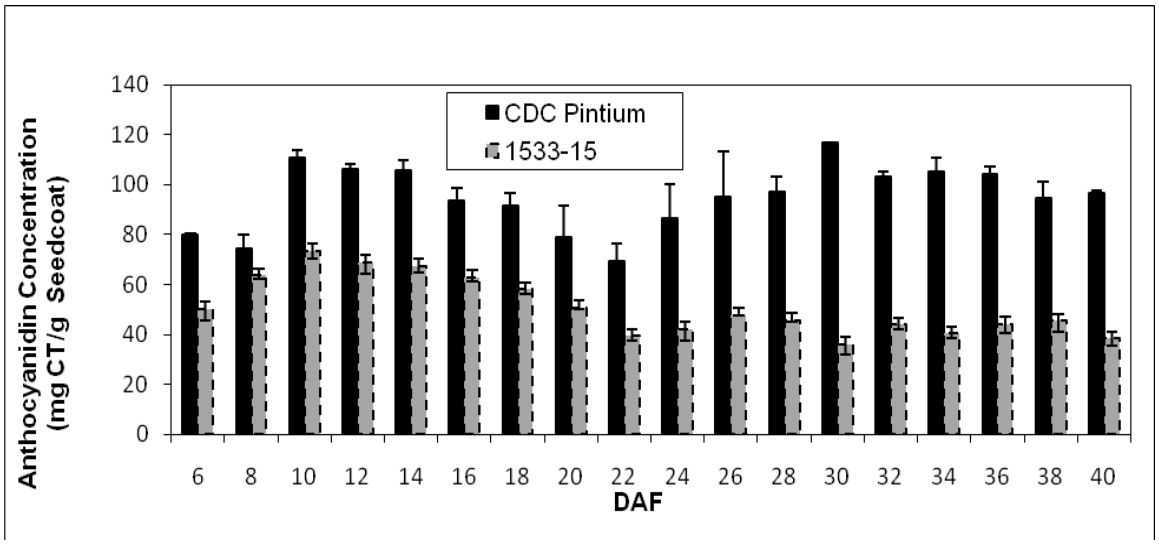


Figure 4.12 Condensed tannin concentration for CDC Pintium and 1533-15 (averaged over three biological replications).

4.3.7 Comparing Condensed Tannin Concentration and Content Trends

Although CT concentration should not be used to evaluate the accumulation of CT, a brief discussion of CT concentration over time will be used to illustrate the problems associated with using CT concentrations to measure accumulation as compared to CT content. Therefore, line charts will be used for CT concentration over time in order to demonstrate how incorrect conclusions of CT accumulation may be generated if CT concentration is used for evaluating accumulation rather than CT content.

Two asymptotes were observed for the CT concentration trend in RIL89 (Figure 4.13). The first asymptote was linear where an increase in CT concentration, from 80 – 150 mg CT g^{-1} tissue, was observed between 6 – 10 DAF. Following this increase, CT concentration stabilized at approximately 150 mg CT g^{-1} tissue from 12 – 40 DAF. Three asymptotes were observed for the CT concentration trends of DOR364 and RIL58. As for DOR364, the first asymptote was a linear increase from 6 – 10 DAF where CT concentration rose from approximately 80 to 130 mg CT g^{-1} tissue. The second asymptote was stabilization at approximately 130 mg CT g^{-1} tissue from 10 – 30 DAF. Finally, CT concentrations increased once again from 130 – 150 mg CT g^{-1}

tissue between 30 – 40 DAF in DOR364. As for RIL58, the first asymptote was also a linear increase from 6 – 10 DAF, however the increase was less than that of DOR364, where CT concentrations only reaching approximately 115 mg CT g^{-1} tissue. The second asymptote was a linear reduction from 10 – 22 DAF where CT concentrations dropped from 115 – 60 mg CT g^{-1} tissue and finally CT concentrations stabilized in RIL58 at 60 mg CT g^{-1} tissue between 22 – 40DAF.

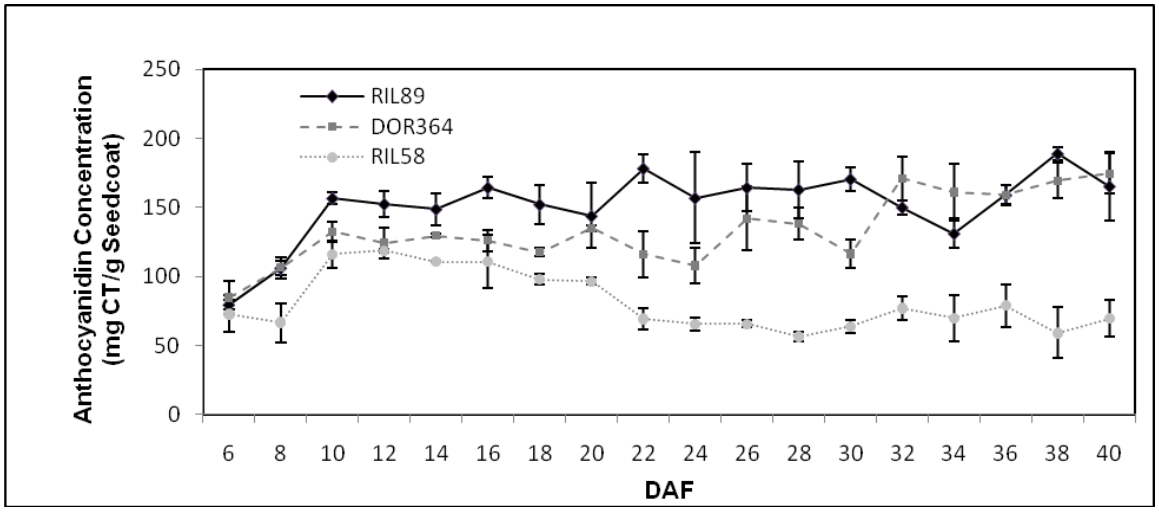


Figure 4.13 Condensed tannin concentration for DOR364, RIL89 and RIL 58 (averaged over three biological replications).

CT concentration increased rapidly from 80 – 110 and 50 – 70 mg CT g^{-1} of tissue between 6 – 10 DAF in CDC Pintium and 1533-15 respectively (Figure 4.14). Following this increase, CT concentrations declined from 110 – 70 and 70 – 40 mg CT g^{-1} of tissue between10 – 22 DAF in CDC Pintium and 1533-15 respectively. CT concentration then stabilized between 22 – 40 DAF in 1533-15 at approximately 40 mg CT g^{-1} tissue. By contrast, there was a second increase in CT concentration, from approximately 70 – 100 mg CT g^{-1} tissue between 22 – 40 DAF in CDC Pintium.

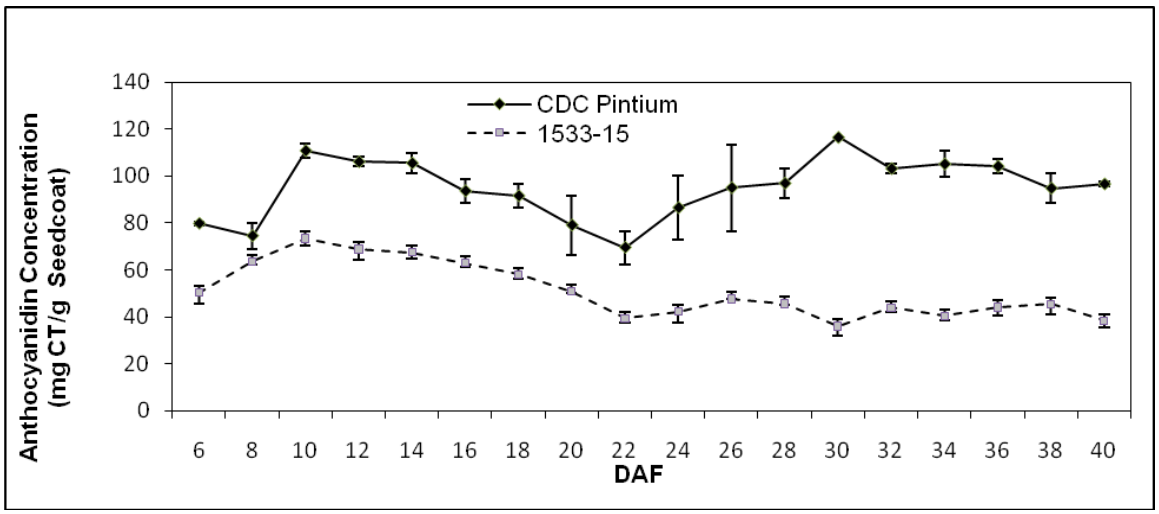


Figure 4.14 Condensed tannin concentration for CDC Pintium and 1533-15 (averaged over three biological replications).

Results demonstrated that CT concentration increased very early on in the developing seed coat of common bean, namely from at least 6 DAF in all five genotypes (Figure 4.13 and 4.14). It was also determined that CT concentration increased quite rapidly from 6 – 10 DAF for all five genotypes. Following this rapid increase one of three observations were recorded: (1) a slower increase or stabilization of CT in the seed coat was apparent from 14 – 40 DAF for those genotypes that were found to have relatively high and moderate levels of CT at maturity, namely RIL89 and DOR364; (2) by contrast, genotypes that were found to have low concentrations of CT at maturity, RIL58 and 1533-15, showed declines in CT concentration from 14 – 40 DAF; or (3) a decrease followed by an increase in CT concentration occurred in the genotype CDC Pintium. As in the case of CT content, genotypes that had relatively higher CT concentrations at maturity tended to have higher concentrations throughout seed coat development.

4.3.8 Environmental Variability

Condensed tannin accumulation in developing seed coats is likely highly influenced by environmental factors such as temperature, precipitation, humidity and light duration and intensity. The environmental conditions for each of the three biological replications for all five genotypes were different; the environmental conditions between the two experiments were also significantly different so treated separately. Seed coat mass and CT accumulation trends varied

slightly from one biological replication likely to the next due to environmental differences between biological replications.

In the DOR364, RIL89 and RIL58 experiment, the first biological replication was planted at the peak of the dry season. The plants emerged and began to flower in the hot dry sunny months of March. Seed coats used for evaluation were harvested from these plants in the hot dry season. The second replication was planted at end of the dry season, plants emerged in the hot dry sunny months of March and began to flower the beginning of the cool wet season; harvesting of this replication was conducted throughout the wet season. The third replication was planted at the beginning of the wet season; the plants emerged in the cool wet month of April and began to flower during the transition period between the cool wet season and the approaching hot dry season. The seed coats from the third replication were harvested during this climate transition where cloud cover and precipitation were intermediate between the first and second replications.

The CDC Pintium and 1533-15 experiment was planted at exactly the same time as the DOR364, RIL89 and RIL58 experiment, but was carried out in a more controlled environment - the quarantined greenhouses at CIAT. The major difference among the replications of the greenhouse growout was sunlight intensity. The first replication was planted, flowered and was harvested during the sunny season; the second replication was planted in the sunny season, however, flowering and harvest commenced in the cloudy season. The third replication was planted in the cloudy season and flowering and harvesting occurred during a period where cloud cover was variable.

On average, screen house temperatures were higher and a larger diurnal range was apparent throughout all three replications when compared to the greenhouse environment. Precipitation in the screen house was more frequent compared to the greenhouse environment for all three replications. The glass walls and roof of the greenhouse permitted more intense sunlight to penetrate the plants of the greenhouse growout when compared to that reaching the screen house growout. Pests were more problematic in the screen house, however, were unlikely to have altered experimental results.

DOR364, RIL89 and RIL58 were grown in a screen house, primarily an outdoor environment, which may have contributed to a higher degree of variability in seed coat mass and CT content and concentration than observed with CDC Pintium and 1533-15, which were grown

in a greenhouse. As for the three genotypes grown in the screen house, the hot, dry, sunny conditions of the first replication resulted in earlier development of the seed coat and a faster rate of CT accumulation when compared to the cool, wet, cloudy conditions of the second biological replication, where seed coat mass and CT content were both delayed by several days. This trend, however, was not as pronounced in the two genotypes grown in the greenhouse where temperature and moisture conditions were more consistent for all three replications. This resulted in a more accurate assessment of genotypic differences between genotypes in the greenhouse when compared to the screen house growout. This trend was evident by (1) the reduced variance around the means of CDC Pintium and 1533-15 when compared to DOR364, RIL89 and RIL58 and (2) the earlier divergence and greater significant difference in CT content and concentration between CDC Pintium and 1533-15 when compared to DOR364, RIL89 and RIL58. The later of the two explanations is particularly noteworthy as DOR364, RIL89 and RIL58 were much more variable in CT concentration at maturity compared to CDC Pintium and 1533-15. Therefore, genotypes grown in the screen house were expected to have CT concentrations and contents that had greater significant differences and diverged at earlier DAF compared to the two greenhouse genotypes. These results illustrate that seed coat biochemistry may change drastically due to changes in the environment.

Fluctuation in CT content and concentration between sampling dates were observed frequently; in some cases the fluctuations were drastic and resulted in large differences in CT trends over a very short period of time. These fluctuations may be a result of several factors, including: (1) environmental variability between DAF, (2) the relative health of plants, (3) the number of developing pods on a single plant (4) the relative position of the pods on the plant – particularly in indeterminate genotypes, (5) the exact time of harvest (i.e. hour after sunrise) of the pods from plants and (6) the amount of time that seed coats were handled outside of the freezer or before freeze drying. These large fluctuations further illustrate the effect of environmental factors on CT accumulation during seed coat development.

4.4 Summary and Conclusion

Phytochemicals found in the seed coat of common bean play an important role in the biochemical and physiological development of this important crop. The biochemical and genetic factors responsible for the accumulation of CT throughout seed coat development are not well understood. There was a need to understand the pattern of CT accumulation throughout seed coat development in order to make inferences on how CT can be phenotypically and genetically characterized in common bean.

CT content describes the amount of CT in an entire seed coat, whereas CT concentration describes the amount of CT in a unit mass of seed coat. CT concentration has been incorrectly used by some researchers to describe accumulation trends. CT concentration is not suited for evaluation of accumulation trends over time; rather, CT content is necessary for describing changes in CT throughout seed coat development. CT concentration is best suited for evaluating CT across genotypes at a single point in time. Because CT content is dependent on the mass of the entire seed coat it is important that investigators measure seed coat mass accurately prior to using it for evaluation of CT or other compounds. Seed coat mass is, therefore, an important tool when measuring CT content with the intent to identify accumulation trends over time. Seed coat mass should always be measured on a dry weight basis when dealing with maturing seed coats because environmental factors may cause rapid changes in seed coat water content between seeds of the same genotype at any given DAF.

CT accumulates as early in low CT genotypes as in high CT genotypes. CT content stabilizes after 14 DAF in low CT genotypes. By contrast, CT content decreases slightly after reaching a peak in high CT genotypes. It is apparent that the production of CT is not delayed in low CT genotypes as was hypothesized at the start of these experiments. Rather, CT were present at very early stages of seed coat development in both high and low CT genotypes and likely became bound to other molecules or stopped being produced as the progression to seed coat maturity resumed. Following this accumulation, stabilization in CT content occurred in the low and moderate CT lines, namely DOR364, RIL58 and 1533-15, and a reduction in CT content in the higher CT lines of each experiment, namely RIL89 and CDC Pintium. The initial accumulation from 6 – 14 DAF in all five genotypes was likely due to a higher ratio of CT production to CT binding or production of CT and an absence of CT binding. Stabilization of CT

content in the moderate and low CT lines is likely due to equilibrium between CT production and binding. Finally, the reduction of CT content at 30 DAF in RIL89 and CDC Pintium was likely due to a higher ratio of CT binding to CT production or an absence of CT production and continuation of CT binding.

It is important to note that CT content is strongly influenced by environmental factors. Sudden or gradual changes in environmental conditions may result in drastic changes in seed coat biochemistry. Because there is a need for a relatively large amount of tissue to conduct CT assays, a large number of seeds and seed coats must be harvested. Therefore, it is often the case that seed is obtained from multiple plants or from the same plant at different days to ensure enough seed coat is available for evaluation. Therefore, it is crucial that environmental conditions are kept as consistent as possible throughout the entirety of experiments to reduce environmental variability. Reducing environmental variation will help identify genotypic differences more accurately. This phenomenon was demonstrated in the CDC Pintium and 1533-15 experiment, where both genotypes were grown in a greenhouse where environmental conditions were much more consistent compared to the outdoor environment of the screen house where DOR364, RIL89 and RIL58 were grown. This resulted in a more accurate assessment of genotypic differences between lines as was observed by an increased variance around the mean of the screen house growouts when compared to the greenhouse grow outs.

CHAPTER 5 : GENERAL DISCUSSION AND CONCLUSIONS

5.1 Seed Coat Post Harvest Darkening: The Quantitative Range

Junk et al. (2007) conducted a quantitative inheritance study of PHD using the RD x SD cross CDC Pintium x 1533-15. In this study seed coats were darkened using UVC light and then quantified on a colorimeter using **L** values (0 = black, 100 = white), as a measurement of the relative darkening of the seed coat. A frequency distribution was generated from this quantitative data for a set of RILs and segregation clearly 1RD:1SD with distinct bimodal distribution (Junk et al. 2007). However, it was noted that within the distinct RD and SD classes there was a fairly wide distribution of **L** values ranging from 28 – 33 for RD RILs and from 35 – 42 for SD RILs (Figure 5.1).

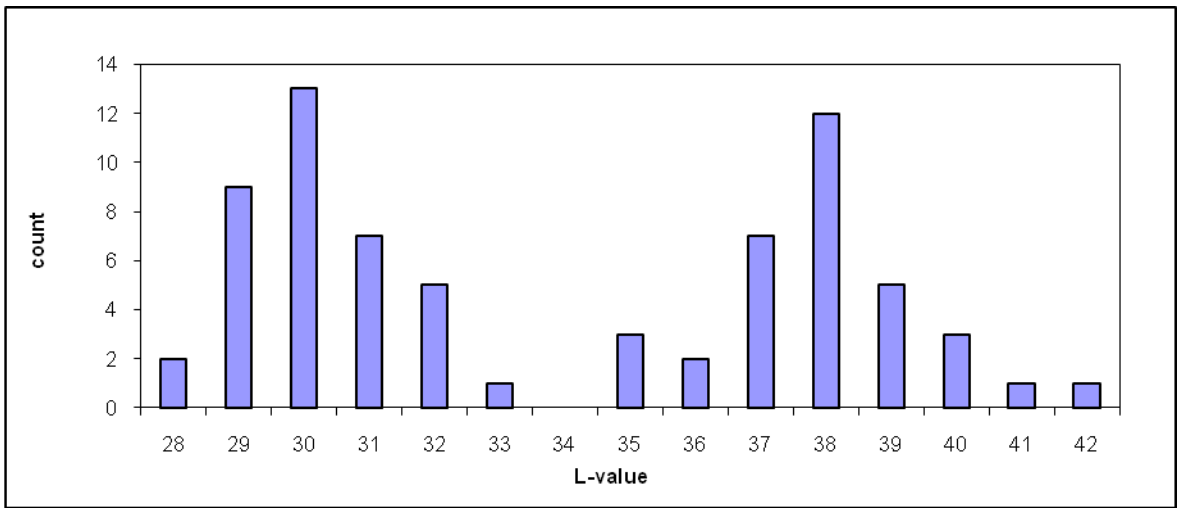


Figure 5.1 Hunter Lab L-value colorimeter value frequency distribution of the RILs from the cross CDC Pintium x 1533-15. Adopted from Junk, 2005.

This distribution indicates that a factor, other than the single gene model for PHD suggested from their inheritance study, was influencing PHD and causing a quantitative range within each PHD class. One reason for this distribution is inconsistent light intensity within the UVC chamber. However, a more likely reason for the range is the occurrence of seed coat compounds that may be causing variability in seed coat colour prior to and after the seed coats have been subject to UVC lights.

5.2 Background Seed Coat Colour in Pinto Beans: Is it a Function of CT Oxidation?

Aged seed coats of CDC Pintium, a RD bean, had reduced concentrations of kaempferol compared to newly harvested seed coats (Beninger et al., 2005). The same study also found that CDC Pintium had higher concentrations of seed coat kaempferol when compared to both aged and non-aged seed of 1533-15, a SD bean. Marles et al. (2008a) showed similar results when comparing CDC Pintium and 1533-15 as well as RILs from this population and observed that the RD seed coat of CDC Pintium and RD RILs had increased levels of kaempferols compared to 1533-15 and the SD RILs. Therefore, they suggested that concentration of kaempferol is at least partly responsible for the degree of PHD; higher concentrations of kaempferol caused beans to darken rapidly while lower levels gave rise to SD beans.

Elevated POD and PPO activities were found in RD beans when compared to SD beans both prior to and after eight months of storage (Moura et al., 1999). Aged, darkened common bean seed coats have also been found to have elevated levels of PPO and POD activity (De Oliveira Rios et al., 2002). Marles et al. (2003) had similar results when comparing PPO activity in the seed coats of CDC Pintium, 1533-15 and the RILs from a cross between these two genotypes. Therefore, PPO and POD activity may be at least partially responsible for the degree of PHD.

There seems to be no direct correlation between CT concentrations and the classification of pinto beans into RD and SD types (Marles et al., 2008a). However, indirect correlations between PHD and CT may be causing the range within each PHD class that was observed by Junk et al. (2007). This indirect correlation may be explained by an interaction between kaempferols, quinone, CT and PPO, which may be undergoing a series of reactions causing the background colour of common bean seed coats (Marles et al., 2008a) to be variable both before and after exposure to UVC light or under high temperature, moisture or natural light conditions.

Flavanols oxidize to form colour byproducts, therefore, seed coat colour changes during storage may be due to kaempferol levels (Jimenez et al., 1998; Jiménez and Garcia-Carmona 1999). Recent experiments of kaempferol, CT and PPO activity in common bean seed coats conducted by (Marles et al. 2008a**)** suggested these biochemicals function in PHD. They found that RD pinto bean genotypes had higher kaempferol and PPO concentrations than did SD pinto bean genotypes. PPO-mediated reactions may be interacting with flavonols and contributing to

post harvest colour changes (Marles et al., 2008a). This may imply that RD genotypes, which had higher kaempferol concentrations at maturity when compared to the SD genotypes, may be converted to darkened pigmentation due to a greater pool of PPO in RD genotypes (Marles et al., 2008a). Decreases in post-harvest kaempferol concentrations in aged parental pinto bean seed coats (Beninger et al., 2005) may explain the results of Marles et al. (2008a). PPO-mediated reactions also give rise to quinone, which may effect seed coat colour changes during storage as they may modify protein moieties and other flavonoid polymers like CT (Hernandez et al., 2006; Mayer, 2006; Guyot et al., 1996).

At this point it can only be speculated that PPO may also be interacting with other compounds and effecting seed coat colour. Such an interaction may be causing the clear colourless CT in the seed coat to turn into a creamy or light brown colour. Therefore, bean lines with elevated concentrations of CT, kampferol and quinone may be darker than those lines with lower concentrations of these compounds in their seed coats prior to exposure to UVC light. Upon exposure to UVC light the darker coloured seed coats would darken more readily when compared to the lighter coloured seed coats. This phenomenon may explain the range within each PHD class that was observed in the findings of Junk et al. (2007).

5.3 Difficulty Phenotyping Post Harvest Darkening Due to Seed Coat Background Colour

Crosses made between ND x ND common bean resulted in F_2 progeny that were all ND; however, a wide range in seed coat background colours was noted in the progeny (Figure 5.2; left). Some of the progeny had very white seed coats, while others had creamy brown seed coats. In several of the crosses made between ND x RD and SD x RD common bean F_1 progeny were all RD, however, a wide range of RD phenotypes were noted in the progeny (Figure 5.2; right). Some of the progeny had RD seed coats that were very dark, while others had RD seed coats that, although were darker than the normal SD phenotype, were still relatively lighter than some of the darker RD progeny.

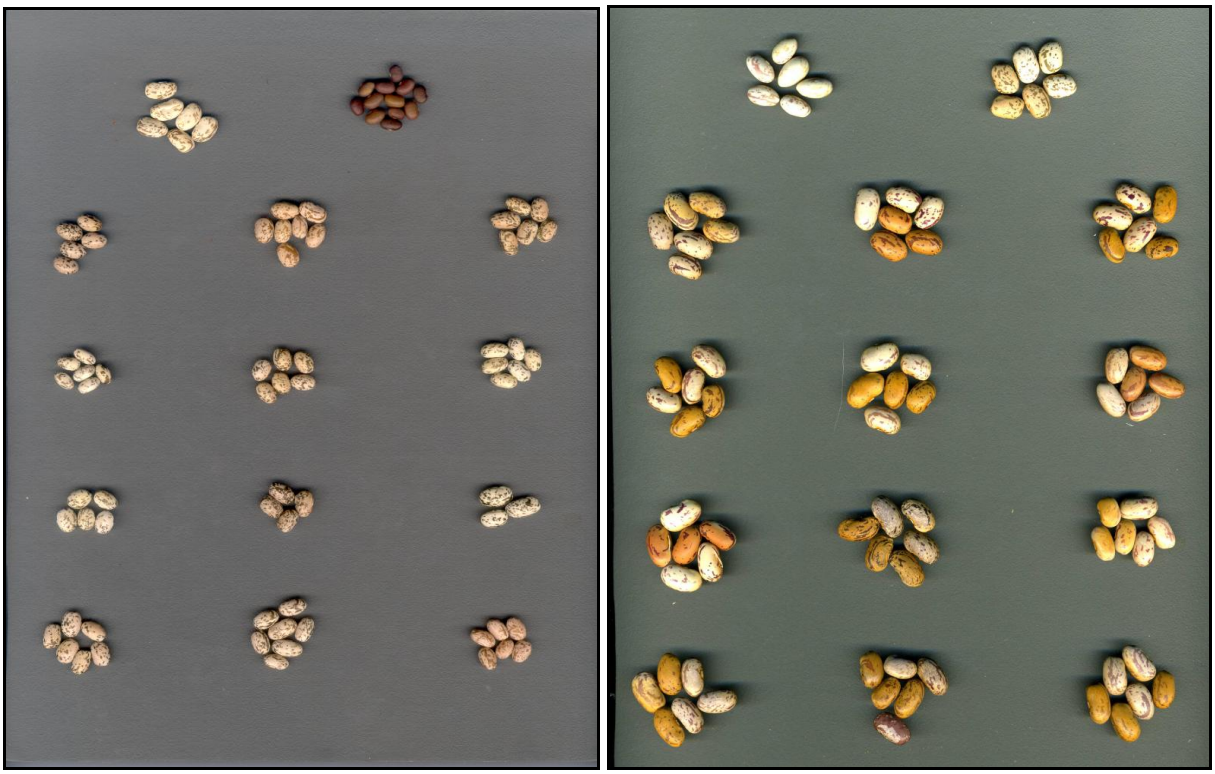


Figure 5.2 F_2 progeny of cross between KVxUI-1 and PI 608688 (ND x jj tester) (left) – all progeny have ND seed coats. F_2 progeny of cross between Wit-rood and 1533-15 (ND x SD) (right) – all progeny have RD seed coats.

The range in ND phenotypes prior to exposure to UVC lights and the range in RD phenotypes after exposure to UVC lights may be correlated. Common bean seed coats that are darker prior to UVC light exposure are expected to have darker seed coats once they have been exposed to UVC lights. It may be possible that the seed coats that were darker prior to exposure to UVC lights had elevated CT and kaempferols or other flavonoids, resulting in elevated oxidative reactions that triggered the oxidation of CT prior to UVC light exposure; therefore, resulting in a range of seed coat darkening phenotypes within the SD and RD phenotypes.

It is, therefore, suggested that CT are indirectly responsible for PHD. The indirect relationship is one that may be visualized prior to UVC light exposure and is only causing variation within each PHD phenotype. Therefore, CT does not seem to be directly correlated to PHD and, presumably, does not cause a large enough effect in seed coats that would result in a mis-identification of the three major PHD phenotypes (RD, SD and ND).

5.4 Reducing Phenotypic Errors in PHD by Choosing Low CT Lines

If CT are responsible for the variation observed within each PHD phenotype then choosing low CT lines would be useful in inheritance studies and in breeding efforts targeted at introducing SD genotypes. The use of low CT lines would enable more accurate phenotyping of the PHD trait, therefore, reducing phenotypic errors associated with mis-identification of PHD phenotypes. This may be particularly useful when evaluating crosses that produce populations with a large range of CT in their seed coats.

It is suggested that a more in depth study of CT content in PHD phenotypes be undertaken. Several ND x ND populations segregating for seed coat background colour are available at the U of S and could be analysed for CT content. Such a study would identify whether the difference between the white and creamy background colours in genotypes prior to exposure to UVC light is due to CT or some other compound(s). If CT is not found to be the cause of the variability in background seed coat colour, an analysis of kaempferols, quinones and PPO activity may explain the difference. However, it is important to note that the range in seed coat background colour may be a function of CT, kaempferols, PPO, quinones and possibly other compounds or enzymes interacting together. Therefore, the analysis may not be simple, particularly if one of the compounds is present in only small amounts and acting as a limiting factor in the progression of the darkening of the background colour. CT, kaempferols, quinones and PPO may all be present in the seed coat in sufficient quantities to trigger the darkening of the background colour. However, if one or more of these compounds are missing, the background darkening reaction of the seed coat may not progress.

A more appropriate tool for reducing phenotypic error during PHD phenotyping would be to develop a molecular marker for the SD PHD phenotype and possibly for the ND phenotype as well. Breeders could then use MAS to identify the different PHD phenotypes and reduce errors due to G x E interactions and the interaction of different compounds and enzymes within the seed coat, which may be indirectly contributing to the PHD trait. Unfortunately, using MAS for selecting desirable PHD phenotypes does not account for the background seed coat colour. This scenario is problematic because, although SD genotypes can be genetically identified, the variability in darkening within the SD phenotype cannot be determined. This would lead to

potentially undesirable variation within the SD phenotype where some seed coats would be much darker than others. Therefore, a certain amount of variability would still have to be eliminated in the selection process in order to standardize the SD seed coat phenotype because most markets expect seed coats that are uniform in colour.

5.5 Future Work: Seed Coat Post Harvest Darkening

5.5.1 Two Major Genes or Quantitative Trait Loci?

Although it appears that PHD is controlled by 2 major genes, it is likely that additional genes or alleles may also be involved in regulating the expression of this trait. Observed phenotypes from reciprocal populations made from the parents Bayo Mochica and Bayo Florida were more indicative of quantitative genes. Future experiments should be designed to ensure even exposure to all phenotypes and should use a colorimeter to quantify the level of PHD as well as qualitative evaluation as a means of confirming the data from this inheritance study.

5.5.2 Alteration of Market Class Due to Reduced Seed Coat Darkening

In some instances crosses between different market classes of beans are necessary in order to incorporate SD or ND phenotypes. It was observed in some of the inter- market class crosses made in this study that progeny were not appropriate for marketing. For example, crosses between Wit-rood and 1533-15 only produced true cranberry beans when RD seed coats were observed; whenever ND phenotypes were observed the classic cranberry patterning was either very pale or a mixture of pinto and cranberry and only a few SD cranberries had normal patterning. In another example, the progeny of crosses between the PI lines and the pinto lines were often discoloured with unmarketable patterning and in many cases impossible to phenotype for PHD. This study identified ND and SD populations that can be easily phenotyped and are more appropriate to use for genetic studies and breeding for the PHD trait when compared to the PI lines. Future genetic studies and breeding associated with PHD of common bean and other crops should take these effects into consideration. Further investigation into the seed coat colour pattern changes and the outcome of other crosses from a variety of market classes may be beneficial to future breeding efforts and genetic studies.

5.5.3 Post Harvest Darkening in Other Crops

Understanding the genetic control of PHD of common bean may help geneticists and breeders trying to understand the genetic control of seed coat or testa darkening in other pulses, wheat, fruits, vegetables, beverage commodities and other crops. It is recommended that a range of genotypes from an assortment of crops, which are subject to seed coat or testa darkening be exposed to UVC lights and evaluated for darkening. A more comprehensive understanding of the genetics of seed coat darkening will be obtained as more crops are evaluated for PHD from a genetic perspective.

5.5.4 Developing a Molecular Marker for the PHD Trait

The molecular characterization of ND, SD and RD parental lines used in this study were useful in identifying a variety of primers which showed polymorphic bands in several SSRs, and STSs (Elsadr et al., data not shown). Primers which were found to be polymorphic between these sets of lines could be used in genetic studies to identify molecular markers for the genes responsible for PHD. Before completion of this study, a series of F_2 progeny from crosses between ND x SD and ND x RD cross classes were generated and plant material was extracted and freeze dried for DNA extraction. This set of material can be used to map segregating populations using primers that were polymorphic between ND, SD and RD parents. Furthermore, several parents segregating for PHD were screened using a series of primers and polymorphisms were identified (Refer to Appendix B).

Identification of a molecular marker for the PHD trait would be extremely beneficial to bean breeders attempting to target markets where consumers are willing to pay a premium for light coloured beans. A marker for PHD would enhance breeding efficiency through the reduction of cost and labor associated with (i) growing, harvesting, threshing and darkening procedures, (ii) land use and (iii) phenotyping and selection.

5.6 Future Work: Seed Coat Condensed Tannins

As part of this research, RNA was obtained from DOR364, RIL89 and RIL58 for gene expression work. The RNA was converted to cDNA at CIAT and can be analyzed for polyphenolic gene expression. This material can be used to evaluate differences in the RNA

composition between contrasting parents. This data could then be used to determine candidate genes for mapping in a segregating population in order to identify genes that co-segregate. Co-segregating genes could then be evaluated for their use as molecular markers for CT. The high (RIL89) and low (RIL58) CT DOR364 x G19833 RILs may also be used to assess gene expression using real time PCR. A correlation may be identified between the accumulation of CT and the genes under investigation. This correlation may increase our understanding of how and when CT is being expressed in developing seed coats. Once CT expression is determined, certain genes could be targeted for potential manipulation to tweak CT concentrations in the seed coats of common bean and other crops for the purpose of enhancing the nutritional benefits of these crops.

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APPENDICES

Appendix A: Seed Coat Post Harvest Darkening

Table A.1 Observed phenotypes and putative genotypes for the F_1 seed coats of ND x ND crosses.

Cross #	Female Parent	Male Parent	Observed F_1Phenotype	Putative F_1 Genotype
4420aS-1	PI 608688	Wit-rood	All ND	jjSD?
4420aS-2	PI 608688	Wit-rood	All ND	jjSD?
4420aS-3	PI 608688	Wit-rood	All ND	jjSD?
4420bS-1	Wit-rood	PI 608688	All ND	jjSD?
4422S-1	Wit-rood	KVxUI-1	All ND	jjSDSD
4422S-2	Wit-rood	KVxUI-1	All ND	jjSDSD
4423S-1	Wit-rood	KVxUI-6	All ND	jjSDSD
4423S-2	Wit-rood	KVxUI-6	All ND	jjSDSD

Table A.2 Observed phenotypes and putative genotypes for the F_2 seed coats of ND x ND crosses

Cross #	Female Parent	Male Parent	Observed F_2 Phenotype	Putative F_2 Genotype
4119aS-1	KVxUI-1	P1608686	24 ND	jjSDSD
4119aS-2	KVxUI-1	P1608686	11 ND	jjSDSD
4119aS-4	KVxUI-1	P1608686	1 ND	jjSDSD
4119bS-2	P1608686	KVxUI-1	33 ND	jjSDSD
4119bS-3	P1608686	KVxUI-1	18 ND	jjSDSD
4119bS-4	P1608686	KVxUI-1	38 ND	jjSDSD
4120S-1	KVxUI-1	P1608688	7 ND	jjSD?
4120S-2	KVxUI-1	P1608688	28 ND	jjSD?
4120S-3	KVxUI-1	P1608688	28 ND	jjSD?
4122S-1	P1608686	KVxUI-6	16 ND	jjSDSD
4122S-2	P1608686	KVxUI-6	27 ND	jjSDSD
4122S-3	P1608686	KVxUI-6	3 ND	jjSDSD
4122S-4	P1608686	KVxUI-6	2 ND	jjSDSD
4420aS-1	PI 608688	Wit-rood	4 ND	jjSD?
4420aS-2	PI 608688	Wit-rood	5 ND	jjSD?
4420aS-3	PI 608688	Wit-rood	4 ND	jjSD?
4420bS-1	Wit-rood	PI 608688	1 ND	jjSD?
4422S-1	Wit-rood	KVxUI-1	5 ND	jjSDSD
4422S-2	Wit-rood	KVxUI-1	5 ND	jjSDSD
4423S-1	Wit-rood	KVxUI-6	16 ND	jjSDSD
4423S-2	Wit-rood	KVxUI-6	22 ND	jjSDSD

Table A.3 Observed phenotypes and putative genotypes for the F_1 seed coats of SD x SD crosses

Cross #	Female Parent	Male Parent	Observed F_1 Phenotype	Putative F_1 Genotype
4086-1	1533-15	SDIP	All SD	JJsdsd
4086-2	1533-15	SDIP	All SD	JJsdsd
CIAT1	Bayo Mochica	1533-15	All SD	JJsdsd
CIAT2	1533-15	Bayo Mochica	All SD	JJsdsd

Table A.4 Observed phenotypes and putative genotypes for the F_2 seed coats of SD x SD crosses

Cross #	Female Parent	Male Parent	Observed F_2 Phenotype	Putative F_2 Genotype
4086-1	1533-15	SDIP	26 SD	JJsdsd
4086-2	1533-15	SDIP	23 SD	JJsdsd

Table A.5 Observed phenotypes and putative genotypes for the F_3 seed coats of SD x SD crosses

Cross #	Female Parent	Male Parent	Observed F_3 Phenotype	Putative F_3 Genotype
4086-1	1533-15	SDIP	25 SD	JJsdsd
4086-2	1533-15	SDIP	23 SD	JJsdsd

Table A.6 Observed phenotypes and putative genotypes for the F_4 seed coats of SD x SD crosses

Cross #	Female Parent	Male Parent	Observed F_4 Phenotype	Putative F_4 Genotype
4086-1	1533-15	SDIP	52 SD	JJsdsd
4086-2	1533-15	SDIP	52 SD	JJsdsd

Table A.7 PHD phenotypes of all progeny for crosses 3257S and W18, W19 and W52 for the F_2 seed coats of ND x SD and SD x ND crosses

Cross #	Female Parent	Male Parent	F_2 PHD Phenotype	Cross #	Female Parent	Male Parent	F_2 PHD Phenotype
3257S-1-1	PI 608688	1533-15	ND	W19-26	Wit-rood	1533-15	RD
3257S-2-1	PI 608688	1533-15	SD	W19-27	Wit-rood	1533-15	SD
3257S-3-1	PI 608688	1533-15	ND	W19-28	Wit-rood	1533-15	RD
3257S-4-1	PI 608688	1533-15	RD	W19-29	Wit-rood	1533-15	ND
3257S-5-1	PI 608688	1533-15	RD	W19-30	Wit-rood	1533-15	RD
3257S-6-5	PI 608688	1533-15	RD	W19-31	Wit-rood	1533-15	RD
3257S-7-1	PI 608688	1533-15	RD	W19-32	Wit-rood	1533-15	RD
3257S-8-1	PI 608688	1533-15	RD	W19-33	Wit-rood	1533-15	RD
W18-1	Wit-rood	1533-15	ND	W19-34	Wit-rood	1533-15	ND
W18-3	Wit-rood	1533-15	ND	W19-35	Wit-rood	1533-15	SD
W18-4	Wit-rood	1533-15	RD	W19-36	Wit-rood	1533-15	RD
W18-5	Wit-rood	1533-15	RD	W19-37	Wit-rood	1533-15	ND
W18-7	Wit-rood	1533-15	RD	W19-38	Wit-rood	1533-15	ND
W18-8	Wit-rood	1533-15	ND	W19-39	Wit-rood	1533-15	RD
W18-9	Wit-rood	1533-15	ND	W19-40	Wit-rood	1533-15	RD
W18-10	Wit-rood	1533-15	RD	W19-41	Wit-rood	1533-15	RD
W18-11	Wit-rood	1533-15	RD	W19-42	Wit-rood	1533-15	RD
W18-12	Wit-rood	1533-15	RD	W19-43	Wit-rood	1533-15	RD
W18-13	Wit-rood	1533-15	RD	W19-44	Wit-rood	1533-15	ND
W18-14	Wit-rood	1533-15	RD	W19-45	Wit-rood	1533-15	RD
W18-15	Wit-rood	1533-15	RD	W19-46	Wit-rood	1533-15	RD
W18-17	Wit-rood	1533-15	ND	W19-47	Wit-rood	1533-15	RD
W18-18	Wit-rood	1533-15	ND	W19-48	Wit-rood	1533-15	ND
W18-19	Wit-rood	1533-15	ND	W19-49	Wit-rood	1533-15	ND
W18-20	Wit-rood	1533-15	RD	W19-50	Wit-rood	1533-15	RD
W18-21	Wit-rood	1533-15	RD	W19-51	Wit-rood	1533-15	SD
W18-22	Wit-rood	1533-15	ND	W19-52	Wit-rood	1533-15	RD
W18-23	Wit-rood	1533-15	RD	W19-53	Wit-rood	1533-15	RD
W18-24	Wit-rood	1533-15	RD	W19-54	Wit-rood	1533-15	RD
W18-25	Wit-rood	1533-15	ND	W19-55	Wit-rood	1533-15	SD
W18-26	Wit-rood	1533-15	RD	W19-56	Wit-rood	1533-15	RD
W18-27	Wit-rood	1533-15	RD	W19-57	Wit-rood	1533-15	RD
W18-28	Wit-rood	1533-15	RD	W19-58	Wit-rood	1533-15	RD
W18-29	Wit-rood	1533-15	ND	W19-59	Wit-rood	1533-15	RD
W18-31	Wit-rood	1533-15	SD	W19-60	Wit-rood	1533-15	RD
W18-33	Wit-rood	1533-15	RD	W19-61	Wit-rood	1533-15	RD
W19-1	Wit-rood	1533-15	ND	W19-62	Wit-rood	1533-15	ND

Cross #	Female Parent	Male Parent	F_2 PHD Phenotype	Cross #	Female Parent	Male Parent	F_2 PHD Phenotype
W19-2	Wit-rood	1533-15	SD	W19-63	Wit-rood	1533-15	ND
W19-3	Wit-rood	1533-15	RD	W19-64	Wit-rood	1533-15	RD
W19-5	Wit-rood	1533-15	RD	W19-65	Wit-rood	1533-15	RD
W19-6	Wit-rood	1533-15	RD	W19-66	Wit-rood	1533-15	RD
W19-7	Wit-rood	1533-15	RD	W19-67	Wit-rood	1533-15	SD
W19-8	Wit-rood	1533-15	RD	W19-68	Wit-rood	1533-15	ND
W19-9	Wit-rood	1533-15	SD	W19-69	Wit-rood	1533-15	RD
W19-10	Wit-rood	1533-15	SD	W19-70	Wit-rood	1533-15	RD
W19-11	Wit-rood	1533-15	RD	W52-1	Wit-rood	1533-15	SD
W19-12	Wit-rood	1533-15	ND	W52-4	Wit-rood	1533-15	RD
W19-13	Wit-rood	1533-15	RD	W52-5	Wit-rood	1533-15	RD
W19-14	Wit-rood	1533-15	RD	W52-6	Wit-rood	1533-15	SD
W19-15	Wit-rood	1533-15	ND	W52-7	Wit-rood	1533-15	RD
W19-16	Wit-rood	1533-15	RD	W52-8	Wit-rood	1533-15	RD
W19-17	Wit-rood	1533-15	RD	W52-9	Wit-rood	1533-15	RD
W19-18	Wit-rood	1533-15	ND	W52-10	Wit-rood	1533-15	RD
W19-19	Wit-rood	1533-15	ND	W52-11	Wit-rood	1533-15	SD
W19-20	Wit-rood	1533-15	RD	W52-12	Wit-rood	1533-15	SD
W19-21	Wit-rood	1533-15	SD	W52-13	Wit-rood	1533-15	RD
W19-22	Wit-rood	1533-15	RD	W52-14	Wit-rood	1533-15	RD
W19-23	Wit-rood	1533-15	ND	W52-15	Wit-rood	1533-15	ND
W19-24	Wit-rood	1533-15	RD	W52-17	Wit-rood	1533-15	SD
W19-25	Wit-rood	1533-15	RD	W52-18	Wit-rood	1533-15	RD

Table A.8 Observed and tested PHD segregation ratios and Chi-Squared, P-values and putative genotypes for the F_3 seed coats of ND x SD and SD x ND crosses

Cross #	Female Parent	Male Parent	Observed F_3 Ratio[1]	Tested F_3 Ratio[1]	X^2	P-value	Putative F_3 Genotype
3257S-1-1	PI 608688	1533-15	0:0:18	0:0:1	-	-	jj__
3257S-2-1	PI 608688	1533-15	0:11:5	0:3:1	0.3333	0.5673	Jjsdsd
3257S-3-1	PI 608688	1533-15	0:0:24	0:0:1	-	-	jj__
3257S-4-1	PI 608688	1533-15	14:5:12	9:3:4	3.1219	0.2099	JjSDsd
3257S-5-1	PI 608688	1533-15	15:10:6	9:3:4	3.7527	0.1531	JjSDsd
3257S-6-5	PI 608688	1533-15	9:4:4	9:3:4	0.2549	0.8803	JjSDsd
3257S-7-1	PI 608688	1533-15	27:0:7	3:0:1	0.4444	0.505	JjSDSD
3257S-8-1	PI 608688	1533-15	21:3:0	3:1:0	2	0.1573	JJSDsd
W18-1	Wit-rood	1533-15	0:0:6	0:0:1	-	-	jj__
W18-3	Wit-rood	1533-15	0:0:6	0:0:1	-	-	jj__
W18-4	Wit-rood	1533-15	6:1:0	3:1:0	0.4286	0.5127	JJSDsd

W18-5	Wit-rood	1533-15	6:1:1	9:3:4	1.6617	0.558	JjSDsd
W18-7	Wit-rood	1533-15	2:1:1	9:3:4	0.1111	0.946	JjSDsd
W18-8	Wit-rood	1533-15	0:0:11	0:0:1	-	-	JJSDSD
W18-9	Wit-rood	1533-15	0:0:11	0:0:1	-	-	jj__
W18-10	Wit-rood	1533-15	2:0:0	1:0:0	-	-	JJSDSD
W18-11	Wit-rood	1533-15	6:0:0	1:0:0	-	-	JJSDSD
W18-12	Wit-rood	1533-15	10:0:0	1:0:0	-	-	JJSDSD
W18-13	Wit-rood	1533-15	6:2:0	3:1:0	-	-	JJSDsd
W18-14	Wit-rood	1533-15	7:0:0	1:0:0	-	-	JJSDSD
W18-15	Wit-rood	1533-15	12:0:0	1:0:0	-	-	JJSDSD
W18-17	Wit-rood	1533-15	0:0:8	0:0:1	-	-	jj__
W18-18	Wit-rood	1533-15	0:0:8	0:0:1	-	-	jj__
W18-19	Wit-rood	1533-15	0:0:2	0:0:1	-	-	jj__
W18-20	Wit-rood	1533-15	5:0:4	3:0:1	1.8148	0.1779	JjSDSD
W18-21	Wit-rood	1533-15	11:0:2	3:0:1	0.641	0.4233	JjSDSD
W18-22	Wit-rood	1533-15	0:0:11	0:0:1	-	-	jj__
W18-23	Wit-rood	1533-15	6:1:2	9:3:4	0.4815	0.786	JjSDsd
W18-24	Wit-rood	1533-15	9:0:1	3:0:1	1.2	0.2733	JjSDSD
W18-25	Wit-rood	1533-15	0:0:6	0:0:1	-	-	jj__
W18-26	Wit-rood	1533-15	9:0:2	3:0:1	0.2727	0.6015	JjSDSD
W18-27	Wit-rood	1533-15	4:1:1	9:3:4	0.2963	0.8623	JjSDsd
W18-28	Wit-rood	1533-15	9:4:0	3:1:0	0.2308	0.631	JJSDsd
W18-29	Wit-rood	1533-15	0:0:7	0:0:1	-	-	jj__
W18-31	Wit-rood	1533-15	0:8:0	0:1:0	-	-	JJsdsd
W18-33	Wit-rood	1533-15	8:0:4	3:0:1	0.4444	0.505	JjSDSD
W19-1	Wit-rood	1533-15	0:0:12	0:0:1	-	-	jj__
W19-2	Wit-rood	1533-15	0:8:0	0:1:0	-	-	JJsdsd
W19-3	Wit-rood	1533-15	9:0:0	0:1:0	-	-	JJSDSD
W19-5	Wit-rood	1533-15	6:4:0	3:1:0	1.2	0.2733	JJSDsd
W19-6	Wit-rood	1533-15	8:0:6	3:0:1	2.381	0.1228	JjSDSD
W19-7	Wit-rood	1533-15	2:0:0	1:0:0	-	-	JJSDSD
W19-8	Wit-rood	1533-15	10:0:3	3:0:1	0.0256	0.8728	JjSDSD
W19-9	Wit-rood	1533-15	0:4:2	0:3:1	0.2222	0.6374	Jjsdsd
W19-10	Wit-rood	1533-15	0:4:0	0:1:0	-	-	JJsdsd
W19-11	Wit-rood	1533-15	10:1:3	9:3:4	1.6508	0.4381	JjSDsd
W19-12	Wit-rood	1533-15	0:0:14	0:0:1	-	-	jj__
W19-13	Wit-rood	1533-15	3:1:1	9:3:4	0.0667	0.9672	JjSDsd
W19-14	Wit-rood	1533-15	9:1:1	9:3:4	2.9394	0.23	JjSDsd
W19-15	Wit-rood	1533-15	0:0:7	0:0:1	-	-	jj__
W19-16	Wit-rood	1533-15	12:0:0	1:0:0	-	-	JJSDSD
W19-17	Wit-rood	1533-15	6:1:0	3:1:0	0.4286	0.5127	JJSDsd

W19-18	Wit-rood	1533-15	0:0:11	0:0:1	-	-	jj__
W19-19	Wit-rood	1533-15	0:0:7	0:0:1	-	-	jj__
W19-20	Wit-rood	1533-15	10:0:0	1:0:0	-	-	JJSDSD
W19-21	Wit-rood	1533-15	0:11:2	0:3:1	0.641	0.4233	Jjsdsd
W19-22	Wit-rood	1533-15	6:2:2	9:3:4	0.1333	0.9355	JjSDsd
W19-23	Wit-rood	1533-15	0:0:11	0:0:1	-	-	jj__
W19-24	Wit-rood	1533-15	9:2:2	9:3:4	0.9847	0.6223	JjSDsd
W19-25	Wit-rood	1533-15	10:3:0	3:1:0	0.0256	0.8728	JJSDsd
W19-26	Wit-rood	1533-15	8:0:3	3:0:1	0.0303	0.8618	JjSDSD
W19-27	Wit-rood	1533-15	0:6:4	0:3:1	1.2	0.2733	Jjsdsd
W19-28	Wit-rood	1533-15	9:0:0	1:0:0	-	-	JJSDSD
W19-29	Wit-rood	1533-15	0:0:9	0:0:1	-	-	jj__
W19-30	Wit-rood	1533-15	4:3:0	3:1:0	1.1905	0.2752	JJSDsd
W19-31	Wit-rood	1533-15	5:0:2	3:0:1	0.0476	0.8273	JjSDSD
W19-32	Wit-rood	1533-15	12:0:0	1:0:0	-	-	JJSDSD
W19-33	Wit-rood	1533-15	10:0:1	3:0:1	1.4848	0.223	JjSDSD
W19-34	Wit-rood	1533-15	0:0:10	0:0:1			jj__
W19-35	Wit-rood	1533-15	0:7:0	0:1:0	-	-	JJsdsd
W19-36	Wit-rood	1533-15	6:3:0	3:0:1	0.3333	0.5637	JJSDsd
W19-37	Wit-rood	1533-15	0:0:11	0:0:1	-	-	jj__
W19-38	Wit-rood	1533-15	0:0:14	0:0:1	-	-	jj__
W19-39	Wit-rood	1533-15	13:0:0	1:0:0	-	-	JJSDSD
W19-40	Wit-rood	1533-15	5:0:0	1:0:0	-	-	JJSDSD
W19-41	Wit-rood	1533-15	8:3:2	9:3:4	0.6752	0.7135	JjSDsd
W19-42	Wit-rood	1533-15	6:2:4	9:3:4	0.4444	0.8007	JjSDsd
W19-43	Wit-rood	1533-15	8:0:4	3:0:1	0.4444	0.505	JjSDSD
W19-44	Wit-rood	1533-15	0:0:9	0:0:1	-	-	jj__
W19-45	Wit-rood	1533-15	9:0:0	1:0:0	-	-	JJSDSD
W19-46	Wit-rood	1533-15	6:1:0	3:1:0	0.4286	0.5127	JJSDsd
W19-47	Wit-rood	1533-15	11:0:0	1:0:0	-	-	JJSDSD
W19-48	Wit-rood	1533-15	0:0:12	0:0:1	-	-	jj__
W19-49	Wit-rood	1533-15	0:0:5	0:0:1	-	-	jj__
W19-50	Wit-rood	1533-15	7:3:0	3:1:0	0.1333	0.715	JJSDsd
W19-51	Wit-rood	1533-15	0:9:2	0:3:1	0.2727	0.6015	Jjsdsd
W19-52	Wit-rood	1533-15	5:0:3	3:0:1	0.6667	0.4142	JjSDSD
W19-53	Wit-rood	1533-15	6:0:2	3:0:1	-	-	JjSDSD
W19-54	Wit-rood	1533-15	7:5:0	3:1:0		0.1824	JJSDsd
W19-55	Wit-rood	1533-15	0:11:0	0:1:0	-	-	JJsdsd
W19-56	Wit-rood	1533-15	9:2:0	3:1:0	0.2727	0.6015	JJSDsd
W19-57	Wit-rood	1533-15	7:2:0	3:1:0	0.037	0.8474	JJSDsd
W19-58	Wit-rood	1533-15	8:3:0	3:1:0	0.0303	0.8618	JJSDsd

W19-59	Wit-rood	1533-15	7:4:0	3:1:0	0.7576	0.3841	JJSDsd
W19-60	Wit-rood	1533-15	8:2:0	3:1:0	0.1333	0.715	JJSDsd
W19-61	Wit-rood	1533-15	4:1:0	3:1:0	0.0667	0.7963	JJSDsd
W19-62	Wit-rood	1533-15	0:0:10	0:0:1	-	-	jj__
W19-63	Wit-rood	1533-15	0:0:9	0:0:1	-	-	jj__
W19-64	Wit-rood	1533-15	10:0:1	3:0:1	1.4848	0.223	JjSDSD
W19-65	Wit-rood	1533-15	7:0:5	3:0:1	1.7778	0.1824	JjSDSD
W19-66	Wit-rood	1533-15	8:2:0	3:1:0	0.1333	0.715	JJSDsd
W19-67	Wit-rood	1533-15	0:11:0	0:1:0	-	-	JJsdsd
W19-68	Wit-rood	1533-15	0:0:10	0:0:1	-	-	jj__
W19-69	Wit-rood	1533-15	6:4:4	9:3:4	1.2381	0.5385	JjSDsd
W19-70	Wit-rood	1533-15	4:3:3	9:3:4	1.2444	0.5368	JjSDsd
W52-1	Wit-rood	1533-15	0:10:0	0:1:0	-	-	JJsdsd
W52-4	Wit-rood	1533-15	7:0:0	1:0:0	-	-	JJSDSD
W52-5	Wit-rood	1533-15	3:0:0	1:0:0	-	-	JJSDSD
W52-6	Wit-rood	1533-15	0:4:3	0:3:1	1.1905	0.2752	Jjsdsd
W52-7	Wit-rood	1533-15	4:2:1	9:3:4	0.6825	0.7109	JjSDsd
W52-8	Wit-rood	1533-15	9:0:0	1:0:0	-	-	JJSDSD
W52-9	Wit-rood	1533-15	15:0:0	1:0:0	-	-	JJSDSD
W52-10	Wit-rood	1533-15	10:0:0	1:0:0	-	-	JJSDSD
W52-11	Wit-rood	1533-15	0:3:0	0:1:0	-	-	JJsdsd
W52-12	Wit-rood	1533-15	0:13:0	0:1:0	-	-	JJsdsd
W52-13	Wit-rood	1533-15	8:0:0	1:0:0	-	-	JJSDSD
W52-14	Wit-rood	1533-15	10:2:2	9:3:4	1.3651	0.505	JjSDsd
W52-15	Wit-rood	1533-15	0:0:8	0:0:1	-	-	jj__
W52-17	Wit-rood	1533-15	0:6:0	0:1:0	-	-	JJsdsd
W52-18	Wit-rood	1533-15	11:0:0	1:0:0	-	-	JJSDSD

[1] Segregation ratios expressed as RD:SD:ND

Table A.9 PHD phenotypes of all progeny for the cross 3254S for the F_2 seed coats of ND x RD and RD x ND crosses

Cross #	Female Parent	Male Parent	F_2 PHD Phenotype
3254S-1-1	PI 608688	Pintium	RD
3254S-2-1	PI 608688	Pintium	RD
3254S-3-1	PI 608688	Pintium	RD
3254S-4-1	PI 608688	Pintium	RD
3254S-5-1	PI 608688	Pintium	RD
3254S-6-1	PI 608688	Pintium	RD
3254S-7-1	PI 608688	Pintium	RD
3254S-8-1	PI 608688	Pintium	RD
3254S-9-1	PI 608688	Pintium	RD
3254S-10-1	PI 608688	Pintium	RD
3254S-11-1	PI 608688	Pintium	ND
3254S-12-1	PI 608688	Pintium	ND

Appendix B: Surveying Parental Genotypes for Potential Markers for the PHD trait

The parental genotypes CDC Pintium, 1533-15, KVxUI-1 and KVxUI-6 were screened with over 100 microsatellite markers (SSR) from the BM-ATA and PV series as well as Sequence-Tagged Site (STS) and Random Amplified Polymorphic DNA (RAPD) markers for micronutrient and colour genes (Cichy et al., 2009; Grisi et al., 2007; Mclean et al., 2002) (Table 8.10).

Table B.1 Microsattellite (SSR), Sequence-Tagged Site (STS) and random amplified polymorphic DNA (RAPD) markers used to survey CDC Pintium, 1533-15, KVxUI-1 and KVxUI-6 for genetic PHD polymorphisms

PV-Brazil		BM-ATA		Mclean
Pv 26	Pv 149	BM-ATA3	BM-ATA234	OL4
Pv 31	Pv 163	BM-ATA4	BM-ATA241	OM9
Pv 35	Pv 167	BM-ATA5	BM-ATA243	OAP2
Pv 45	Pv 168	BM-ATA6	BM-ATA244	OM19
Pv 46	Pv 169	BM-ATA7	BM-ATA247	OJ17
Pv 53	Pv 172	BM-ATA9	BM-ATA248	OAM10
Pv 54	Pv 173	BM-ATA10	BM-ATA268	OAP7
Pv 60	Pv 181	BM-ATA13	BM-ATA269	OU14
Pv 61	Pv 182	BM-ATA16	BM-ATA289	OW17
Pv 67	Pv 185	BM-ATA20	BN	OD12
Pv 69	Pv 188	BM-ATA22	PV CAC1	OAP3
Pv 78	Pv 191	BM-ATA24	PV GA15	
Pv 82	Pv 198	BM-ATA26	PV GA16	
Pv 83	Pv 199	BM-ATA27	HRG	
Pv 87	Pv 201	BM-ATA32	ME 1	
Pv 92	Pv 213	BM-ATA71	PG2	
Pv 93	Pv 215	BM-ATA74	PV CA5	
Pv 94	Pv 218	BM-ATA 76		
Pv 101	Pv 228	BM-ATA108		
Pv 102	Pv 229	BM-ATA112		
Pv 106	Pv 233	BM-ATA116		
Pv 107	Pv 235	BM-ATA133		
Pv 109	Pv 236	BM-ATA150		
Pv 112	Pv 242	BM-ATA154		
Pv 113	Pv 243	BM-ATA173		
Pv 124	Pv 250	BM-ATA138		
Pv 125	Pv 251	BM-ATA143		
Pv 128	Pv 255	BM-ATA145		
Pv 133	Pv 259	BM-ATA180		
Pv 139		BM-ATA217		

The parents used in this survey represented the full range of PHD phenotypes, and therefore, polymorphisms found between pairs of parents could be used to make inferences on expected polymorphisms in many populations segregating for PHD. Parental surveying has also helped determine which populations could be mapped to identify molecular markers associated with RD, SD and ND phenotypes.

The surveying procedure was conducted at CIAT. Bean seeds of DOR364, G19833, CDC Pintium, 1533-15, KVxUI-1 and KVxUI-6 were allowed to germinate in paper towels in the dark. DOR364 and G19833 were used as checks as these genotypes had been previously assessed using these same primers. Ten etiolated first true leaves from each genotype were removed from the plants six days after germination using tweezers. Leaves from each genotype were packaged in individual 10 ml size? microfuge tubes and placed in a -80 ºC freezer for 2 hours. The tissue was then freeze dried at -50 ºC at a partial pressure of 53 mBars for 36 hours and manually ground using a pestle grinder. DNA was extracted using the Viogene plant genomic DNA extraction mini prep system, according to manufacturer's instructions and then the DNA was quantified.

The PCR cocktails, programs and agarose and polyacrilamide gel electrophoresis and staining procedures for the BM-ATA, PV Brazil and McClean primers were as described by Cichy et al. (2009), Grisi et al. (2007) and McClean et al. (2002), respectively. Upon staining, each gel was scanned and then scored for presence or absence of a band or for the relative position of the band on the gel.

Several polymorphisms were identified from the molecular survey of the parental genotypes (Table 8.11). Polymorphic primers will be used to map populations made between 1533-15 x CDC Pintium, 1533-15 x KVxUI-1, 1533-15 x KVxUI-6, Wit-rood x 1533-15 and CDC Pintium x KVxUI-1.

Table B.2 Scoring data for the PV Brazil, BM-ATA and McClean primers of various RD, SD and ND genotypes of common bean

Primer Name (PV)	Pintium x 1533	Pintium x KV-1	Pintium x KV-6	1533 x KV-1	1533 x KV-6	KV-1 x KV-6	# of polymorphisms
Pv 26	M	M	M	M	M	M	0
Pv 31	P	M	P	P	M	P	4
Pv 35	P	P	P	P	P	M	5
Pv 45	M	P	P	P	P	M	4
Pv 46	M	P	P	P	P	M	4
Pv 53	P	M	P	P	P	P	5
Pv 54	M	M	M	M	M	M	0
Pv 60	M	M	M	M	M	M	0
Pv 61	P	M	M	P	P	M	3
Pv 67	M	M	M	M	M	M	5
Pv 69	NA	NA	NA	na	NA	M	0
Pv 78	P	M	M	P	P	M	3
Pv 82	M	P	P	P	P	M	4
Pv 83	P	P	P	P	P	M	5
Pv 87	M	P	P	P	P	P	4
Pv 92	M	M	M	M	M	M	0
Pv 93	P	P	P	P	P	M	5
Pv 94	P	M	M	P	P	M	3
Pv 101	M	P	P	P	P	M	4
Pv 102	P	P	P	P	P	M	5
Pv 106	M	M	M	M	M	M	0
Pv 107	M	P	P	P	P	M	4
Pv 109	P	P	P	P	P	M	5
Pv 112	M	P	P	P	P	M	4
Pv 113	P	P	P	P	P	M	5
Pv 124	P	P	P	P	P	M	5
Pv 125	NA	P	NA	NA	NA	NA	1
Pv 128	NA	M	M	P	P	M	3
Pv 133	M	P	P	P	P	M	4
Pv 139	M	M	M	M	M	M	0
Pv 149	P	P	P	P	P	M	5
Pv 163	M	P	P	P	P	M	4
Pv 167	M	M	M	M	M	M	0
Pv 168	M	M	M	M	M	M	0
Pv 169	M	P	P	P	P	M	4
Pv 172	NA	NA	NA	NA	NA	NA	0
Pv 173	M	M	M	M	M	NA	0
Pv 181	M	P	P	P	P	M	4
Pv 182	M	M	M	M	M	M	0
Pv 185	P	P	P	P	P	M	5
Pv 188	NA	NA	NA	NA	NA	M	0
Pv 191	NA	M	NA	NA	NA	NA	0
Pv 198	P	P	P	P	P	M	5

Primer Name (PV)	Pintium x 1533	Pintium x KV-1	Pintium x KV-6	1533 x KV-1	1533 x KV-6	KV-1 x KV-6	# of polymorphisms
Pv 199	M	M	M	M	M	M	0
Pv 201	M	M	M	M	M	M	0
Pv 213	M	M	P	M	M	M	1
Pv 215	M	M	M	M	M	M	0
Pv 218	M	P	P	P	P	M	4
Pv 228	M	M	M	M	M	M	0
Pv 229	NA	NA	NA	NA	NA	NA	0
Pv 233	NA	P	P	NA	NA	NA	2
Pv 235	P	P	P	P	M	P	5
Pv 236	M	M	M	M	M	M	0
Pv 242	M	P	P	P	P	M	4
Pv 243	P	M	M	P	P	M	3
Pv 250	M	M	M	M	M	M	0
Pv 251	P	P	P	P	P	M	5
Pv 255	P	P	P	M	M	P	3
Pv 259	NA	NA	NA	NA	NA	NA	0

Primer Name (BM ATA)	Pintium x 1533	Pintium x KV-1	Pintium x KV-6	1533 x KV-1	1533 x KV-6	KV-1 x KV-6	# of polymorphisms
BM-ATA3	M	P	P	P	P	M	4
BM-ATA4	M	P	P	P	P	M	4
BM-ATA5	M	M	M	M	M	M	0
BM-ATA6	M	P	NA	P	NA	NA	2
BM-ATA7	M	M	M	M	M	M	0
BM-ATA9	M	P	P	P	P	M	4
BM-ATA10	P	P	P	P	P	M	5
BM-ATA13	M	M	M	M	M	M	0
BM-ATA16	M	M	M	M	M	M	0
BM-ATA20	P	M	M	P	P	M	2
BM-ATA22	M	P	P	P	P	M	4
BM-ATA24	P	P	P	P	P	M	4
BM-ATA26	M	P	P	P	P	M	4
BM-ATA27	P	M	M	P	P	M	3
BM-ATA32	M	NA	P	NA	P	M	2
BM-ATA71	M	P	M	M	M	M	1
BM-ATA74	M	M	P	M	M	M	1
BM-ATA 76	P	P	P	P	P	M	5
BM-ATA108	NA	M	NA	NA	NA	NA	0
BM-ATA112	M	NA	P	NA	P	NA	2
BM-ATA116	M	M	M	M	M	M	0
BM-ATA133	NA	P	P	P	P	M	5
BM-ATA150	NA	NA	NA	P	P	M	2
BM-ATA154	M	P	P	P	P	M	4
BM-ATA173	M	P	P	P	P	M	4
BM-ATA138	M	M	M	M	M	M	0
BM-ATA143	M	M	M	M	M	M	0
BM-ATA145	M	NA	M	NA	M	NA	0

Primer Name (BM ATA)	Pintium x 1533	Pintium x KV-1	Pintium x KV-6	1533 x KV-1	1533 x KV-6	KV-1 x KV-6	# of polymorphisms
BM-ATA180	P	M	P	P	M	P	4
BM-ATA217	M	M	M	M	M	M	0
BM-ATA234	M	M	M	M	M	M	0
BM-ATA241	P	M	M	M	P	M	2
BM-ATA243	NA	NA	NA	NA	NA	NA	0
BM-ATA244	M	M	M	M	M	M	0
BM-ATA247	M	P	P	M	P	M	3
BM-ATA248	M	M	M	M	M	M	0
BM-ATA268	NA	NA	NA	NA	NA	NA	0
BM-ATA269	M	M	M	M	M	M	0
BM-ATA289	P	M	P	P	P	P	5
BN	P	M	M	M	P	M	2
PV CAC1	NA	NA	NA	NA	NA	NA	0
PV GA15	P	M	M	M	P	M	2
PV GA16	M	P	P	M	P	M	3
HRG	M	M	M	M	M	M	0
ME 1	M	NA	P	NA	P	NA	2
PG2	NA	NA	NA	NA	NA	NA	0
PV CA5	M	P	P	P	P	P	5

Primer Name (Mclean)	Pintium x 1533	Pintium x KV-1	Pintium x KV-6	1533 x KV-1	1533 x KV-6	KV-1 x KV-6	# of polymorphisms
OL4	P	P	P	P	P	M	5
OM9	M	M	M	M	M	M	0
OAP2	P	M	P	P	M	P	4
OM19	M	M	M	M	M	M	0
OJ17	M	P	P	P	P	M	4
OAM10	M	P	P	P	P	M	4
OAP7	P	M	M	P	P	M	3
OU14	M	M	M	M	M	M	0
OW17	M	M	M	M	M	M	0
OD12	M	M	M	M	M	M	0
OAP3	P	P	M	M	P	P	4
Total Polymorphisms	32	47	54	55	59	10	257