SEEDCOAT DARKENING IN PINTO BEAN (*PHASEOLUS VULGARIS* L.)

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By
Donna C. Junk

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ABSTRACT

Post-harvest seedcoat darkening is a major problem in many pulses, including common bean (*Phaseolus vulgaris* L.). In some bean market classes, such as pinto, beans that have a darkened seedcoat are discounted in the market place as it is assumed that the beans are old and will be hard-to-cook (HTC). Pinto genotypes that darken more slowly than conventional pinto beans would be more desirable and have been identified in the bean breeding program at the University of Saskatchewan.

To study the slow-darkening trait, a quick, reliable, and inexpensive screening method that would not affect seed germination would be beneficial. Three potential protocols to accelerate seedcoat darkening were examined. The greenhouse protocol was conducted in the greenhouse by placing the bean seeds in polybags with a 1 cm^2 piece of moistened felt. For the UV light protocol, bean seeds were placed 10 cm below an UV lamp which had a wavelength of 254 nm. For the cabinet protocol, bean seeds were placed in a cabinet set at 30˚C, 80% relative humidity, and full fluorescent lights. Color measurements were taken routinely using a Hunter Lab colorimeter. All three methods were successful in distinguishing darkening beans from slow-darkening beans although the UV light protocol was considered to be superior to the greenhouse and cabinet protocol as the UV light protocol was quick, consistent over years, and the most economical. Unlike the greenhouse and the cabinet protocols, the UV light protocol did not affect seed germination following accelerated darkening.

The stability of the slow-darkening trait was further investigated in genotype by environment (g x e) studies across different indoor and outdoor environments. In the g x e study across different field environments, it was found that prior to accelerated seedcoat darkening the g x e interaction was significant. Following accelerated seedcoat darkening, environment and genotype were both significant and g x e was not. The slow-darkening genotypes had lighter seedcoats than the darkening genotypes and those field sites that had more favorable weather had lighter seedcoats. For the g x e study across indoor and outdoor environments, when the genotypes were split into either slow-darkening or darkening, the g x e interaction was not significant and the slow-darkening genotypes had lighter seedcoats.

Genetic control of the slow darkening trait was determined. For crosses between slow-darkening genotypes and CDC Pintium, the F_2 populations segregated 3 darkening : 1 slow-darkening with distinct bimodal distribution. This indicated that seedcoat darkening was controlled by a single gene and darkening was dominant over slow-darkening. For both slow-darkening by slow-darkening crosses, the F_2 populations' L* values were unimodal, normal distributions, indicating there may be modifying genes for the slow-darkening trait.

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1. INTRODUCTION

Common dry bean (*Phaseolus vulgaris* L.) is an ancient pulse crop originating in Central and South America. From these regions arose a wide array of beans differing in color, shape, and size (Gepts and Debouck, 1991). These visual characteristics are the basis for the characterization of today's bean market classes. Some of the market classes of beans in Canada include pinto, small red, pink, great northern, black, navy, red kidney, light red kidney, white kidney, and cranberry. The pinto market class has plump, medium sized seeds (300-400 mg) and seedcoats have a cream background with brown mottling.

Dry bean production in Canada has increased by nearly 100% from 1991-1992 and 2000-2001, with most of the growth in Manitoba and Saskatchewan (Skrypetz, 2002). In 1991-1992 Saskatchewan produced 136 thousand tonnes of dry beans and by 2002-2003 Saskatchewan was producing 345 thousand tonnes (Skrypetz, 2002). With shorter season bean varieties becoming available, Agriculture and Agri-Food Canada expect Saskatchewan to become one of the main dry bean producing provinces (Skrypetz, 2002).

In many of these market classes, including pinto, the seedcoat color of the bean slowly changes to a darker brown color after the seed is physiologically mature. It has been shown that post-harvest seedcoat darkening occurs more rapidly in environments that have high temperature, humidity, and light (Park and Maga, 1999). These conditions that cause the beans to darken more rapidly also cause beans to develop the hard-to-cook (HTC) phenomenon (Barrón et al., 1996; Hincks and Stanley, 1986; Richardson and Stanley, 1991; Sievwright and Shipe, 1986; Srisuma et al., 1989; Stanley, 1992; Stanley et al., 1989). Thus, consumers presume that beans with dark seedcoats are HTC. HTC beans are undesirable as they require longer soaking and cooking times compared to fresh beans and are less palatable. Merchants have expressed interest in beans that would maintain their seedcoat color and not darken.

At the Crop Development Centre (CDC), University of Saskatchewan, several pinto bean genotypes have been identified that maintain their seedcoat color better than currently grown cultivars and do not darken as fast even when exposed to conditions that

favor darkening. These new genotypes are referred to as slow-darkening genotypes and traditional cultivars are referred to as darkening genotypes.

The slow-darkening genotypes were identified during a visit to Mexico in 2002. Drs. K.E. Bett and A. Vandenberg discovered that merchants preferred one of their samples of pinto beans compared to any other sample that was presented to them as this sample of pinto beans had a very white background and was slow-darkening. Upon return to Canada, the sample seed was seeded and re-selected producing slow-darkening lines SC11743-3 P8 and SC11743-3 P9. The original sample of SC11743-3 was crossed to CDC Pintium to improve the agronomic performance and seed shape characteristics. Two selected breeding lines, 1533-14 and 1533-15 were field tested in 2002-2004 and the superior line, 1533-15, is currently being released to seed growers on contract. As well, during this study the Saltillo Agricultural Experiment Station of the Instituto Nacional de Investigaciones Forestales Agricolas y Pecuarias (INIFAP) of Mexico released a short day cultivar, Pinto Saltillo, that the developers claim will not darken after one or two years of storage (Sanchez-Valdez et al., 2004).

In the near future, bean traders in Canada speculate that growers selling slow-darkening beans could receive a two cent per pound (CDN) price premium over regular darkening beans (Gildardo Silva, personal communication August 2003). Further into the future, Saskatchewan pinto beans could become world renowned and branded as having lighter colored beans thereby creating a higher demand for Saskatchewan pinto beans and a more stable selling market for Saskatchewan pinto bean producers.

To introgress the slow-darkening trait into pinto cultivars a quick method to identify the slow-darkening trait would be useful. By traditional means determining if a genotype is slow-darkening or darkening is a lengthy process as one needs to grow the plant to maturity, harvest the seed, and then let the seeds age to determine the phenotype. As well, knowing the stability of the slow-darkening trait over environments and the genetic control of the trait is important for the plant breeders who are trying to incorporate the slow-darkening trait into their breeding program.

Thus, the objectives of this study were to: i) develop an accelerated darkening protocol that can quickly differentiate slow-darkening and darkening genotypes; ii)

determine if slow-darkening is influenced by genotype by environment interactions; and iii) determine the genetic control of the slow-darkening trait.

2. LITERATURE REVIEW

2.1. Introduction

2.1.1. Common Bean (*Phaseolus vulgaris* L.)

Common bean (*Phaseolus vulgaris* L.) is a dicotyledonous plant that belongs to the family Fabaceae. The species evolved from a wild-growing vine ancestor in the highlands of Middle America and the Andes (Gepts and Debouck, 1991). Middle America is the origin of races Durango, Jalisco, and Mesoamerica, and the Andes is the origin of races Chile, Nueva Granada, and Peru (Singh et al., 1991). There are two types of common bean: dry and snap. Dry beans are harvested once the seeds are fully mature and dry whereas snap beans are harvested while the pods are immature and fleshy. For dry beans, there are market classes developed based on the color, shape, and size of the bean. Voysest and Dessert (1991) list 59 known market classes of dry beans. Pinto is one market class with a plump, medium sized seed with a cream background and brown mottling.

2.1.2. Nutritional Benefits of Common Bean

Beans have been consumed for thousands of years and have an excellent nutritional profile as Geil and Anderson (1994) found after conducting an extensive review. Dry beans contain 21-25% crude protein, are rich in amino acids such as lysine, but are moderately deficient in sulfur containing amino acids such as methionine and tryptophan. The carbohydrate content of dry beans is 60-65%, composed mainly of starch with small amounts of monosaccharides and disaccharides. Carbohydrate in the form of fiber is 3-7% in cooked beans, composed primarily of cellulose and hemicellulose. Beans are cholesterol free and very low in fats. Of the fat that is present, 16% is saturated and 84% is unsaturated. Beans are an excellent source of minerals. A single cup serving of cooked dry beans contains 29% of the of the US recommended dietary allowance of iron for females, and 55% for males, 20-25% of phosphorus, magnesium, manganese, potassium, and copper, and 10% of calcium and zinc.

Geil and Anderson (1994) found the consumption of bean to be related to decreased health concerns such as coronary heart disease, diabetes mellitus, obesity, and cancer. For coronary heart disease, it has been proven that the consumption of beans significantly lowers serum lipid concentrations in humans. Beans have a low glycemic index as they are digested slowly and produce very low blood glucose and insulin responses. Diabetic patients are encouraged to consume at least half a cup of cooked beans daily. For similar reasons and because beans delay the return of hunger sensations, prolonging feelings of satiety, beans can be used in a weight loss or weight maintenance diet for obese patients. The consumption of beans reduces of the risk of cancer, especially breast and colon cancer. This could be in part due to the significant amount of antioxidant activity found in the phenolic compounds in the seedcoat of beans (Beninger and Hosfield, 2003). Beans are a rich source of folic acid which is especially important for women of child bearing age as low levels of folic acid during pregnancy can lead to neural tube defects in their infants (Gupta and Gupta, 2004).

2.2. Seedcoat Darkening in Common Bean

2.2.1. Seedcoat Darkening is a Result of Time and Storage Conditions

Seedcoats turning to a dark brown color is a major problem for many bean market classes, including pinto. White seeded market classes such as navy do not experience seedcoat darkening. For beans that do darken, the rate of seedcoat darkening is a result of storage time and conditions. As storage time of beans increases, the seedcoat color becomes darker (Brackmann et al., 2002a; Hughes and Sandsted, 1975; Paredes-López et al., 1989; Park and Maga, 1999). Darkening accelerates when beans have high moisture content and/or are stored under high relative humidity and high temperatures (Park and Maga, 1999). Exposure to ultraviolet and cool-white light also augments seedcoat darkening (Hughes and Sandsted, 1975; Brackmann et al., 2002a). Atmospheres with high carbon dioxide and oxygen coupled with low nitrogen increase the rate of seedcoat darkening (Brackmann et al. 2002a; Brackmann et al. 2002b; Sartori, 1982).

2.2.2. Seedcoat Darkening is Associated with the Hard-to-Cook Phenomenon

When beans are stored in conditions with high temperature, high humidity, and light and/or for extended periods of time, not only do the beans become darker but the hard-to-cook (HTC) phenomenon occurs (Aguilera and Steinsapir, 1985; Brackmann et al., 2002b; Michaels and Stanley, 1991; Mills et al., 1995; Reyes-Moreno et al., 1994; Richardson and Stanley, 1991; Rozo et al., 1990; Stanley et al., 1989). Unlike fresh cooked beans that have a moist mouth feel and disintegrate easily into a smooth paste when squeezed, HTC beans are drier, are more prone to fracturing, and contain hard, grainy, persistent pieces (Stanley et al., 1989). Compared to freshly harvested beans, HTC beans require longer periods of time for both soaking and cooking (Paredes-López et al., 1989; Stanley et al., 1989). HTC beans have reduced water absorption, as if there is a barrier for water penetration during soaking and cooking (Gesto and Vazquez, 1976; Hincks and Stanley, 1986; Paredes-López et al., 1989). One possible explanation is that the beans lose solids during soaking and as a result, the concentration gradient between the beans and the soak/cook water is lowered. As a result less water moves into the seed (Richardson and Stanley, 1991). Thus, even after very prolonged soaking and cooking, HTC beans have a harder Instron hardness and a higher puncture force (Paredes-López et al., 1989; Stanley et al., 1989).

The seedcoat influences the texture of fresh and HTC beans. As beans age, seedcoats become harder and take longer to soften during cooking (Stanley et al., 1989). Like the seedcoats, the cotyledons become harder with age, yet the cotyledons soften faster with soaking and cooking than do the seedcoats (Stanley et al., 1989). Removing the seedcoat of a bean results in a decrease in human sense of hardness and chewiness as well as a decrease in instrumental puncture force, although the role of seedcoat for texture and hardness is relatively more important for fresh beans compared to HTC beans (Hincks and Stanley, 1986; Stanley et al., 1989).

2.2.3. Seedcoat Darkening is Associated with Poor Germination

When pinto beans have been aged, germination decreases (Barrón et al., 1996; Gesto and Vazquez, 1976). Beans stored at 80% relative humidity and 24°C for 12 months

have a 0% germination (Hughes and Sandsted, 1975). Beans stored at room temperature for five years have a slight but significant loss of viability (Gesto and Vazquez, 1976). The seeds that remain viable have lower rates of germination and seedling growth with the seedlings having smaller leaves and retarded chlorophyll development (Gesto and Vazquez, 1976). Barrón and colleagues (1996) found that seed stored at high temperature (40°C) and humidity (30, 45, and 60% relative humidity) resulted in poor germination, short root and shoot lengths, and low seedling weights. Surprisingly, when the next two generations of seed were stored in refrigerated conditions (<5°C), the seed had good germination and yield, but seedlings still had shorter main root and shoot lengths and low seedling weights (Barrón et al., 1996). Beans darkened with ultraviolet or cool-white light, without high temperatures or high humidity, have little loss in germination (Hughes and Sandsted, 1975). To maintain high germination, Brackmann et al. (2002b) recommend that beans be stored in an inert atmosphere with low oxygen levels and low temperature. In western Canada, if initial high storage temperatures at harvest are avoided, storage of beans over the winter in bins in Manitoba does not adversely affect seed germination (Mills et al., 1995).

2.2.4. Economic Importance of Seedcoat Darkening

Bean seedcoat color is a major concern for buyers and sellers. Bean processing plants often have electronic color sorters that are able to separate discolored beans. The Canadian Grain Commission uses color as part of their primary and export grade determinants with "good natural color" beans being classified as Extra No. 1 Canada and "off–color" beans being classified as No 4 Canada (Table 1.1).

Table 1.1 The Canadian Grain Commission's primary and export grades and grade determinants for dry field bean (Canadian Grain Commission, 2004).

Grade Name	Standard of Quality
Extra No. 1 Canada	Uniform size, good natural color
Canada No. 1 Select	Fairly good color
No. 1 Canada	Reasonably good color
No. 2 Canada	Fairly good color
No. 3 Canada	Fairly good color
No. 4 Canada	Off-color

2.3. Prevention of Seedcoat Darkening

2.3.1. Cultural Practices

Currently, few inexpensive, practical cultural techniques are available to successfully prevent seedcoat darkening and the HTC effect. One cultural technique to prevent seedcoat darkening is to store the beans in a controlled, nitrogen enriched atmosphere, as is done for horticulture crops such as apples. Sartori (1982) found that after six months of storage at 24°C and 75% relative humidity, pinto beans that were stored in an enriched nitrogen atmosphere showed no change in color while pinto beans stored in a natural atmosphere began to significantly darken after two months of storage. The pinto beans stored under forced nitrogen that did not darken still developed long cooking times, became hard, and produced poor flavor, all of which was similar to the natural atmosphere pinto beans.

Nene et al. (1975) investigated the use of radiation to retard or stop seedcoat darkening and the HTC effect. Although radiation could reduce cooking time by 12 and 18% for every Mrad for white and red kidney beans, respectively, with higher doses reducing the cooking time further, the higher doses caused excessive browning and off odors rendering the beans unacceptable (Nene et al., 1975).

Park and Maga (1999) found that low moisture content prevented seedcoat darkening. In Chile, Aguilera and Steinsapir (1985) attempted to reduce moisture content by heating the beans in an 86 L metal drum to the temperature of 105°C for three minutes followed by rapid cooling of the beans to room temperature with fans. When canned, the beans were softer compared to the control canned beans but the beans were also darker. A second treatment, which heated beans in layers in an oven set at 70°C for one hour followed by rapid cooling of the beans to room temperature with fans, did not darken the seedcoats of the beans like the metal drum treated beans but the canned beans were harder than the metal drum treated beans.

Alternatively, in Arkansas where canning processors are located close to farmer's fields, harvesting beans at the semi-dry stage (50% of the pods on the plants are yellow) followed by 24 hours at 27°C rather than harvesting the beans at the dry stage (100% of the pods dry) was found to improve the color, flavor, texture, and general

acceptability of canned beans (Gonzalez et al., 1982). As well, harvesting beans in the field earlier at the semi-dry stage reduces the risk of seed discoloration in the field.

In Manitoba, beans are harvested into large bins at the end of the summer months before the onset of the cold winter. Although there are yearly differences inside bins, temperatures are initially high and fall during the winter months (Mills and Woods, 1994). Beans subjected to simulated bin temperatures that reached 44°C in the first month resulted in 0% germination and very long cooking times (Mills et al., 1995). However, if initial high bin temperatures are avoided, the germination rate and cooking quality of the beans will not be adversely affected (Mills et al., 1995).

2.3.2. Genetic Improvement

There is little published research on the prospects of breeding for varieties that resist seedcoat darkening. However, there has been published work on improving the cooking and nutritional quality of beans. Since seedcoat darkening is associated with cooking and nutritional quality, this work could infer what could be possible with seedcoat darkening.

Since the HTC phenomenon is especially problematic in developing countries where fuel supplies are low, Elia et al. (1997) investigated the possibility of shortening cooking time through plant breeding in Africa. From examining crosses from 16 genotypes, both variance and narrow sense heritability was found to be high for protein content, tannin content, water absorption and cooking time, suggesting that cooking time could be shortened through plant breeding. Since a negative correlation between water absorption and cooking time was found, water absorption could be used as a predictor for cooking time.

For navy, black and pinto beans grown in Saskatchewan, most canning quality traits were highly influenced by the genotype (Balasubramanian et al., 1999). The genotype by year by environment interaction predominated the corresponding genotype by year and genotype by environment (g x e) and thus first order interactions were considered not as important (Balasubramanian et al., 1999). For black turtle soup beans grown in Michigan, there was a larger season by genotype variance than genotypic variance for hydration coefficient, clumping and splitting (Hosfield et al., 1984).

However, for texture and washed drain weight, more variance was due to genotype although genotype by year variance was still significant (Hosfield et al., 1984). Interestingly, Michaels and Stanley (1991) found that for bean texture there was a large variance due to g x e interaction and heritability estimates were low with high standard error.

Elia et al. (1997) suggested that new varieties would cook faster if plant breeders selected for low tannin beans as a positive correlation was found between cooking time and tannin content. In Mexico, it was found that the greatest contribution to variance for tannins, lectin activity, and trypsin inhibitors was from the genotype, with smaller components of variance attributed to environment and g x e (De Mejía et al., 2003). This suggests that plant breeders could change the tannin profile of beans.

Recently, two new varieties of beans have been developed that reportedly do not darken in storage. 'Pinto Saltillo' is a new pinto bean cultivar developed at Saltillo Agricultural Experiment Station of the Instituto Nacional de Investigaciones Forestales Agricolas y Pecuarias (INIFAP) of Mexico (Sanchez-Valdez et al., 2004). According to the developers, Pinto Saltillo will not darken after one or two years of storage (Sanchez-Valdez et al., 2004). 'BRS Requinte' is a carioca cultivar developed in Brazil by Embrapa Rice and Beans (De Faria et al., 2004). Developers of BRS Requinte report that no major alterations in seedcoat color are evident and that cooking time decreases with BRS Requinte compared to check cultivars 'Perola' and 'Iapar 81'. For both Pinto Saltillo and BRS Requinte, neither the genetic control nor the rate of slow-darkening is known.

2.4. Chemistry and Physiology of Seedcoat Darkening

2.4.1. Chemistry

Two chemical groups that are widely accepted to be associated with bean darkening are phenols and phytates. For total phenols, studies claim that phenols increase with bean aging (Gesto and Vazquez, 1976) while other studies indicate that phenols decrease with aging (Hincks and Stanley, 1986; Martin-Cabrejas et al., 1997). The discrepancy could be due to differences in extraction procedures. Srisuma et al. (1989) found that there is an increase in phenols in the seedcoat over time, specifically methanol soluble

phenolic acid esters, free phenolic acids, and cell wall bound phenolic acids. In the cotyledon, free phenolic acids increase but a decrease occurs for methanol soluble phenolic acid esters. Later, Garcia et al., (1998) found that free phenolic acids increased over time, methanol soluble esters decreased, and phenols bound to cell walls or pectin in the cotyledon increased over time.

Some studies show an increase in condensed tannins in bean with age (Martin-Cabrejas et al., 1997) while another study showed a decrease (Stanley, 1992). This discrepancy may be explained by a study conducted by Sievwright and Shipe (1986) who found that condensed tannins increase over time, reach a plateau, and then decline when stored over time at elevated temperatures. The increase in level of condensed tannins is thought to be due to small molecular weight non-tannin compounds developing into tannins, and the decline of condensed tannins is thought to occur as condensed tannins bind to macro-molecules found in the cotyledon (Sievwright and Shipe, 1986; Stanley 1992) The movement of condensed tannins from the seedcoat to the cotyledon over time occurs in kabuli chickpeas (Reyes-Moreno et al., 2000).

As beans age, phytic acid levels decrease (Martin-Cabrejas et al., 1997; Sievwright and Shipe, 1986; Hincks and Stanley, 1986; Chitra and Singh, 1998). Hincks and Stanley (1986) studied phytic acid levels in beans and found that over time phytic acid decreases. This decrease over time is thought to be due to the increasing activity of phytase.

Hincks and Stanley (1986) found that individually, phenols and phytic acid have significant positive and negative correlation, respectively, to bean hardness although the correlation coefficients were low. When both phenol and phytic acid are considered together, 97-100% of the variation is accounted for at each storage period. Hincks and Stanley (1986) speculated that changes in the beans during storage initially are due to phytate but as storage time increase, changes in phenols are the main contributor to bean hardness.

A recent study of bean seedcoat chemistry by Beninger et al. (2005) may help to explain the difference between darkening and slow-darkening genotypes and what happens as beans age. Non-aged seedcoats of CDC Pintium, a normal darkening genotype, were found to contain significantly more kaempferol than aged seedcoats of

CDC Pintium as well as aged or non-aged seedcoats of 1533-15, a slow-darkening genotype. Analysis of the overall level of condensed tannins demonstrated that aged and non-aged seedcoats of CDC Pintium had significantly higher levels of tannins than aged and non-aged 1533-15 seedcoats. Interestingly, in both lines kaempferol-catechin adducts formed and their concentration increased with seedcoat age.

In Brazil, the recent emphasis in darkening research is phenols and phenol oxidizing enzymes: peroxidase (POD) and polyphenol oxidase (PPO). Esteves et al., (2002) found a negative relationship between polyphenol concentration, lignin concentration, and POD activity with water absorption. Moura et al. (1999) found that POD activity increases over storage and is higher in darker beans. Although PPO activity was found to decrease after eight months of tropical storage, PPO activity was higher before and after storage for those beans that darkened faster. As well, higher levels of phenolics were found before and after aging in beans that darkened faster. Like Moura et al. (1999), De Oliveira Rios et al., (2002) found that darkened beans have higher levels of phenols, PPO activity, and POD activity.

2.4.2. Physiology

As beans age, the cells in the seeds change physically. The seedcoats of old beans lose permeability, preventing water from entering the seed (Gesto and Vazquez, 1976). When water does pass through the seedcoat, water collects between the seedcoat and the cotyledons (Paredes-López et al., 1989; Stanley et al., 1989). Although old beans initially have an increased water absorption rate compared to fresh beans, older beans have lower total water absorption (Hincks and Stanley 1986; Paredes-López et al., 1989; Richardson and Stanley, 1991).

There are two explanations for the reduced total water absorption of old beans. The first is that old beans are losing solutes to the soak water, creating a low concentration gradient for water (Hincks and Stanley 1986; Richardson and Stanley, 1991). Light microscope work found that old beans had starch granules that were fused together while fresh beans had distinct starch granules (Hincks and Stanley, 1986). Richardson and Stanley (1991) found that the membranes of old beans had a higher phase transition temperatures as well as a higher percentage of saturated fatty acids and

a lower percentage of unsaturated fatty acids, both of which indicate a lower bulk lipid fluidity of the membrane indicating a loss of functionality. Thus, as beans become old the membranes break down, releasing starch to the soak water, which creates a low water potential, causing less water to move into the beans.

The second possible explanation is that old beans have lower total water absorption because it is difficult for water to physically penetrate the cells. When viewed with a scanning electron microscope, old beans have dense packing of cotyledon cells compared to fresh beans (Paredes-López et al., 1989; Hincks and Stanley 1986). Not only are the cells densely packed, but the cells of old beans do not expand or separate like fresh beans, leading to cell wall puckers and ruptures (Aguilera and Steinsapir 1985; Hincks and Stanley, 1987; Hincks and Stanley, 1986). Hincks and Stanley (1987) stained cotyledon cells with potassium permanganate to test for lignin, a hydrophobic compound that can tightly bind cell walls together thereby reducing water permeability, and found more lignin in old beans than fresh beans. Unlike fresh beans, old beans had lignin in the corners of the intercellular spaces and more lignin in the middle lamella. In old beans, the distinction between the primary and secondary walls could be detected as the secondary wall was staining positive for lignin. Martin-Cabrejas et al. (1997) found increases in lignin and lignified proteins when beans are stored in tropical conditions. Rozo et al. (1990) had earlier tired to quantify lignin through chemical extraction and were unable to find significant differences between old and fresh beans. However, it was found that neutral detergent residue, which measures cell wall content or hemicellulose, increased as well as the nitrogen content of the neutral detergent residue in the cotyledons of old beans. The increase in cell wall content reported by Rozo et al. (1990) agreed with the results of Hincks and Stanley (1987), who observed through scanning electron microscopy, that old beans have several more layers in their cell walls than fresh beans. Thus, old beans have cells that are not physically separating from one another due to lignin, which prevent water from reaching the cells, and when the water does reach the cells, the cell walls are thicker and it is more difficult for water to enter the cell.

Low amounts of water being absorbed by the cells leads to low gelatinization of starch, with damaged starch compounding the problem even further (Hincks and

Stanley, 1987). Scanning electron micrographs showed that fresh beans have complete gelatinization whereas old beans have ungelatinized starch granules (Aguilera and Steinsapir, 1985). As well, scanning electron microscope work demonstrated that starch granules have a wrinkled surface, indicating starch damage (Hincks and Stanley, 1986). Analysis of bean flour showed that, although no differences were evident between old and fresh bean flour for gelatinization temperatures, old bean flour had a higher paste consistency than fresh bean flour (Paredes-López et al., 1989).

2.5. Other Crops Affected by Darkening

2.5.1. Pulse Crops

Similar seedcoat darkening phenomena and associated darkening problems occur with other pulses. Lentil (*Lens culinaris* Medik.) seedcoats of most market classes become darker naturally with aging, when exposed to higher humidity, and when subjected to accelerated aging (38°C, 30% RH) (Kulkarni et al., 1989; Mills et al., 1999; Vaillancourt and Slinkard, 1985). As lentil seedcoats become darker, germination decreases, solute leakage increases, and papads made from lentil dhal have a very soft texture and are no longer acceptable in the market place (Kulkarni et al., 1989; Mills et al., 1999). In kabuli chickpea (*Cicer arietinum* L.) high temperature and relative humidity cause darkening as well as the HTC phenomenon (Reyes-Moreno et al., 2000). In cowpea (*Vigna unguiculata*), high temperature and relative humidity during storage leads to darkening as well hard texture (Sefa-Dedeh et al., 1979). In faba bean (*Vicia faba* L.) tannin-free cultivars do not darken unlike tannin containing faba beans which clearly darken after six months of room temperature storage (Crofts et al., 1980).

2.5.2. Cereal Crops

Discoloration of cereals can occur in storage, leading to lower consumer acceptance and lower prices. Rice and rice flour becomes more yellow when temperature and water activity increase, with temperature accounting for most of the variance (Chrastil 1990; Soponronnarit et al., 1998). Barley is well known to darken in the field when exposed to precipitation and pathogens (Edney et al., 1998; Miles et al., 1987; Petr and Capouchová, 2001). Reuss (2001) found that after 104 weeks of storage

barley does become darker, more red, and more yellow, with higher temperatures (45°C) causing more discoloration than lower temperatures (25 and 35°C). In wheat, it was found that increasing the temperatures and storage time unfortunately causes the color of milled flour to increase in color grade value (Srivastava and Rao, 1994).

2.5.3. Fruits, Vegetable, and Beverage Crops

The browning of foods such as prunes, raisins, cider, wine, coffee, cocoa, and black tea is crucial for consumer acceptance (Walker, 1995; Whitaker and Lee, 1995). However browning in other food products is undesirable especially in fruits (apple, apricot, banana, grape, peach, pear, and strawberry), vegetables (lettuce and potato), mushrooms, and shrimp (Walker, 1995; Whitaker and Lee, 1995). Apple, banana juice, cocoa, lettuce, mushroom, peach and nectarine skin, potato, raisins, tea, and white wine all become brown in part due to phenolic substrates reacting with PPO producing oxidized phenolic compounds (Broadbent et al., 1997; Buta and Moline, 2001; Cheng and Crisosto, 1995; Heimdal et al., 1997; Jinap et al., 2003; Mahanta et al., 1993; Rajarathnam et al., 2003; Rocha and Morais 2001; Sakharov and Ardila 1999; Sims et al., 1991; Sims et al., 1994; Singleton et al., 1985).

2.6. Color and Color Measurement

2.6.1. How the Human Eye Perceives Color and L*a*b* Color Scale

The human eye has rod and cone cells that absorb wavelengths between 400 and 700 nm (Zollinger, 1999). Rod cells are used for night vision while cone cells are used for light vision (Zollinger, 1999). For the cone cells, the human eye has three different types of cones; cones that can absorb short blue wavelengths, medium green wavelengths and long red wavelengths. Ganglion cells then compare signals from numerous cones so we are able to sense the amount of green or red, the amount of blue or yellow, and the amount of lightness to darkness (Zollinger, 1999). The three ways that ganglion cells report color is the basis for the Commission Internationale de L'Eclairage (CIE) recommending in 1976 the use of the L*a*b color system which uses three axes to describe color: L*, a*, and b* (Marcus, 1998). The L* runs on the z-axis and an L* value of 100 is perfect white while an L* value of 0 is perfect black, while the

a* component is the x-axis with positive values being more red and negative values being more green, and the b* component is the y-axis with positive values being more yellow and negative values being more blue (Marcus, 1998). The L*a*b* color system is advantageous as it has an approximately uniform color scale and provides a way to compare color values between different samples (Marcus, 1998).

3. MATERIALS AND METHODS

3.1. The Slow-darkening Genotypes

The slow-darkening genotypes were identified during a visit to Mexico in 2002. Drs. K.E. Bett and A. Vandenberg discovered that merchants preferred one of their samples of pinto beans compared to any other sample that was presented to them as this sample of pinto beans had a very white background and was slow-darkening. Upon return to Canada, the sample seed was seeded and re-selected producing slow-darkening lines SC11743-3 P8 and SC11743-3 P9. The original sample of SC11743-3 was crossed to CDC Pintium to improve the agronomic performance and seed shape characteristics. Two selected breeding lines, 1533-14 and 1533-15, were field tested in 2002-2004 and the superior line, 1533-15, is currently being released to seed growers on contract. As well, the Saltillo Agricultural Experiment Station of the Instituto Nacional de Investigaciones Forestales Agricolas y Pecuarias (INIFAP) of Mexico released a short day cultivar, Pinto Saltillo, that the developers claim will not darken after one or two years of storage (Sanchez-Valdez et al., 2004)

3.2. Darkening Protocols

The objective of this experiment was to find a fast, reliable, and inexpensive protocol to darken pinto beans so that slow-darkening genotypes could be rapidly distinguished from darkening genotypes.

Eighteen genotypes, three slow-darkening and 15 darkening, were grown in the field in 2003 and 2004 (Table 3.1). Of the 15 darkening genotypes, CDC Pintium, CDC Minto, and CDC Camino are commercially released cultivars and the remaining 12 genotypes were entries in the 2003 Dry Bean Co-operative Registration Trials. One of the entries, 786-2, was a mixture of both slow-darkening and darkening genotypes and line 999s-2a had black mottling rather than brown mottling. As a result, both 786-2 and 999s-2a were excluded from the data analysis.

The experiment was grown as a randomized complete block design with the first replicate grown at Saskatoon in 2003 and four replicates grown at two locations in 2004:

one in Saskatoon at the Preston Avenue plot area and the second at the Saskatchewan Pulse Growers (SPG) research farm near Floral, Saskatchewan. The SPG site in 2004 had multiple hail-storms and a mid-August frost. The plants were unable to produce enough seed for the experiment. Thus, the total number of replicates used in this experiment was three, one from 2003 and two from 2004 grown in Saskatoon. The field growing conditions in 2003 were warmer and drier with a long growing season and 2004 had a wet, cool, short growing season with an early frost in August.

Table 3.1 Slow-darkening and darkening pinto bean genotypes grown in the field in 2003 and 2004 for the accelerated seedcoat darkening protocol experiment.

Genotype	Post-harvest darkening
CDC Pintium	Darkening
CDC Minto	Darkening
CDC Camino	Darkening
SC11745-3	Darkening
999s-2A	Darkening Black Pinto*
828B-9	Darkening
828B-3	Darkening
786-2	Both Darkening & Slow-Darkening*
841-8	Darkening
841-1	Darkening
955s-1	Darkening
1091M-57	Darkening
954S-952S	Darkening
1073M-42	Darkening
1073M-46	Darkening
SC11743-3 P8	Slow-Darkening
1533-14	Slow-Darkening
1533-15	Slow-Darkening

* Not included in data analysis

Three protocols were tested. The amount of seed tested for each treatment was equal to the volume of seed that could cover a 100 mm petri dish in a single layer. Prior to testing the three protocols, the seed was stored in a dark cold room set at 8°C to allow the moisture content of the beans to equilibrate to one another.

The first protocol was based on the results of Park and Maga (1999) who found that darkening accelerates when beans have high moisture contents and/or are stored under high relative humidity and high temperatures. To achieve this in an economically feasible way, the greenhouse protocol was created. Seeds were placed in polybags (Nasco Whirl-Pak®, 16.5 cm x 9.5 cm) with a 1 cm^2 piece of moistened felt and then the

bags were placed on benches in the greenhouse. A preliminary test using a single droplet of water in the bag without the piece of felt revealed that the felt is needed for even humidity in the bag and that the felt prevents seedcoat wrinkling and seed germination.

The second protocol was designed to maximize the effect of light, which was partially blocked in the greenhouse protocol by the greenhouse glass and the polybag plastic. For second protocol, called the UVC light protocol, beans were placed in open 100 mm petri dishes directly under germicidal 254 nm UV lights (model G40T10, Ushio America, Inc., Cypress, California, USA). In the light standards, a UV light bulb alternated with a fluorescent light bulb every 12 cm. The two UV and two fluorescent light bulbs were raised 10 cm above the beans and emitted a light intensity of 4.06 mW cm^{-2} at the 254 nm light spectrum.

Preliminary work using a UV transilluminator from a gel imaging system indicated that exposing beans to UV light darkens beans more than not exposing the beans to UV light and that UVC light darkens them more than UVA and UVB light. For genotype 1533-15, beans darkened for 12 hours under UVA light were not significantly different than untreated beans (Figure 1). Surprisingly, for genotype CDC Pintium, beans treated with UVA light were lighter than the untreated beans. For both CDC Pintium and 1533-15, beans treated for 12 hours under either UVB or UVC light were significantly darker than untreated beans and UVC treated beans were significantly darker than UVB treated beans.

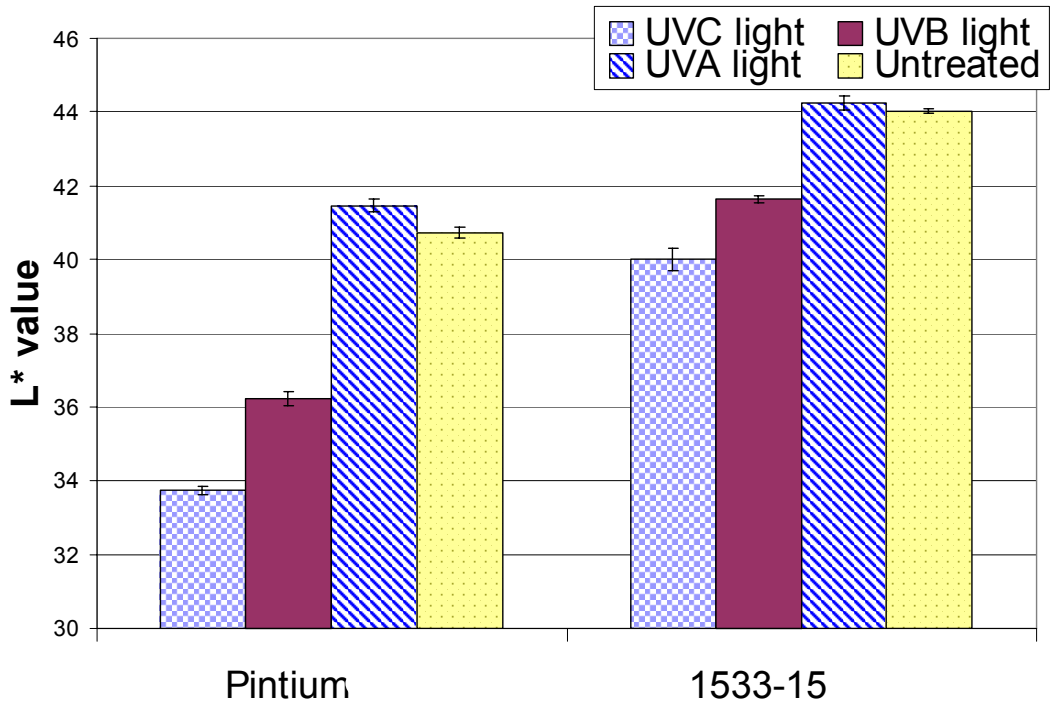

Figure 1. The L* color values and standard errors of darkening genotype CDC Pintium and slow-darkening genotype 1533-15 when untreated or exposed to UVA, UVB, and UVC light for 12 hours.

The third protocol was similar to how researchers create the HTC effect (Barrón et al., 1996; Hincks and Stanley, 1986; Richardson and Stanley, 1991; Sievwright and Shipe, 1986; Srisuma et al., 1989; Stanley, 1992; Stanley et al., 1989). For this protocol the seeds were placed in open 100 mm petri dishes in a seed germination cabinet set at 30°C, 80% relative humidity and full fluorescent light.

Color values L*, a*, and b* were measured using a Hunter Lab colorimeter (model No 45/0-L MiniScan XE, Hunter Associates Lab Inc., Reston, Virginia, USA). The L* value measures brightness with an L* value of 100 being perfectly white while a L* value of 0 is perfectly black. The a* value measures red to green with positive values being more red and negative values being more green, and the b* value measures yellow to blue with positive values being more yellow and negative values being more blue (Marcus, 1998). Color values were measured every seven days for bean seeds from the greenhouse and cabinet protocol and every eight hours for bean seeds from the UV

protocol. During each color measurement, the beans were randomly turned over and re-oriented in the petri dish or polybag.

Statistics were completed using The SAS System 8.2 (SAS Institute Inc., Cary, North Carolina, USA, 1985). Analyses of variance were conducted on the L*, a*, and b* color values according to the outline in Table 3.2. The basic analysis was a split-block with the factors being time and genotype. When the time by genotype interaction was significant, it was partitioned to determine the linear, quadratic, and cubic significance. If there were significant differences in the linear, quadratic, and/or cubic components, time by genotype was subdivided into linear, quadratic and cubic to determine if the genotypes differed at any of these components. The appropriate polynomial equations and regression coefficients were determined. To compare regression coefficients, an analysis of variance was conducted and means were compared using Fisher's Protected Least Significant Difference (LSD) test with a $P \leq 0.05$ significance level.

When both the L* and a* values could distinguish slow-darkening genotypes from darkening genotypes, correlation coefficients were determined for the beginning, middle, and end time point for each of the protocols using SAS. Replicates two and three from year two were averaged and pooled with replicate one in year one after the chi-square homogeneity tests were conducted.

Table 3.2 Outline of the analyses of variance and the F-tests used for the accelerated pinto bean seedcoat darkening protocol experiment.

Variance	df	MS	F
Years (Y)	1	m1	m1/m2
Replicates in Years (R)	1	m2	m2/m11
Genotypes (G)	15	m3	m3/m4
G x Y	15	m4	m4/m5
G x R	15	m5	m5/m11
Time (T)	8	m6	m6/m11
T x Y	8	m7	m7/m8
T x R	8	m8	m8/m11
T x G	120	m9	m9/m10
T x G x Y	120	m10	m10/m11
T x G x R	120	m11	

Following darkening, seed germinations tests were conducted on undarkened seed and darkened seed from the three protocols. Seed germination tests were conducted in

accordance with Canadian Food Inspection Agency Methods and Procedures for Testing Seed (Anonymous, 2004). Prior to germination, seeds were surface sterilized with 70% ethanol for one minute and 1.25% bleach for 10 minutes followed by three rinses with water. Seeds were placed between two 38 lb rolled towels (Anchor Paper Company, St. Paul, Minnesota) and the rolled towels were placed inside a pail in a seed germination cabinet (model 2015, VWR Signature Diurnal Growth Chamber) set at 20°C with eight hours of light every 24 hours. Three sub-samples of 10 seeds were taken from each of the replications and germinated consecutively. Replication one and its three sub-samples were germinated in 2004 and replication two and three with their three sub-samples were germinated in 2005. Data analysis was conducted using The SAS System 8.2 (SAS Institute Inc., Cary, North Carolina, USA, 1985) with data subjected to analysis of variance (ANOVA) and protocol and treatments means compared using Fisher's Protected LSD test with a $P \leq 0.05$ significance level.

3.3. Genotype by Environment Interaction for Seedcoat Darkening

3.3.1. Field Environments

A genotype by environment (g x e) study was conducted to determine if the slow-darkening trait was stable over different field environments. Nine darkening cultivars and three slow-darkening genotypes were grown in a randomized complete block design at four locations in Saskatchewan - Saskatoon, Davidson, Oxbow, and Outlook in 2004 (Table 3.3). These sites were chosen as the Crop Development Centre (CDC) bean breeding program tests beans at all of these locations as these sites vary in their location coordinates, soil type, and growing degree days (Table 3.4). The dates the beans were seeded are also shown in Table 3.4. The Outlook site was irrigated while the other sites were dryland. The site at Saskatoon experienced a hail storm in July 12th. Saskatoon and Oxbow had an early frost in August 20th and Davidson had an early snowfall on October 10th and was harvested following the snowfall (Table 3.4).

Table 3.3 Slow-darkening and darkening pinto bean genotypes grown for the genotype by environment field experiment in 2004.

Genotype	Seedcoat Type
CDC Altiro	Darkening
Bill Z	Darkening
Buster	Darkening
Maverick	Darkening
CDC Minto	Darkening
Othello	Darkening
CDC Pinnacle	Darkening
CDC Pintium	Darkening
Pintoba	Darkening
SC11743-3	Slow-Darkening
1533-14	Slow-Darkening
1533-15	Slow-Darkening

Table 3.4 The locations and their descriptions for the genotype by environment field experiment in 2004.

Location	Latitude & Longitude	Elevation	Soil Type	Growing Degree Days in 2004*	Date Seeded	Date & Temperature of First Frost
Saskatoon	52° 9'N 106° 33'W	510.00m	Dark Brown	413	May 26	August 20 -0.7°C
Outlook	51° 28'N 107° 3'W	541.00m	Dark Brown	569	May 28	September 2 -0.2°C
Davidson	51° 16'N 105° 58'W	618.70m	Dark Brown	506	May 24	September 21 -1°C
Oxbow	49° 13'N 102° 10'W	582.20m	Black	420	June 4	August 20 -0.5°C

* Calculated as the sum of ((Minimum daily temperature + Maximum daily temperature)/2) – Base Temperature of Bean (10°C) from date seeded to date of first frost.

The L* color values of the beans were measured before and after artificially darkening the beans via UV light for 120 hours as described in section 3.1. The amount of seed tested for each treatment was equal to the volume of seed that could cover a 60mm petri dish in a single layer. Prior to darkening, the seed was stored in a dark, cold room set at 8°C to allow the moisture content of the beans to equilibrate to one another. During darkening, the beans were not disturbed in the petri dish. Data analysis was conducted using The SAS System 8.2 (SAS Institute Inc., Cary, North Carolina, USA, 1985) with data subjected to analysis of variance (ANOVA) and means compared using standard errors.

3.3.2. Indoor and Outdoor Environments

Beans are grown in indoor (greenhouse and phytotron) and outdoor (field and polyhouse) environments at different stages of the breeding program. The objective of this study was to determine if there are significant interactions between these different genotypes, growing environments, and darkening protocols. The same genotypes as described in section 3.2.1, excluding 1533-14 and 1533-15, were grown in the phytotron, greenhouse, polyhouse, and field at Saskatoon in 2003 (Table 3.3). Prior to darkening, the seed was stored in a dark, cold room set at 8°C to allow the moisture content of the beans to equilibrate to one another. Seed was darkened via the greenhouse, UV, and cabinet protocols as described in section 3.1. Data analysis was conducted in SAS (SAS Institute, 1985) with data subjected to analysis of variance and means compared using standard errors.

3.4. Genetic Control of Post-Harvest Darkening

3.4.1. Slow-darkening Genotype

Genotype 1533-15 is a slow-darkening pinto bean. To determine the genetic control of the slow-darkening trait, 1533-15 was crossed to darkening pintos HR99 and CDC Pintium and the F_2 plants and $F_{2:5}$ families were phenotyped for darkening. One F_2 population of HR99 x 1533-15 and two F_2 populations of CDC Pintium x 1533-15 were grown in the field in 2003 at Saskatoon and the harvested seed was darkened for 33 days in a seed germination cabinet as described in section 3.1. Prior to darkening, the seed was stored in a dark, cold room set at 8°C to allow the moisture content of the beans to equilibrate to one another. In 2004, CDC Pintium x 1533-15 and 1533-15 x CDC Pintium F_2 populations as well as the parents were grown in the field at Saskatoon and the harvested seed was darkened via the UV method as described in section 3.1. Prior to darkening, the seed was stored in a dark, cold room set at 8°C to allow the moisture content of the beans to equilibrate to one another. During the accelerated darkening procedure, beans were not disturbed in the petri dish. Heterogeneity tests were conducted each year to confirm homogeneity among the populations and chi-square tests were conducted to determine if the slow-darkening trait is controlled by a single recessive gene.

The three F_2 populations grown in the field in 2003 were advanced in the greenhouse and grown in the field at Saskatoon in 2004 as $F_{2:5}$ families. Seedcoats were darkened via the UV protocol as described in section 3.1. Prior to darkening, the seed was stored in a dark, cold room set at 8°C to allow the moisture content of the beans to equilibrate to one another. Those families that were still segregating for seedcoat darkening were not used in data analysis. A heterogeneity test was conducted to confirm homogeneity among the populations and a chi-square test was conducted to determine if the slow-darkening trait is controlled by a single gene.

3.4.2. Pinto Saltillo and Other Slow-Darkening Genotypes

Other genetic sources of slow-darkening pinto beans were identified by other researchers during the course of this research. Pinto Saltillo is a short day cultivar developed at Saltillo Agricultural Experiment Station of the Instituto Nacional de Investigaciones Forestales Agricolas y Pecuarias (INIFAP) of Mexico (Sanchez-Valdez et al., 2004). The CDC pulse breeding program has re-selected the genotype SC11743-3 P9 that darkens more slowly than 1533-15. SC11743-3 P8 is another re-selected genotype that has slow-darkening properties similar to 1533-15 but has poorer agronomic characteristics. An F_2 population from SC11743-3 P9 x SC11743-3 P8 and the parents were grown in the greenhouse in 2003. The F_2 populations of CDC Pintium x Pinto Saltillo, Pinto Saltillo x CDC Pintium, SC11743-3 P9 x CDC Pintium, Pinto Saltillo x 1533-15, and their parents were grown in the field in 2004 at Saskatoon. Following harvest, all of the seed was darkened via the UV protocol described in section 3.1. Prior to darkening, the seed was stored in a dark, cold room set at 8°C to allow the moisture content of the beans to equilibrate to one another. For the slow-darkening by darkening populations, chi-square tests were conducted to determine if the slow-darkening trait is controlled by a single recessive gene. For the slow-darkening by slow-darkening populations, Shapiro-Wilk normal distribution, skewness, and kurtosis were tested.

4. RESULTS AND DISCUSSION

4.1. Assessment of Darkening Protocols

For all the seedcoat darkening protocols, the Hunter Lab L* and a* color responses over time were similar for the two years whereas the b* color response was erratic. For the b* value, the response over time varied greatly for the different darkening protocols and years. The UV light protocol had a more positive b* color response over time although the response could be negative for certain time intervals and the cabinet protocol had both positive and negative b* color responses over time. In 2003 the greenhouse protocol had a negative b* color response over time whereas in 2004 the b* color response was positive. None of the b* color response curves for the different protocols or years could distinguish the slow-darkening genotypes from the darkening genotypes.

The greenhouse protocol was terminated after 57 days in 2003 as the seeds had become infected with fungi and would have been destroyed had the darkening continued longer. At this time point, one could seed a color difference of roughly ten L* values and four a* values between the slow-darkening genotypes and the darkening genotypes. The ten L* values and four a* values were then used as a guideline for ending the other two darkening protocols.

Figure 1 is representative of the three darkening protocols and replicates for the L* color value response over time. Initially, all of the genotypes have similar L* color values and over time the L* values decrease indicating that the beans are becoming darker. At the end, the slow-darkening genotypes had higher L* values than the darkening genotypes indicating the slow-darkening genotypes were lighter. For all genotypes, the slope or rate of darkening was negative with a slight positive quadratic response over time. Thus, the rate of seedcoat darkening was higher at initial darkening times than later darkening times. The slope or rate of darkening for the slow-darkening genotypes was not as great as the darkening genotypes.

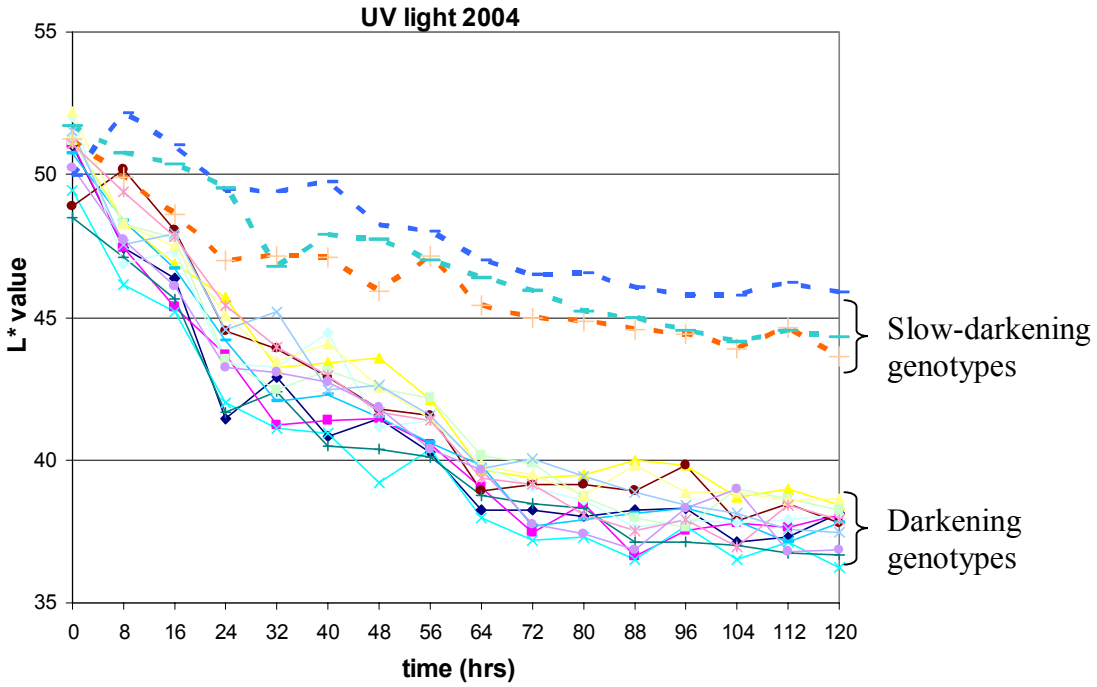

Figure 2. The L* color value response of the darkening and slow-darkening genotypes during exposure to UV light darkening in 2004 for replicate 2.

Figure 2 is representative of the three darkening protocols and replicates for the a* color value response over time. Like the L* color value, initially all of the genotypes have similar color values but over time the a* value increase indicating that the beans are becoming redder. At the end, the slow-darkening genotypes have lower a* values than the darkening genotypes indicating the darkening genotypes are redder. For all genotypes, the slope or rate of darkening was positive with a slight negative quadratic response over time. Thus, the rate of seedcoat darkening was higher at initial darkening times than later darkening times. The slope or rate of darkening for the slow-darkening genotypes was not as great as the darkening genotypes.

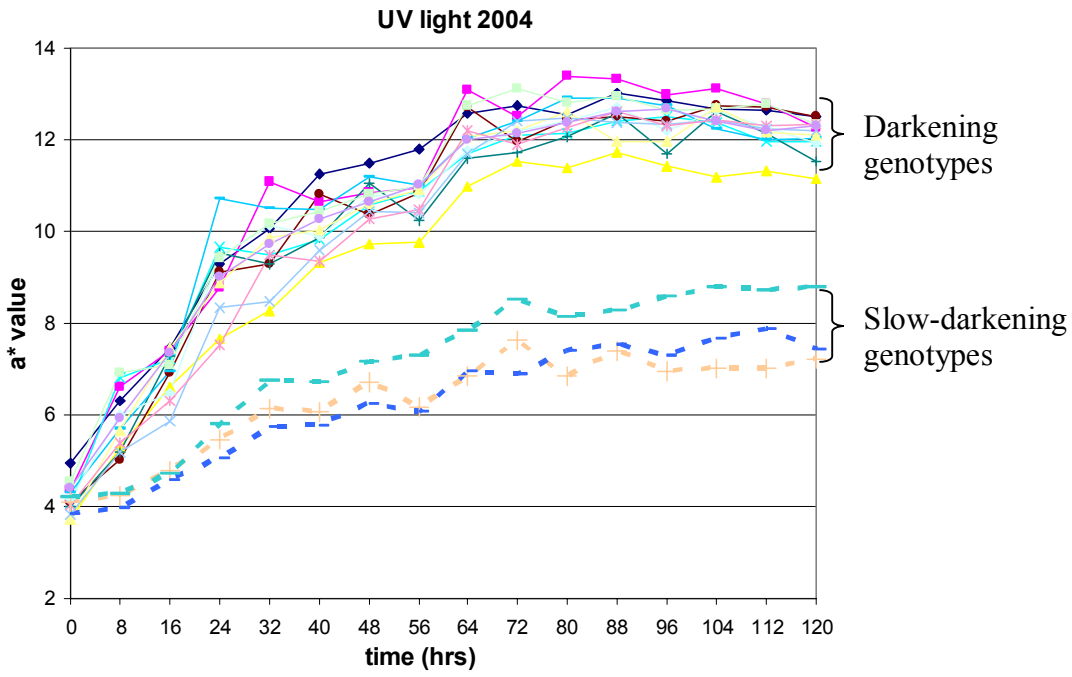

Figure 3. The a* color value response of the darkening and slow-darkening genotypes during exposure to UV light darkening in 2004 for replicate 2.

4.1.1. Greenhouse Protocol

Analysis of the data gathered from the greenhouse protocol indicates that for the L*, a*, and b* values there was a significant year effect (Appendix I). The differences observed between the two years could be due to differences in the conditions during both the growing and the post-harvest seedcoat darkening seasons. In 2003, the growing season was warm, dry, and relatively long; whereas in 2004, the growing season was wet, cool, and relatively short due to an early frost in August. For the 2003 darkening season, average daily accumulated light levels were approximately 2.40 $mol.m^{-2}.day^{-1}$ compared to the 2004 darkening season which had roughly 2.73 $mol.m^{-2}.day^{-1}$. The average air temperature inside the greenhouse was comparable in both years with temperatures ranging between 16°C (night) and 26°C (day).

The data for the greenhouse darkening protocol also indicates that the time by year interaction was highly significant for all color values. Again, this could be a result of the differences in the conditions during the growing and darkening seasons.

Time was significant for all color values, having both linear and polynomial responses. For the L* value, the genotypes differed only in their linear components but they differed in both their linear and quadratic components for the a* and b* values.

With the greenhouse protocol, the L*, a*, and b* color values cannot be used to distinguish the slow-darkening genotypes from the darkening genotypes based on the intercept of the color by time curves, which was the color prior to darkening (Table 4.1). There were significant differences among the genotypes, but the slow-darkening genotypes could not be clearly distinguished from the darkening genotypes.

Table 4.1 Comparison of the intercepts of the color response curves for each pinto bean genotype as measured by the color values L*, a*, and b* for the greenhouse darkening protocol.

Genotype	L*	a*	b*
Darkening:			
CDC Pintium	46.55	8.67	5.34
CDC Minto	45.79	8.24	4.89
CDC Camino	47.36	7.52	4.09
SC11745-3	46.29	7.69	4.55
828B-3	45.88	8.07	4.72
828B-9	48.37	8.09	4.64
841-8	48.15	7.75	4.34
841-1	47.54	7.93	4.65
954S-952S	47.80	7.91	4.36
955s-1	48.08	8.34	5.08
1073M-42	47.84	7.97	4.89
1073M-46	47.75	8.01	5.20
1091M-57	47.81	8.35	5.52
Slow-darkening:			
SC11743-3	48.67	5.82	4.10
1533-14	50.95	5.61	3.82
1533-15	49.60	5.46	3.75
LSD	4.64	0.60	2.11

The linear component for both the L* and a* color value curves are better able to distinguish the slow-darkening genotypes from the darkening genotypes than the b* values (Table 4.2). With the b* values, the slow-darkening slopes overlap with the darkening slopes. The slopes of the slow-darkening genotypes were larger and smaller for the L* and a* values respectively. For the L* value, there was no overlap of the slopes of the slow-darkening and darkening genotypes and there were differences among the darkening genotypes. With the a* value, only slow-darkening SC11743-3 P8

overlapped with darkening SC11745-3. Perhaps if the beans had been allowed to darken for an extended period of time in the greenhouse a better separation of the slow-darkening and darkening genotypes may have been observed with the a* value. However, the experiment had to be terminated after 57 days in 2003 due to fungal infection.

Table 4.2 Comparison of the linear component of the color response curves for each pinto bean genotype as measured by the color values L*, a*, and b* for the greenhouse darkening protocol.

Genotype	L*	a*	b*
Darkening:			
CDC Pintium	-0.40	0.27	0.18
CDC Minto	-0.35	0.22	0.10
CDC Camino	-0.33	0.23	0.11
SC11745-3	-0.32	0.21	0.14
828B-3	-0.37	0.26	0.15
828B-9	-0.39	0.25	0.16
841-8	-0.35	0.23	0.16
841-1	-0.35	0.23	0.13
954S-952S	-0.36	0.22	0.14
955s-1	-0.38	0.24	0.15
1073M-42	-0.39	0.25	0.14
1073M-46	-0.41	0.29	0.17
1091M-57	-0.39	0.27	0.15
Slow-darkening:			
SC11743-3	-0.19	0.12	0.02
1533-14	-0.24	0.10	0.03
1533-15	-0.19	0.07	-0.01
LSD	0.08	0.09	0.14

Examining the quadratic component of the curves, only the means of the a* and b* values were compared as the ANOVA indicated that the genotypes did not significantly differ for the quadratic component of the L* values (Appendix I and Table 4.3). For both the a* and b* values, although there were significant difference among the genotypes, the slow-darkening genotypes were not significantly different from the darkening genotypes.

Table 4.3 Comparison of the quadratic component of the color response curves for each pinto bean genotype as measured by the color values a* and b* for the greenhouse darkening protocol.

Genotype	a*	b*
Darkening:		
CDC Pintium	-0.0027	-0.0030
CDC Minto	-0.0017	-0.0011
CDC Camino	-0.0018	-0.0010
SC11745-3	-0.0016	-0.0014
828B-3	-0.0026	-0.0022
828B-9	-0.0022	-0.0022
841-8	-0.0019	-0.0019
841-1	-0.0020	-0.0014
954S-952S	-0.0018	-0.0015
955s-1	-0.0022	-0.0019
1073M-42	-0.0025	-0.0023
1073M-46	-0.0034	-0.0031
1091M-57	-0.0029	-0.0023
Slow-darkening:		
SC11743-3	-0.0010	0.0004
1533-14	-0.0006	0.0002
1533-15	-0.0003	0.0006
LSD	0.0011	0.0018

4.1.2. UV light Protocol

The UV light protocol ended after 120 hours as slow-darkening genotypes could clearly be distinguished from darkening genotypes. Similar to the results from the greenhouse protocol, data from the UV protocol data indicates that for the L*, a*, and b* values there was a significant year effect (Appendix II). For the UV protocol, the differences observed between the years could be due to differences in the growing conditions only and not the darkening seasons. The darkening seasons for the two years were nearly identical. As mentioned previously, in 2003, the growing season was warm, dry, and relatively long; whereas in 2004, the season was wet, cool, and relatively short due to an early frost in August. The differences in the growing seasons may also explain the highly significant time by year interaction for all the color values.

Time was significant for all color values, having both linear and polynomial forms. For the L* and a* values, the genotypes differ in their linear and quadratic components but for the b* values, the genotypes differ only in their quadratic component.

With the UV light protocol, the intercepts of the L*, a* or b* values could not be used to distinguish the slow-darkening genotypes from the darkening genotypes (Table 4.4). For the L* and b* intercepts, the genotypes were not even significantly different. For the a* intercepts, the genotypes were significantly different, but there was no difference between the slow-darkening genotypes and the darkening genotypes.

Table 4.4 Comparison of the intercept of the color response curves for each pinto bean genotype as measured by the color values L*, a*, and b* for the UV light darkening protocol.

Genotype	L*	a*	b*
Darkening:			
CDC Pintium	46.13	4.82	12.37
CDC Minto	46.04	4.74	12.50
CDC Camino	45.78	3.92	11.68
SC11745-3	45.47	4.31	11.17
828B-3	45.21	4.33	12.01
828B-9	46.41	3.90	11.17
841-8	47.18	4.45	12.00
841-1	47.06	4.41	11.83
954S-952S	46.79	3.93	11.31
955s-1	46.59	4.84	12.13
1073M-42	47.01	4.00	11.61
1073M-46	46.38	4.55	11.92
1091M-57	47.17	4.21	12.28
Slow-darkening:			
SC11743-3	48.05	3.48	11.66
1533-14	48.87	3.54	11.67
1533-15	48.58	3.73	11.28
LSD	4.88	0.46	1.97

For the UV light protocol, the linear component of both the L* and a* values can be used to clearly distinguish the slow-darkening genotypes from the darkening genotypes (Table 4.5). The genotypes were not significantly different in the linear components of their b* value (Appendix II). The slopes of the L* and a* values were significantly larger and smaller, respectively, for the slow-darkening genotypes than the darkening genotypes.

Table 4.5 Comparison of the linear component of the color response curves for each pinto bean genotype as measured by the color values L* and a* for the UV light darkening protocol.

Genotype	L*	a*
Darkening:		
CDC Pintium	-0.23	0.15
CDC Minto	-0.21	0.15
CDC Camino	-0.18	0.14
SC11745-3	-0.20	0.14
828B-3	-0.21	0.15
828B-9	-0.19	0.15
841-8	-0.22	0.16
841-1	-0.22	0.16
954S-952S	-0.19	0.16
955s-1	-0.20	0.15
1073M-42	-0.21	0.16
1073M-46	-0.22	0.15
1091M-57	-0.22	0.16
Slow-darkening:		
SC11743-3	-0.11	0.07
1533-14	-0.11	0.07
1533-15	-0.10	0.08
LSD	0.04	0.04

For the UV light protocol, the genotypes were significantly different in the quadratic component of the L*, a*, and b* values (Table 4.6). The slow-darkening genotypes could not be differentiated from the darkening genotypes based on the L* quadratic component, however, based on the a* quadratic component, the slow-darkening genotypes were different from the darkening genotypes, with the slow-darkening genotypes having a significantly smaller quadratic component than the darkening genotypes. The b* value quadratic component could not distinguish the slow-darkening genotypes from the darkening genotypes.

Table 4.6 Comparison of the quadratic component of the color response curves for each pinto bean genotype as measured by the color values L*, a*, and b* for the UV light darkening protocol.

Genotype	L*	a*	b*
Darkening:			
CDC Pintium	0.00109	-0.00080	0.00120
CDC Minto	0.00097	-0.00080	0.00107
CDC Camino	0.00073	-0.00077	0.00110
SC11745-3	0.00092	-0.00077	0.00110
828B-3	0.00094	-0.00087	0.00120
828B-9	0.00081	-0.00080	0.00123
841-8	0.00100	-0.00090	0.00120
841-1	0.00099	-0.00083	0.00120
954S-952S	0.00079	-0.00083	0.00127
955s-1	0.00088	-0.00083	0.00127
1073M-42	0.00092	-0.00080	0.00113
1073M-46	0.00100	-0.00080	0.00113
1091M-57	0.00101	-0.00087	0.00110
Slow-darkening:			
SC11743-3	0.00046	-0.00030	0.00067
1533-14	0.00042	-0.00030	0.00070
1533-15	0.00035	-0.00037	0.00070
LSD	0.0003	0.0002	0.0005

4.1.3. Cabinet Protocol

For the cabinet protocol, the darkening data for 2003 and 2004 were not combined. In 2003, a Conviron model 630 cabinet was used but due to its malfunction in 2004, a Conviron model PGR15 cabinet was used instead. Although the cabinets were made by the same manufacturer and the temperature, relative humidity, and light settings were set exactly the same, the beans darkened much faster in model PGR15 compared to the model 630. In 2003 model 630 required 120 days for the slow-darkening genotypes and the darkening genotypes to have an approximate L* value color difference of 10 whereas in 2004 model PGR15 produced the same difference in L* color values between the darkening and slow-darkening genotypes in only 35 days. The models did vary in the positions of the lights, with model 630 having the lights positioned on the sides and model PGR15 having the lights positioned above, and this most likely explains the difference in darkening time. Regardless, the data from 2003 and 2004 were analyzed separately with 2003 having one replicate and 2004 having two replicates.

For the cabinet protocol in 2003, it appeared that the slow-darkening genotypes had slightly higher L* value intercepts and slightly lower a* value intercepts when compared with the darkening genotypes (Table 4.7). For the b* value intercepts, there was no difference between the slow-darkening and darkening genotypes. The L* and a* value linear components were larger and smaller respectively for the slow-darkening genotypes compared to those of the darkening genotypes. There does not appear to be a difference between the linear components for the b* values for the slow-darkening and darkening genotypes. For the L*, a*, and b* value quadratic component, there appears to be no difference between the slow-darkening genotypes and the darkening genotypes.

Table 4.7 Comparison of the intercepts, linear components, and quadratic components of the color response curves for each pinto bean genotype as measured by the color values L*, a*, and b* for the cabinet darkening protocol in 2003.

	Intercept			Linear			Quadratic		
Genotype	L*	a*	b*	L*	a*	b*	L*	a*	b*
Darkening:									
CDC Pintium	40.46	3.83	7.99	-0.16	0.094	0.0141	0.0011	-0.0004	-0.00011
CDC Minto	40.38	3.75	8.35	-0.18	0.097	0.0003	0.0010	-0.0004	-0.00008
CDC Camino	37.77	3.59	7.82	-0.15	0.096	0.0031	0.0012	-0.0005	-0.00006
SC11745-3	38.39	3.65	4.98	-0.15	0.082	0.0653	0.0011	-0.0004	-0.00041
828B-9	39.28	3.73	7.80	-0.15	0.091	0.0131	0.0009	-0.0004	-0.00011
828B-3	39.14	3.84	8.55	-0.16	0.091	-0.0136	0.0013	-0.0004	0.00005
841-8	39.87	3.72	8.00	-0.16	0.089	0.0083	0.0008	-0.0004	-0.00009
841-1	40.46	4.00	8.63	-0.17	0.099	0.0006	0.0012	-0.0005	-0.00008
955s-1	39.87	4.01	8.33	-0.16	0.098	0.0102	0.0010	-0.0004	-0.00012
1091M-57	39.74	3.72	8.25	-0.16	0.099	0.0005	0.0010	-0.0004	-0.00007
952S952S	40.35	3.82	8.48	-0.16	0.093	-0.0020	0.0013	-0.0004	0.000002
1073M-42	38.59	4.03	8.20	-0.15	0.091	-0.0014	0.0009	-0.0005	-0.00006
1073M-46	40.46	4.14	8.68	-0.15	0.089	-0.0060	0.0011	-0.0004	-0.00001
Slow-darkening:									
SC11743-3	42.03	3.19	8.44	-0.09	0.057	-0.0062	0.0009	-0.0002	0.00012
1533-14	44.33	3.08	8.87	-0.10	0.075	-0.0019	0.0011	-0.0003	0.00007
1533-15	42.55	3.39	8.39	-0.09	0.053	-0.0107	0.0009	-0.0002	0.00014

In 2004, differences in genotype and time were significant for all three measured color values, with time having significant linear and polynomial forms (Appendix III). For the L* value, the genotypes differed in their linear components but with the a* and b* values the genotypes differed in both their linear and quadratic component.

The genotypes were significantly different for the L*, a*, and b* value intercepts, but the slow-darkening genotypes were not significantly different from the darkening genotypes (Table 4.8).

Table 4.8 Comparison of the intercept of the color response curves for each pinto bean genotype as measured by the color values L*, a*, and b* for the cabinet darkening protocol in 2004.

Genotype	L*	a*	b*
Darkening:			
CDC Pintium	49.81	4.81	13.45
CDC Minto	48.33	4.81	13.02
CDC Camino	52.53	4.00	13.32
SC11745-3	50.20	4.23	11.90
828B-3	49.82	4.14	12.83
828B-9	50.49	4.09	12.17
841-8	52.85	4.29	12.12
841-1	52.13	4.45	12.29
954S-952S	51.53	3.96	11.53
955s-1	52.48	4.56	12.66
1073M-42	51.27	4.38	11.96
1073M-46	50.98	4.57	12.49
1091M-57	53.18	4.01	13.12
Slow-darkening:			
SC11743-3	51.41	4.02	13.94
1533-14	52.03	3.75	12.67
1533-15	52.18	3.94	12.57
LSD	1.65	0.28	1.04

For the cabinet protocol in 2004, the linear component of both the L* and a* values can be used to clearly distinguish the slow-darkening genotypes from the darkening genotypes as the slow-darkening genotypes have larger L* value slopes and smaller a* value slopes (Table 4. 9). For the L* and a* value linear components, there were significant differences within the darkening genotypes which indicates variation occurred in the rate of darkening among those genotypes. There were significant differences between the slow-darkening and darkening genotypes for the b* value linear component, but there was some overlap between the slow-darkening genotype 1533-15 and the darkening genotypes CDC Minto, CDC Camino, and SC11745-3 and between the slow-darkening genotype 1533-14 and darkening genotype SC11745-3.

Table 4.9 Comparison of the linear component of the color response curves for each pinto bean genotype as measured by the color values L*, a*, and b* for the cabinet darkening protocol in 2004.

Genotype	L*	a*	b*
Darkening:			
CDC Pintium	-0.63	0.245	0.29
CDC Minto	-0.57	0.240	0.24
CDC Camino	-0.57	0.260	0.22
SC11745-3	-0.56	0.225	0.18
828B-3	-0.61	0.275	0.33
828B-9	-0.57	0.260	0.30
841-8	-0.62	0.250	0.32
841-1	-0.59	0.245	0.29
954S-952S	-0.56	0.265	0.33
955s-1	-0.63	0.260	0.32
1073M-42	-0.64	0.250	0.41
1073M-46	-0.63	0.225	0.32
1091M-57	-0.68	0.265	0.32
Slow-darkening:			
SC11743-3	-0.32	0.120	0.02
1533-14	-0.32	0.120	0.07
1533-15	-0.33	0.140	0.14
LSD	0.07	0.017	0.10

For the cabinet protocol, neither the a* nor the b* quadratic component could be used to clearly distinguish the slow-darkening genotypes from the darkening genotypes (Table 4.10). The L* quadratic component was not tested as the ANOVA had indicated that the genotypes were not significantly different in the quadratic response for the L* value (Appendix III). There was overlap in the a* value quadratic component of the slow-darkening genotype 1533-15 and the darkening genotypes SC11745-3 and 841-1. For the b* value quadratic component there was overlap between slow-darkening genotype 1533-15 and darkening genotypes CDC Minto, CDC Camino, and SC11745-3.

Table 4.10 Comparison of the quadratic component of the color response curves for each pinto bean genotype as measured by the color values a* and b* for the cabinet darkening protocol in 2004.

Genotype	a*	b*
Darkening:		
CDC Pintium	-0.0039	-0.0056
CDC Minto	-0.0042	-0.0042
CDC Camino	-0.0038	-0.0039
SC11745-3	-0.0026	-0.0021
828B-3	-0.0053	-0.0071
828B-9	-0.0045	-0.0052
841-8	-0.0045	-0.0059
841-1	-0.0033	-0.0052
954S-952S	-0.0037	-0.0056
955s-1	-0.0044	-0.0069
1073M-42	-0.0072	-0.0105
1073M-46	-0.0068	-0.0088
1091M-57	-0.0070	-0.0080
Slow-darkening:		
SC11743-3	-0.0008	0.0003
1533-14	-0.0006	-0.0011
1533-15	-0.0017	-0.0030
LSD	0.0018	0.0027

In summary, all darkening protocols could distinguish slow-darkening from darkening genotypes on the basis of L* and a* linear components. For the UV light and cabinet protocols, the a* value quadratic component could also distinguish the slow-darkening from the darkening genotypes.

Since both the L* and a* value could distinguish slow-darkening from darkening genotypes, further analysis was conducted to determine if there was a correlation between the L* and a* values. For the greenhouse protocol, the last day (35), correlation coefficients from year 1 and 2 were not pooled as the years were heterogeneous (df = 1, $\chi^2 = 6.16$, p = 0.01) with year one and two and having a correlation coefficient of -0.60 and -0.93, respectively. Yet, for both years the correlation coefficients were high and negative. For the midpoint day (28), the correlation coefficients for the two years were pooled as the data for the two years were homogeneous (df = 1, $\chi^2 = 0.63$, p = 0.43) and the pooled correlation coefficient was -0.82. For the first day (0), the correlation coefficients for the two years were not pooled as the years were heterogeneous (df = 1, $\chi^2 = 3.93$, p = 0.047) and the correlation coefficients were -0.37 and +0.37 for years one and two, respectively.

For the UV light protocol, the correlation coefficients were pooled for the end time (hour 120) (df = 1, $\chi^2 = 2.57$, p = 0.11) and the middle time (hour 64) (df = 1, $\chi^2 = 1.87$, p = 0.17) and the correlation coefficients were high and negative being -0.89 and -0.96 respectively. Year one and year two were not homogeneous (df = 1, $\chi^2 = 11.60$, p = 0.0006) for initial time (0 hours) and the correlation coefficients were -0.48 and -0.95 for years one and two, respectively.

For the cabinet protocol, the correlation coefficients were not pooled over years due to the different darkening rates. For the final time, the correlation coefficients were -0.84 and -0.89 for year one and two, respectively. The midpoint time (day 21 and 63) and last measured time (day 35 and 119) also had negative correlations, but the values were much higher. In both years the midpoint correlation was -0.79. For day 0, the correlation coefficients were negative and low at -0.48 and -0.41 for year one and two, respectively.

Thus it appears that the L* and a* values are negatively correlated for the middle and final time points but not for the initial time point.

The seed germination study indicated that the genotypes did not differ in percent germination (Table 4.11). This was interesting since if the seedcoats of the slow-darkening genotypes have less condensed tannins than the darkening genotypes, (Beninger et al., 2005), one might expect the slow-darkening genotypes to have lower germination than the darkening genotypes, based on experience in other crops. Tannin-free faba beans and zero-tannin lentil are more susceptible to soil-borne pathogens than tannin containing lines and therefore have poor germination in the field (Kantar et al., 1996; Matus Munoz, 1991). However, the seeds in this germination study were surface sterilized prior to germination indoors in clean, sterile conditions. Gesto and Vazquez (1976) found that fresh seeds with lower levels of phenolic compounds germinated readily indoors while five year old seeds with considerably high levels of phenolic compounds had a low rate of germination indoors. For the weed shattercane (*Sorghum bicolor* (L.) Moench), caryopsis tannin content was negatively correlated with seed germination indoors but positively correlated with seed germination in the field (Fellows and Roeth, 1992).

Table 4.11 Summary of the ANOVA for percent seed germination from untreated pinto bean seed as well as pinto bean seed aged via the greenhouse, UV light, and cabinet protocols.

Source	df	SS	MS	F	P
Block	2	18.23	9.12	857.73	<0.0001
Genotype	15	1.39	0.09	0.66	0.8200
Protocol	3	23.93	7.98	57.13	<0.0001
Exp Error	171	23.87	0.14	13.13	<0.0001
Subsampling Error	384	4.08	0.01		
Total	575	71.50			

The protocol used greatly affected seed germination (Table 4.11). Seed darkened using the UV protocol had a high percentage of seed germination (94%) that was not significantly different than the percentage of seed germination for the un-aged seed (93%) (Table 4.12). Both the cabinet and the greenhouse protocol seed had low germination: 53 and 52%, respectively, and they were not significantly different from one another.

Table 4.12 Comparison of the percent germinated seed from untreated pinto bean seed and darkened pinto bean seed for each of the darkening protocols.

Protocol	Percent Germinated
UV	94
Untreated	93
Cabinet	53
Greenhouse	52
LSD	2

A possible explanation for the difference in percent germination between the protocols was that the conditions experienced during the cabinet and the greenhouse protocols are not only darkening the seedcoats of the beans, but also aging the cotyledons and embryos of the seed while the UV protocol was only darkening the seedcoats of the beans and not aging the cotyledons and embryos of the seed. It has been shown that when beans have been stored under high humidity, high temperature, and light, the germination declines (Barrón et al., 1996; Gesto and Vazquez, 1976) whereas beans that are subjected to ultraviolet or cool-white light, without high temperatures or high humidity, have little loss in germination (Hughes and Sandsted, 1975). As well, a previous study showed that the cooking time for UV light darkened beans was not significantly different than untreated, fresh beans (Junk, unpublished) which again

suggests that the UV light protocol was only affecting the bean seedcoat and not the cotyledons. Perhaps the UV light breaks has the ability to down the membranes of vacuoles in seedcoat cells that contain reactants and thus the reactants are free to mix with one another and/or other enzymes in the cell, thereby creating a pigmented color.

The estimated cost per sample (in Canadian dollars) for each of the protocols varied greatly with the cabinet protocol being the most expensive and the UV light protocol being the least expensive. In 2003, the cabinet rental was $3/day and was rented for 120 days costing $360 in total. The cabinet held 144 samples which resulted in a cost of $2.50/sample. Each sample required either the bottom or top portion of a 100 x 15mm petri dish which cost $0.04 each. Thus, the total cost per sample in 2003 was $2.54/sample taxes excluded. In 2004, the cost per sample for the cabinet protocol was $0.70 excluding taxes. The cabinet rental was $3/day and was rented for only 35 days costing $105 in total. The cabinet held 160 samples at a cost of $0.66/sample. Each sample required either the bottom or top portion of a 100 x 15mm petri dish which cost $0.04.

The greenhouse protocol was more affordable. Greenhouse rental was $1.00/foot^2/month and the protocol required two months resulting in $2.00/ft^2. One square foot holds six samples resulting in a cost of $0.33/sample. Each sample required one cm^2 piece of felt which were free remnants from the greenhouse flood benches and a Whirl-Pak® bag which cost $0.13/ bag. Thus, the total cost per sample for the greenhouse protocol was $0.46 excluding taxes.

The UV light protocol was by far the most economical. According to the manufacturer, the expected longevity of the bulb is 8000 hours. To darken the beans requires 120 hours, meaning 66 batches per bulb. Each bulb costs $69.00 resulting in $1.05/batch. Forty five samples can be darkened in each batch costing $0.02/sample. Each sample required either the bottom or top portion of a 100 x 15mm petri dish which cost $0.04 each. Thus, the total cost per sample for the UV light protocol, excluding the initial cost of the light fixture, was $0.06/sample.

In summary, even though all darkening protocols can distinguish slow-darkening genotypes from darkening genotypes, the UV light protocol was the most favorable protocol as it was the most reliable over years, it was the fastest, the seed had a high

percentage of germination following darkening, and it was the least expensive. When compared to the UV light protocol, the cabinet protocol was unfavorable as the period to darken the beans was long and depended on the cabinet model used, the seed had a low percentage of germination following darkening, and the cost was very high. The greenhouse protocol was the most unfavorable protocol as the period to darken the beans was greater than the UV light protocol, the seed had a low percentage of germination following darkening, and the cost was higher than the UV protocol. As well, the greenhouse protocol was subject to seasonal darkening conditions, and the seed can become infected with fungi during darkening as occurred in 2003.

Preliminary work has also been conducted using UV light to darken lentils, and perusal of the results suggests that it was effective (Junk, unpublished).

For future darkening experiments, the following darkening protocol is suggested as a means of differentiating between darkening and/or slow-darkening genotypes that darken at different rates. First, grow the beans to be tested so that the harvest date is the roughly the same for each genotype to prevent natural seedcoat darkening from affecting the results. While harvesting the genotypes, store all of the harvested beans together, preferably in cold, dark, dry conditions, in so that they are subjected to the same temperature, humidity, and light conditions. Allow all of the beans to equilibrate in these conditions but do not prolong artificial darkening. Just prior to darkening, record the L* color values of the beans to be darkened with a Hunter Lab colorimeter. Best color measurements are obtained when the color of the sample is read three times and the color results are averaged. Place the beans in open petri dishes, ten centimeters below a 254 nm UV lamp (suggested model G40T10, Ushio America, Inc., Cypress, California, USA) for 120 hours or more without any disruption to the beans. Longer periods of darkening may be required to differentiate genotypes that have similar darkening rates. After darkening, record the L* color value of the beans again with a Hunter Lab colorimeter. Again, best color measurements are obtained when the color of the sample is read three times and the results are averaged. For data analysis, one can compare initial seedcoat color and final seedcoat color, and/or rate of seedcoat color change.

4.2. Genotype x Environment Interaction for Seedcoat Darkening

4.2.1. Field Environments

Analysis of the L* color values of the undarkened seed revealed that genotype, environment, and the genotype by environment (g x e) interaction were all significant (Table 4.13).

Table 4.13 ANOVA showing the proportion of phenotypic variation for seedcoat color (L* value) before the seedcoats were darkened for the twelve pinto bean genotypes grown in four field environments in 2004.

Variance	df	SS	MS	F	P
Environment	3	337.00	112.33	66.77	<0.0001
Block (Environment)	8	13.46	1.68	4.27	0.0002
Genotype	11	201.89	18.35	46.61	<0.0001
G x E	33	56.30	1.71	4.33	<0.0001
Error	85	33.47	0.39		

When examining the data for the genotypes grown at the four different locations, it was found that differences among the L* value means at each location were minimal (Table 4.14). Thus, the slow-darkening genotypes could not always be distinguished from the darkening genotypes at harvest. For instance early maturing, well adapted CDC Pintium often had very light seedcoat color (high L* values). Thus, good seedcoat color at harvest could be a result of adaptation and vigor as well as possessing the slow-darkening trait. As well, the L* value differences among the darkening genotypes was minimal and the relative ranking was different across the different environments. These results may explain why there is an ongoing debate in the pinto bean industry as to which of the currently sown varieties produces the lightest seed.

Table 4.14 The mean seedcoat colors (L* values) ± standard errors for the seedcoats of each pinto bean genotype at each field environment before accelerated seedcoat darkening via UV light.

Saskatoon		Oxbow	
Genotype	L*	Genotype	L*
1533-15	43.77 ± 1.16	1533-15	45.10 ± 0.05
CDC Pintium	41.78 ± 1.17	1533-14	44.00 ± 0.79
SC11743-3	41.43 ± 1.64	SC11743-3	43.42 ± 0.69
Maverick	41.26 ± 1.04	Maverick	42.94 ± 0.59
Othello	41.17 ± 0.81	Buster	41.89 ± 1.93
1533-14	41.09 ± 3.73	CDC Pintium	41.87 ± 0.61
CDC Minto	40.84 ± 1.02	Othello	41.53 ± 1.33
Buster	40.62 ± 0.53	CDC Minto	41.18 ± 0.46
CDC Altiro	40.41 ± 1.29	Bill Z	41.15 ± 0.46
Bill Z	39.98 ± 1.02	CDC Pinnacle	40.87 ± 0.40
Pintoba	39.89 ± 0.95	Pintoba	40.85 ± 1.85
CDC Pinnacle	39.08 ± 1.31	CDC Altiro	40.02 ± 1.95

Outlook		Davidson	
Genotype	L*	Genotype	L*
1533-15	45.96 ± 1.51	1533-14	43.07 ± 1.52
1533-14	44.98 ± 0.54	1533-15	42.10 ± 1.93
SC11743-3	44.57 ± 0.50	SC11743-3	41.72 ± 0.22
Maverick	43.79 ± 1.69	Maverick	39.65 ± 1.60
CDC Pintium	43.53 ± 0.64	CDC Minto	38.53 ± 2.63
Bill Z	43.21 ± 0.83	CDC Pintium	38.50 ± 1.63
Buster	43.20 ± 1.08	Buster	38.47 ± 0.46
CDC Pinnacle	42.95 ± 2.04	CDC Altiro	38.42 ± 1.22
CDC Minto	42.84 ± 0.26	Bill Z	38.02 ± 1.93
Othello	42.33 ± 0.50	CDC Pinnacle	37.34 ± 0.22
Pintoba	42.32 ± 0.24	Pintoba	37.21 ± 0.60
CDC Altiro	42.24 ± 2.17	Othello	37.13 ± 1.06

Analysis of the L* values of the seedcoats after accelerated darkening using UV light, revealed that environment and genotype effects were significant but the g x e interaction was no longer significant (Table 4.15). Thus, regardless of the environment that the genotypes were grown in, the relative ranking of the genotypes remained stable. The least square means and standard errors for the genotypes show that the slow-darkening genotypes are significantly lighter (higher L* values) (Table 4.16) when contrasted to the darkening genotypes.

Table 4.15 ANOVA showing proportion of phenotypic variation for seedcoat color (L* value) after UV darkening for the twelve pinto bean genotypes grown in four field environments in 2004.

Variance	df	SS	MS	F	P
Environment	3	189.06	63.02	29.55	<0.0001
Block (Environment)	8	17.06	2.13	2.94	0.006
Genotype	11	1120.38	101.85	140.26	<0.0001
G x E	33	27.76	0.84	1.16	0.29
Error	85	61.73	0.73		

Table 4.16 The mean seedcoat color (L* value) $\pm$ the standard errors of the twelve pinto bean genotypes grown in four field environments after accelerated darkening via UV light.

Genotype	Mean L* value
1533-15	39.07 $\pm$ 0.31
1533-14	38.40 $\pm$ 0.23
SC11743-3	38.18 $\pm$ 0.25
Maverick	32.70 $\pm$ 0.28
CDC Pintium	32.46 $\pm$ 0.25
CDC Altiro	32.38 $\pm$ 0.25
Buster	31.85 $\pm$ 0.26
Bill Z	31.81 $\pm$ 0.24
CDC Minto	31.76 $\pm$ 0.26
Othello	31.65 $\pm$ 0.25
CDC Pinnacle	31.44 $\pm$ 0.25
Pintoba	31.11 $\pm$ 0.25

Examination of the means and standard errors of the data indicated that the environment at Outlook produced beans with the lightest colored seedcoats followed by the Oxbow environment (Table 4.17). Thus, in 2004 the irrigated site at Outlook produced lighter beans than the dryland locations. In contrast, Balasubramanian et al. (1999) found that canning quality of dry bean seed from irrigated sites differed very little from the seed from dryland sites. Beans from Saskatoon and Davidson had darker seedcoats than those from Outlook and Oxbow, each of which were significantly darker from one another. The differences observed at the Saskatoon site may be a result of the multiple hail storms and the early frost. Similarly an early snowfall causing delayed harvest at Davidson may explain the even darker seeds produced there compared to Saskatoon.

Table 4.17 The mean pinto bean seedcoat color (L* value) ± standard errors of the four field environments in 2004 after accelerated seedcoat darkening via UV light.

Location	Mean L* value
Outlook	34.99 ± 0.15
Oxbow	34.28 ± 0.14
Saskatoon	33.20 ± 0.15
Davidson	31.80 ± 0.15

These results differ from the findings of a study involving seedcoat darkening of lentil, where Vaillancourt and Slinkard (1985) found environment, genotype and g x e to be significant. However, Vaillancourt and Slinkard (1985) suggested that the g x e interaction was significant for their study as the Hagen location received rain in August, causing those lentils that had already matured to darken while the late maturing genotype, Laird, which had not fully matured at that time, did not darken. As a result, Laird had a much lower rate of darkening at their Hagen location compared to other cultivars at that location.

These results also differ from a bean quality study by Michaels and Stanley (1991) who found that for initial bean hardness, g x e was significant and for final hardness genotype and g x e were significant. Similar results were found for other seed quality characteristics such as 100 seed weight, testa content per seed, protein content, water absorption, conductivity, and cooking times for dry beans of Polish origin (Boros and Wawer, 2004) and for trypsin inhibitor content and tannin content of beans from the Jalisco and Durango races (De Mejía et al., 2003).

4.2.2. Indoor and Outdoor Environments

ANOVA was conducted on the L* values of the slow-darkening and darkening genotypes grown in the field, the polyhouse, the phytotron, and the greenhouse. Analysis of the data indicated that time, protocol, genotype, environment, g x e, protocol by genotype, and protocol by environment were all significant (Table 4.18). It was suspected that the differences among the darkening genotypes had created significant g x e interaction so the analysis was repeated again but with the genotypes classified as either slow-darkening or darkening. The g x e interaction was found to be not significant. Thus, the slow-darkening trait was stable across indoor and outdoor

environments (Table 4.19). The significance of the protocol, protocol by genotype interaction, and protocol by environment interaction were to be expected as each of the protocols darkened the beans to a different final color at the time of color scoring.

Table 4.18 ANOVA showing proportion of phenotypic variation for seedcoat color (L* value) of the 10 pinto bean genotypes grown in indoor and outdoor environments in 2004 and then darkened after harvest via three different protocols.

Variance	df	SS	MS	F	P
Time	22	11070.89	503.22	561.74	<0.0001
Protocol	2	144.55	72.28	80.68	<0.0001
Genotype	9	3747.30	416.37	464.78	<0.0001
Environment	3	1052.45	350.82	391.61	<0.0001
G x E	27	141.55	5.24	5.85	<0.0001
P x G	18	95.12	5.28	5.90	<0.0001
P x E	5	60.81	12.16	13.58	<0.0001
Error	1163	1041.85	0.90		

Table 4.19 ANOVA showing proportion of phenotypic variation for seedcoat color (L* value) for the slow-darkening and darkening pinto bean genotypes grown in indoor and outdoor environments in 2004 and then darkened after harvest via three different protocols.

Variance	df	SS	MS	F	P
Time	22	11070.89	503.22	332.43	<0.0001
Protocol	2	48.37	24.18	15.98	<0.0001
Genotype	1	3215.78	3215.78	2124.32	<0.0001
Environment	3	355.54	118.51	78.29	<0.0001
G x E	3	1.71	0.57	0.38	0.7702
P x G	2	12.51	6.26	4.13	0.0163
P x E	5	60.81	12.16	8.03	<0.0001
Error	1211	1833.20	1.51		

For the means of the L* values of the slow-darkening and darkening genotypes, the slow-darkening genotypes were significantly lighter than those of the darkening beans, as expected (Table 4.20). The outdoor environments produced significantly lighter beans than the indoor environments, with the greenhouse producing the darkest beans (Table 4.21). Seed produced in the greenhouse and phytotron most likely had seedcoats darker than the others as these seeds were harvested later than the other environments. Thus, the seeds were physiologically mature in the pods and were beginning to darken in the phytotron and the greenhouse whereas the seeds did not have as much time to darken in the field or polyhouse.

Table 4.20 The mean pinto bean seedcoat color (L* value) ± standard errors of slow-darkening genotypes and darkening pinto bean genotypes which were grown in indoor and outdoor environments in 2003 and then darkened after harvest via three different protocols.

Genotype	Mean L* value
Slow-darkening	34.34 ± 0.22
Darkening	28.78 ± 0.11

Table 4.21 The mean pinto bean seedcoat color color (L* value) ± standard errors for the indoor and outdoor growing environments in 2003 which were subjected to darkening via three different protocols.

Environment	Mean L* value
Polyhouse	30.23 ± 0.27
Field	29.83 ± 0.22
Phytotron	28.96 ± 0.20
Greenhouse	28.53 ± 0.19

Gonzalez et al. (1982) found that kidney and pinto beans harvested at the dry stage had lower L* values or were darker than beans harvested when the plants were harvested at the semi-dry stage. As well, dry harvested beans had more split beans after canning, were not as firm, and had less acceptability by the sensory panel than semi-dry harvested beans. In lentil, similar results have been found. When lentils are harvested when the plants are completely dry, cooking time increases (Iliadis, 2000). However, Forney et al. (1990), in New York State, found that harvest time had little effect on subsequent canning quality of beans. Harvesting too early can have negative effects as well. De Oliveira Rios et al. (2002 and 2003) found that an early harvest results in a higher phenolic content, POD activity, water absorbing capacity, and protein content in beans (De Oliveira Rios et al., 2002 and 2003).

4.3. Genetic Control

4.3.1. Slow-Darkening Trait

The F_2 bean seedcoats segregated in a bimodal distribution of 3 darkening to 1 slow-darkening for all crosses between the slow-darkening parent, 1533-15, and the darkening parents, CDC Pintium or HR99 (See figure 3 for an example). This suggested that slow-darkening was controlled by a recessive allele at a single locus in these crosses.

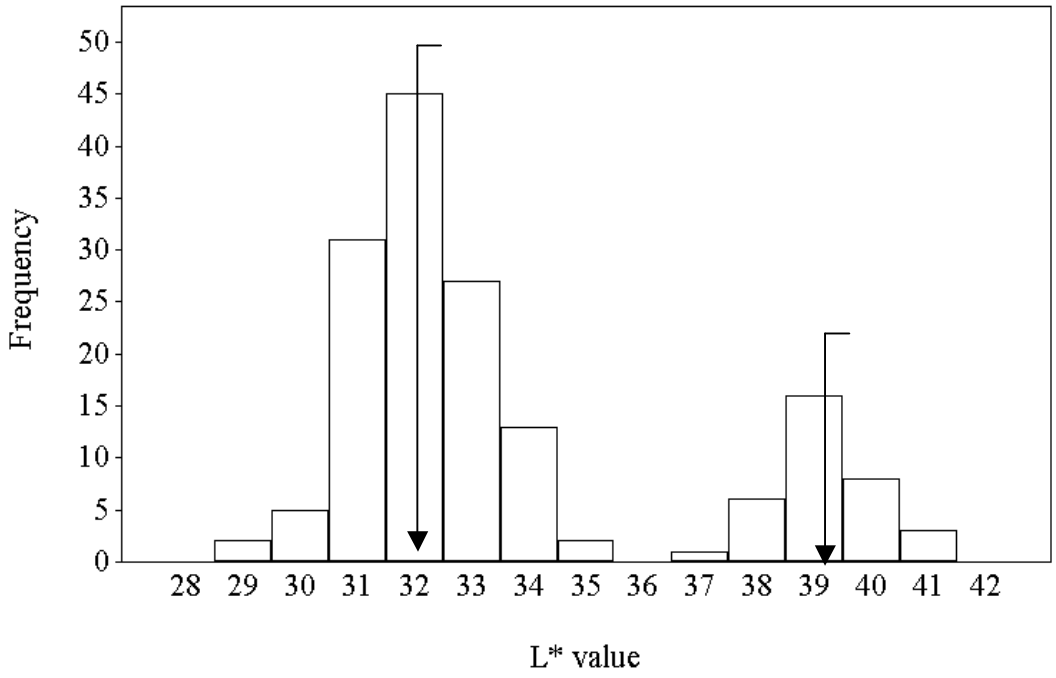

Figure 4 The frequency distribution of L* values for the parents and individual F_2 pinto bean plants from the homogenous crosses CDC Pintium x 1533-15 and 1533-15 x CDC Pintium (df=1, χ^2=1.08, p= 0.58) segregating 125 darkening : 33 slow-darkening.

In 2003, one F_2 population of HR99 x 1533-15 and two F_2 populations of CDC Pintium x 1533-15 were combined because a chi-square heterogeneity test determined that the populations were homogeneous (df= 2, χ^2=0, p= 1.00). The combined homogenous populations segregated 186 darkening to 60 slow-darkening. The measured ratio was compared to a 3:1 ratio. The chi-square results indicated that the measured ratio was not significantly different from the tested 3:1 ratio (df=1, χ^2= 0.05, p= 0.82) suggesting that slow-darkening was controlled by a recessive allele at a single locus.

In 2004, the CDC Pintium x 1533-15 and 1533-15 x CDC Pintium F_2 populations were combined as the chi-square heterogeneity test determined that the populations were homogeneous (df=1, χ^2=1.08, p= 0.58). The combined populations segregated 125 darkening: 33 slow-darkening. This ratio was similar to a 3:1 ratio. Darkening parent, CDC Pintium had L* values similar to the darkening group and slow-darkening parent, 1533-15, had L* values similar to the slow-darkening group (Figure 3). The chi-square results indicated that the measured ratio was not significantly

different from the 3:1 ratio (df=1, χ^2=1.43, p=0.23) suggesting again that slow-darkening was controlled by a recessive allele at a single locus.

The segregating F_2 populations in 2004 tended towards lower frequency for the homozygous recessive class. In 2004, some of the F_2 plants were lost to early fall frost. If the slow-darkening trait is linked to maturity, as might be expected given that it originated in a late maturing line from CIAT, this could explain why the number of slow-darkening phenotypes was lower than expected.

The three F_2 populations grown in the field in 2003 were advanced by single seed descent and grown in the field in 2004 as $F_{2:5}$ families. Those $F_{2:5}$ families that were segregating for slow-darkening were discarded. Due to the early frost, the number of plants harvested per family was low for many of the families. When the data were analyzed regardless of the number of plants per family, the populations were found to be homogeneous (df= 2, χ^2= 0.60, p= 0.74). The combined populations segregated 108 darkening families : 87 slow-darkening families which was not significantly different from a 1:1 ratio (df = 1, χ^2= 2.26, p= 0.13). This supports the F_2 data that suggests that slow-darkening was controlled by a single locus.

The data were then re-analyzed including only those families which had 11 or more plants per family. Again, the populations were homogeneous (df =2, χ^2= 3.52, p= 0.17) and the population segregated 20 darkening families : 19 slow-darkening families which was not significantly different from a 1:1 ratio (df= 1, χ^2= 0.03, p= 0.86). These results again agreed with the previous data that found that slow-darkening was controlled by a single locus.

These results agree with seedcoat studies in both lentil and faba bean (Crofts et al., 1980, Vaillancourt et al., 1986). Both lentil and faba bean seedcoats darken after harvest due to the presence of tannins and resist darkening due to the absence of tannins. When high tannin genotypes were crossed to low tannin genotypes, the F_2 populations segregated 3 high tannin : 1 low tannin, indicating the low tannin trait is controlled by a single recessive gene. This agrees with studies on tannin herbage content in birdsfoot trefoil (*Lotus corniculatus* L.) (Miller and Ehlke, 1997). When low tannin herbage genotypes were crossed with high tannin herbage genotypes, the F_2 population

segregated 3 high tannin : 1 low tannin, again suggesting that low tannin content is controlled by a single recessive gene.

4.3.2. Pinto Saltillo and Other Slow-darkening Genotypes

The phenotypes of the F_2 populations from crosses between slow-darkening and darkening genotypes as well as slow-darkening and slow-darkening genotypes are shown in Table 4.22. All slow-darkening by darkening F_2 populations segregated in a 3 darkening : 1 slow-darkening ratio, with darkening parents having L* values similar to the darkening group and slow-darkening parents having L* values similar to the slow-darkening group, indicating that there was one gene for darkening and that slow-darkening was controlled by a recessive allele. This is in agreement with the results found in section 4.3.1. It is plausible that the same slow-darkening gene occurs in SC11743-3 P8, SC11743-3 P9, and Pinto Saltillo as they are all derived from parents from CIAT, although their pedigrees differed. All F_2 populations derived from slow-darkening by slow-darkening crosses resulted in normal distributions of the L* values. Unfortunately only one plant each of Pinto Saltillo, SC11743-3 P9, and SC11743-3 P8 were grown for this study so the variance of the parents cannot be compared to the F_2 populations. However, for parent 1533-15, more parental plants were grown and the measured L* value variance was 0.48, which was small when compared to the Pinto Saltillo x 1533-15 F_2 population L* value variance of 1.67. This suggests that slow-darkening may also be quantitatively expressed due to environmental interactions or interaction with other genes.

Table 4.22 The phenotypes of the F_2 populations derived from slow-darkening and darkening pinto bean parents.

Cross	Segregation
CDC Pintium and Pinto Saltillo*	22 darkening : 6 slow-darkening For 3:1 $\chi 2$=0.19 , p=0.89
SC11743-3 P9 x CDC Pintium	35 darkening : 14 slow-darkening For 3:1 $\chi 2$=0.33, p=0.86
Pinto Saltillo x 1533-15	Normal distribution all slow-darkening Shapiro-Wilk=0.83; p=0.01; Skewness=-1.44; Kurtosis=1.61
SC11743-3 P9 x SC11743-3 P8	Normal distribution all slow-darkening Shapiro-Wilk=0.96; p=0.02; Skewness= 0.67; Kurtosis=1.03

*Heterogeneity test df=1 $\chi 2$=0.10 p=0.75

The results suggesting that slow-darkening was controlled by a recessive allele at a single locus agree with the results of Vaillancourt et al. (1986) who found that in lentil the zero tannin trait appears to be controlled by a single recessive gene when examining a F_2 population derived from a zero tannin and high tannin parents. They found that the tannin trait also appears to be quantitatively inherited when examining an F_2 population derived from a medium tannin and high tannin parents. Miller and Ehlke (1997) found that for birdsfoot trefoil tannin content in foliage appears to be controlled by additive, quantitative effects as indicated by a significant general combining ability factor, yet one of their diallels found specific combining ability to be significant indicating that tannin content may be controlled by major genes with complementary gene action. Dalrymple et al. (1984) found that low tannin in foliage of birdsfoot trefoil is controlled by a single recessive gene. Dabholkar and Baghel (1982) found that with sorghum grain, the general combining ability component was significant indicating additive gene action.

In the case of slow-darkening seedcoats of pinto bean, we detected a single major gene effect, but the slow-darkening trait was not the result of the complete absence of specific compound(s). The fact that color change does occur, indicates that the phenotype was not the result of a complete biochemical pathway blockage, as found in lentil (Vaillancourt et al., 1986). Slow-darkening genotype 1533-15 still contains

flavonols and tannins although the amount was significantly lower than that present in the darkening genotype CDC Pintium (Beninger et al., 2005).

5. CONCLUSION

5.1. Summary and Conclusion

There is a need for a quick, reliable, inexpensive, non-destructive protocol to accelerate seedcoat darkening as a way to improve selection for improved seed appearance in pinto bean. Three potential protocols were examined. The first protocol tested was the greenhouse protocol. This protocol was conducted in the greenhouse by placing the bean seeds in polybags with a one cm^2 piece of moistened felt. The UV light protocol placed the bean seeds 10 cm under a UV lamp with a wavelength of 254 nm. The cabinet protocol placed the bean seeds in a cabinet set at 30˚C, 80% relative humidity, with fluorescent lights. Color measurements of the bean seeds were taken routinely using a Hunter Lab colorimeter with L*, a*, and b* values being recorded.

The color response over time curves of the darkening genotypes differed from the slow-darkening genotypes in their L* and a* linear component for all three protocols. For the linear component, correlation coefficients between the L* and a* values were found to be highly negative at the middle and final time periods. The a* quadratic component for the UV light and cabinet protocols, could also distinguish the slow-darkening from the darkening genotypes.

The UV light protocol was considered to be superior to the greenhouse and cabinet protocol as the UV light protocol was quick, consistent over years, and the most economical. Unlike the greenhouse and the cabinet protocols, the UV light protocol did not affect seed germination following accelerated darkening. The greenhouse protocol was the most unfavorable protocol as the period to darken the beans was greater than the UV light protocol, the seed had a low percentage of germination following darkening, and the cost was higher than the UV light protocol. As well, the greenhouse was subject to seasonal darkening conditions, and the seeds were prone to fungal infection during darkening. Although the cabinet protocol is used by researchers to create the HTC effect, it is not as effective as the UV light protocol for seedcoat darkening. The cabinet protocol required a long period to darken the beans and was dependent on the cabinet used. Also, the seed had a low percentage of germination following darkening, and the cost was very high.

To determine the stability of the slow-darkening trait, genotype by environment (g x e) studies were conducted across different field environments and across indoor and outdoor environments. For the g x e study across different field environments it was found that prior to seedcoat darkening the g x e interaction was significant. The slow-darkening genotypes could not always be distinguished from the darkening genotypes as the color differences among the darkening genotypes was minimal and the ranking changed across the environments. This may explain why there is an ongoing debate in the pinto bean industry as to which of the currently sown varieties produces the lightest seed. After accelerated seedcoat darkening, both environment and genotype were significant whereas the g x e interaction was not significant. As expected, the slow-darkening genotypes had lighter seedcoats than the darkening genotypes. For the field sites, Outlook and Oxbow produced beans with lighter seedcoats most likely because they had the more favorable growing conditions. The beans grown at Saskatoon and Davidson were the darkest most likely due to unfavorable late season weather conditions at those sites.

For the g x e study across indoor and outdoor environments, initial analysis indicated that there was a significant g x e interaction. However, when the genotypes were split into either slow-darkening or darkening, the g x e interaction was not significant. The slow-darkening genotypes had lighter seedcoats than the darkening genotypes as expected. For the environments, the outdoor environments had lighter seedcoats than the indoor environments but this could be due to differences in harvest times relative to maturity.

The genetic control of various slow-darkening genotypes was determined. All of the F_2 populations derived from slow-darkening genotypes and darkening genotypes, segregated 3 darkening : 1 slow-darkening with distinct bimodal distribution for the L* value color. When the populations were advanced and grown as $F_{2:5}$ families, the populations segregated 1 darkening : 1 slow-darkening. This indicated that seedcoat darkening was controlled by a single gene and darkening was dominant over slow-darkening. For both slow-darkening by slow-darkening crosses, the F_2 population phenotypes were a unimodal distribution, normally distributed for L* value colour,

indicating there may be modifying genes and environmental effects for the slow-darkening trait.

5.2. Future work

This study indicated that UV light can darken pinto bean seedcoats and preliminary work indicated that the UV light can darken lentil seedcoats (Junk, unpublished). Since seedcoat darkening is a problem in other pulses, it would be sensible to determine if the UV light protocol could distinguish darkening from slow-darkening genotypes in the seedcoats of other tannin containing pulses such as faba bean and lentils.

Other market classes of bean are pre-disposed to seedcoat darkening. The integration of the slow-darkening trait into other market classes would be ideal. However, the CDC bean breeding program has observed that when the slow-darkening trait is incorporated into the market class Flor de Mayo, the traditional pink seedcoat pattern changes to a blue-grey seedcoat pattern. Further investigation into this seedcoat color pattern change and the outcome of other crosses would be interesting.

It has been demonstrated that zero-tannin lentil and low-tannin faba bean have a more fragile seedcoat than their high-tannin counterparts (Vaillancourt and Slinkard, 1985). When the seedcoat thickness of lentil was measured, it was found that the zero-tannin lentil had a significantly thinner seedcoat than tannin containing lentil (Vaillancourt and Slinkard, 1985; Crofts et al., 1980). A comparison of seedcoat thickness and other seedcoat physical structural differences between the darkening and slow-darkening pinto genotypes may provide a partial explanation for the differences observed between the genotypes.

Brazilian researchers are very interested in bean seedcoat darkening and are investigating a possible role of peroxidase (POD) and polyphenol oxidase (PPO) (De Oliveira Rios et al., 2002; Esteves et al., 2002; Moura et al., 1999). A study comparing POD and PPO activity during darkening for the slow-darkening and darkening genotypes would be able to determine if POD and PPO activity have a role in seedcoat darkening.

There is a demand for fast cooking beans, especially in developing countries where fuel is scarce. Preliminary work indicates 1533-15 has a higher hydration coefficient and cooks faster than CDC Altiro and CDC Pintium when the seed is fresh

and aged. Further cooking and canning quality should be studied on 1533-15 and other slow-darkening genotypes.

During this study, three $F_{2:5}$ populations which were derived from slow-darkening and darkening parents were developed. These populations could now be used to map and sequence the gene for seedcoat darkening which may help in determining the mechanism for seedcoat darkening.

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7. APPENDICES

Appendix I Summary of the greenhouse darkening protocol ANOVA for the pinto bean seedcoat color values L*, a*, and b*.

Variance	df	Mean Square		
		L*	a*	b*
Years (Y)	1	10751.62**	770.84*	5294.32*
Replicates in Years (R)	1	0.17	3.80**	28.03**
Genotypes (G)	15	236.95**	27.62**	6.03
G x Y	15	20.17	4.87**	7.91**
G x R	15	14.37**	1.33**	1.52**
Time (T)	8	2101.86**	220.23**	35.84
Time linear	(1)	16701.62**	1609.43**	166.00**
Time quadratic	(1)	24.19**	143.01**	87.22**
Time cubic	(1)	62.36**	0.16	0.24
Time lack-of-fit	(5)	5.35*	1.85	6.66**
T x Y	8	28.14**	28.04**	77.36**
T x R	8	1.93	0.66*	2.61**
T x G	120	6.72**	0.87**	0.89**
T x G linear	(15)	45.10**	4.69**	2.89**
T x G quadratic	(15)	2.61	1.58**	2.95**
T x G cubic	(15)	1.13	0.20	0.38
T x G lack-of-fit	(75)	0.98	0.087	0.19
T x G x Y	120	1.59	0.27*	0.43
Residual or T x G x R	120	2.11	0.26	0.50

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

Appendix II Summary of the UV light darkening protocol ANOVA for the pinto bean seedcoat color values L*, a*, and b*.

Variance	df	Mean Square		
		L*	a*	b*
Years (Y)	1	13189.25*	950.77*	11602.03*
Replicates in Years (R)	1	13189.25**	0.19	8.29**
Genotypes (G)	15	253.24**	84.54**	19.30
G x Y	15	8.84	5.78**	12.40**
G x R	15	4.47**	0.55**	1.38**
Time (T)	15	593.53**	218.82**	146.35**
Time linear	(1)	8057.52**	2660.68**	692.03**
Time quadratic	(1)	775.36**	588.83**	1284.38**
Time cubic	(1)	10.67**	14.75**	21.83**
Time lack-of-fit	(12)	4.95**	1.51**	16.41**
T x Y	15	5.49**	6.75**	40.10**
T x R	15	0.70	0.10	0.46**
T x G	225	1.60**	0.65**	0.39**
T x G linear	(15)	15.92**	5.35**	0.64
T x G quadratic	(15)	3.63**	3.08**	3.00**
T x G cubic	(15)	0.50	0.30	0.37
T x G lack-of-fit	(180)	0.31	0.08	0.15
T x G x Y	225	0.41	0.17**	0.17
Residual or T x G x R	225	0.43	0.10	0.17

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

Appendix III Summary of the cabinet darkening protocol 2004 ANOVA for the pinto bean seedcoat color values L*, a*, and b*.

Variance	df	Mean Square		
		L*	a*	b*
Replicates (R)	1	0.44	1.86**	14.39**
Genotypes (G)	15	76.49**	14.27**	5.87**
G x R	15	1.10	0.15	0.42*
Time (T)	5	1702.71**	311.49**	74.45**
Time linear	1	8287.38**	1435.47**	146.67**
Time quadratic	1	1.48	309.33**	76.74**
Time cubic	1	184.93**	317.68**	78.35**
Time lack-of-fit	22	19.88**	98.40**	35.25**
T x R	5	3.26**	0.55**	1.77**
T x G	75	5.44**	1.13**	0.95**
T x G linear	15	23.19**	4.56**	2.34**
T x G quadratic	15	1.44	0.71**	1.42**
T x G cubic	15	1.00	0.09	0.33
T x G lack-of-fit	30	0.79	0.15	0.32
Residual or T x G x R	75	75.83	0.15	0.19

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.