AN ABSTRACT OF THE THESIS OF

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Title: The Cosmetic Stay-Green Trait in Snap Bean and the Event Cascade that Reduces Seed Germination and Emergence

Abstract approved:

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The *persistent color* (*pc*) trait in snap bean (*Phaseolus vulgaris* L.) belongs to a member of the cosmetic stay-green gene family. It is considered very desirable by snap bean breeders for its impacts on pod quality. Persistent color imparts a uniform dark-green color to pods and expresses in seeds as a light green color. Commercially, *pc* genotypes are reported on approximately 40% of the total snap bean acreage in the USA. However, cultivars from *pc* lines have a lower field germination and emergence rate compared to white- and colored-seeded genotypes. Therefore, examination of the *pc* genotypes comparing the other seed genotypes either by anatomical, physical or chemical structure will give insight into why *pc* types have a lower germination and emergence rates. Soil borne pathogens may also be involved because fungicide treatment helps mitigate germination and emergence problems in the field. The research began with production of seeds in the same environment to obtain uniform samples for the future trials. The seeds involving *pc*, white-, and colored-seeded lines were grown first in greenhouse and then increased in the field. Then we tested seed germinability in both the lab and the field. In the process of producing the seeds, from greenhouse, we discovered mixtures in lines that allowed us to develop paired genotypes that differed only in their seed type (*pc* or white). Subsequent analyses were modified to exploit these genotype pairs and allowed us to

make inferences about the effects of *pc* without the complicating factor of genotype. This body of research consists of a series of experiments that include **1**. Germination in a laboratory setting without the presence of soil borne pathogens; **2**. The field emergence percentage determined by planting a diverse set of cultivars of fungicide treated and nontreated seed grown in a randomized complete block design; **3**. Evaluation of a molecular marker for *pc* in *pc* lines that have individuals with both white and green cotyledon expression; **4**. Comparison of leaf colors among *pc* and white-seeded genotypes; **5**. Evaluation of seed viability by tetrazolium assay; **6**. Comparison by isogenic or sister lines for seed water uptake; **7**. Evaluation of seed cracking after water-uptake; **8**. Determination of different genotypic response to mechanical injuries of seed by the drop test; **9**. Verification of seed moisture content; **10**. Measurement of electrical conductivity rates of isogenic pairs to quantify solute leakage from seeds; **11**. Examination of seed coat thickness by studying anatomical structure and lastly; **12**. Comparison of amounts of sugar compounds in different genotypes. The lab germination tests exhibited no significant differences in germination among seed types whereas in the field we observed higher infection rates on seedlings of *pc* genotypes. Some *pc* lines had a few individual plants that produced green cotyledons rather than the bleached white cotyledons normally observed for this seed type. In most cases, this effect was transmissible to the progeny. The *pc* lines that expressed green cotyledon color were verified to be *pc* by molecular marker analysis. These green cotyledon types are an interesting variant that should be investigated further to determine what role this trait may play in seed physiology. Evaluation of leaf color revealed differences across individual plants with upper leaves lighter than older, more mature leaves, but no consistent differences among genotype pairs were observed. Therefore, leaf color cannot be used to predict whether a line has the *pc* gene or not. There was more rapid water uptake in seeds of *pc* lines, and this was associated with higher rates of the imbibitional cracking. All snap bean types showed increases in water uptake after being mechanically injured in the drop test, but seeds of *pc* bean lines were particularly sensitive to mechanical

injury. Even uninjured *pc* seeds leaked more solutes as shown by the electrical conductivity-test. Anatomically, *pc* seeds exhibited thinner seed coats with all three outer layers of the testa showing reduced thickness, but the osteosclereid layer in particular was significantly thinner. The sugar compounds were not statistically significant different among seed type for soil infection, only fructose was higher in *pc* seeds, but not raffinose or sucrose. Thus, it appears that in beans the seed coat structure and integrity is important for soil borne pathogen defense. Fungicide treatment is used in commercial production to maintain germination and emergence rates of *pc* cultivars, but finding ways to reduce seed cracking could be beneficial.

The Cosmetic Stay-Green Trait in Snap Bean and the Event Cascade that Reduces
Seed Germination and Emergence

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ACKNOWLEDGEMENTS

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THE COSMETIC STAY-GREEN TRAIT IN SNAP BEAN AND THE EVENT CASCADE THAT REDUCES SEED GERMINATION AND EMERGENCE

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1.   INTRODUCTION

Snap beans (*Phaseolus vulgaris* L.) are an important vegetable crop in many countries around the world with a production of over 1.8 million tons in 2017 (FAOSTAT, 2018). Because of their popularity, a number of breeding programs work on genetic improvement. These programs focus on increasing productivity and efficiency of production as well as quality traits. Traits that improve productivity would include growth habit, germination and emergence percentage, while those that affect quality might include seed color, pod color, seed size, pod size, shape, thickness, and stringlessness.

The majority of contemporary snap bean varieties have white seed color. White-seeded beans are preferred by most processors because flavonoids of colored seeded beans negatively affect quality. Flavonoids are water soluble and will change the color of the liquid in canned green beans or remain as a shaded band where testa is revealed in cut beans in frozen product (Myers and Baggett, 1999). Another seed type being used for snap bean is *persistent color* (*pc*) which produces a very uniform, dark and green pod color compared to white-seeded beans. Used in conjunction with the *p* gene which conditions white seed color, the *pc* genotype is increasingly used in new cultivars with major US commercial breeding programs having incorporated the trait into around 40% of snap bean cultivars (Myers et al., 2018).

The *pc* trait is controlled by a single recessive gene that was first identified in French flageolet types (Bouwkamp and Honma, 1970). Properties of this gene include dark, uniform, green, pods that stay green even during senescence as well as green seeds, leaves that also stay-green instead of turning yellow during senescence and cotyledons that are white upon emergence (Dean, 1968; Bouwkamp and Honma, 1970). A deleterious trait associated with *pc* is a reduction of germination and emergence in the field. All these properties with the uniform green pods make the *pc* genotype snap beans valuable from a commercial standpoint. However, research is needed to understand and mitigate the deleterious effects of the *pc* genotype (Davis et al., 2009).

1.2. LITERATURE AND REVIEW

The snap bean (*Phaseolus vulgaris* L.) will be examined to answer the question of why germination and emergence rates are reduced in *persistent color* (*pc*) seed types when compared to white- or colored-seeded cultivars. To answer this question, we will begin by taking a closer look at snap bean crop information, including the organization of the genus in relationship to other species, its domestication and crop specific requirements for germination and growth. I then present information about seed types, associated traits and economic importance either in the World or the USA. Subsequently, I describe genetic control of seed color and morphological characteristics as well as literature on germination and emergence response as related to differences in seed types.

1.2.a. Snap Bean Crop Information

The common bean (*Phaseolus vulgaris*) is among the major grain legumes in the family Fabaceae (formerly Leguminosae) used for human consumption (Gepts and Debouck, 1991). The

55 species of this genus belong to the subtribe Phaseolinae (Lackey, 1977; Maréchal and Baudoin, 1978; Lackey, 1981), in the tribe Phaseoleae, within the sub family Papilionideae (Isely and Polhill, 1980; Polhill, 1981). Six *Phaseolus* species were recently recognized as being most closely related to *Phaseolus vulgaris*: *P. albescens, P. coccineus, P costaricensis, P. dumosus, P. parvifolius and P. persistentus* (Broughton et al., 2003; Delgado-Salinas et al., 2006; Pickersgill et al., 2007; Bellucci et al., 2014). Two of which are most closely related to *P. vulgaris,* (*P. dumosus* [year-long bean], and *P. coccineus* [scarlet runner]) are cultivated along with *P. acutifolius* (tepary bean) and *P. lunatus* (lima bean) (Lioi and Piergiovanni, 2013; Bellucci et al., 2014).

The snap bean is known to have close lineages to dry beans due to being a member of same genus and species (Wallace et al., 2018). Yet, the snap bean is consumed as a fresh vegetable wherein green immature stages are eaten before pod fiber develops (Gepts, 1988a; Gepts, 1988b, Gepts and Debouck, 1991); thus this class is primarily determined by pod characteristics and plant type more than seed characteristics (Myers and Baggett, 1999).

Both dry and snap beans are referred to as common beans. Variations in terminology of snap beans include: "French bean" as often described in Europe, "string bean" referring to a pod type where the pod suture fiber (string) is removed, or "green bean" with green pods and "wax bean" with yellow pods. In our study, the term "snap bean" will be used (Myers and Kmiecik, 2017).

Snap beans were most likely derived from dry bean. More than one independent derivation of the snap bean type has been documented based on phaseolin type, but, the exact geographic origins of snap beans are unknown. Wallace et al., (2018) conducted a structure analysis that provided estimates of what gene pools contributed to the origins of snap beans. Some

types of snap beans may have been derived in Europe after the beginning of the Colombian exchange, but some derivations were likely pre-Colombian and occurred in the Americas and then were subsequently taken to Europe. For example, small-seeded black beans may have been introduced from Middle America to the eastern U.S. via the Guanahatabey tribe of Western Cuba (Rouse, 1992). These resulted in one gene pool of snap bean (Song et al., 2015). In contrast, the large flat podded Andean form is believed to have originated in South America and was introduced to Europe and other countries around the world (Wallace et al., 2018).

These independent domestications in different subgroups of the same species resulted in distinct adaptations with different traits and brought different genetic backgrounds to the whole genome among these gene pools. The main characteristics that sere intensely affected by human selection but may not have been under conscious selection included seed size, leaf size, heliotropism and phaseolin seed storage protein type. Additionally, with this long history of domestication, the fundamental changes in characteristic traits that were consciously selected may have included higher yields and biotic and abiotic stress tolerances because the less fit genotypes were likely not planted by ancestors, eliminating them from the gene pool (Singh, 1989).

Common bean grows best at moderate air temperatures with warm soils (10° C is the minimum soil temperature for germination (McCormack, 2004)) and moderate soil water availability (Wallace, 1980). During the reproductive stage, bean is more sensitive to temperature stress, thus, imposing heat or drought conditions results in greater yield losses (Laing et al., 1984). Persistent high temperatures reduce pod and seed set, and interfere with pollen-stigma interaction, pollen germination, pollen tube growth, and fertilization (Stobbe et al., 1966; Dickson and Boettger, 1984; Weaver et al., 1985; Weaver and Timm, 1988; Monterroso and Wien, 1990; Li et al., 1991; Ofir et al., 1993; Gross and Kigel, 1994).

1.2.b. Types of Common Bean and Economic Importance in the World and USA

Among the snap bean varieties, types are differentiated into market classes either for processing or fresh market based on growth habit (determinate or indeterminate), and pod and seed characteristics. Whether due to its nutritional value as a source of the amino acids, proteins, vitamins (C-K), minerals, and beneficial phytochemicals (Leterme, 2002), or flavor and productivity, the crop has always maintained its importance to the world due to consumer demand. In the past 50 years, rapid acreage increases of green beans worldwide and expanding markets in both in Europe and the U.S. resulted in 18,017,731 MT in 2007 growing to 24, 221,252 MT in 2017 (FAOSTAT, 2018). Looking at just U.S. snap bean production, the crop was worth $303,480.000 USD in 2019, including $26,202,000 dollars for production in Oregon (USDA, 2020).

1.2.c. Seed Color Including Preferences and Genetic Control

The physical appearance of seed in terms of color, size, shape, and surface texture can influence pod characteristics of snap bean which are in turn important for pod quality for processing and fresh market. In terms of commercial importance, seed size may vary from 17 to 60 g per 100 seeds and is related to pod size. Seed shape varies from round to oblong to cylindrical and may affect bean pod smoothness. Selection for long, smooth, fleshy pods has resulted in long cylindrical seed. Seed surface texture varies from shiny to opaque, which may affect germination, and when combined with different colors and patterns may affect pod color and quality (Van Schoonhoven and Voysest, 1991). Since the early 20th century, seed color has been the subject of genetic research (Prakken, 1972; Bassett, 1994). Seed coat color has been studied by many

breeders to understand the inheritance, interactions among color genes and relation to agronomic traits that play an important role in consumer preferences. Common bean seeds may be classified by color (varying from white through yellow, brown, red, purple, black) each of which is representative of one or more dry bean market classes (Zhu et al., 2017).

The color of seed and plant parts is influenced by eight major loci: *P*, [*CM*], *Z, J, G, B, V* and *Rk* (Prakken, 1970; Prakken, 1972). The *ground factor* for the basal seed coat color genotypes (other than colored patterns) is the *P* locus (Emerson, 1909). The dominant *P* allele is necessary for the expression of other color genes with the recessive form preventing color expression in seeds, flower, leaves and stems (Bassett, 2007). There are several alleles of *P* that allow partial expression of color; the most important for our purposes being the grayish white color factor first reported as an independent locus and given the *gri* symbol by Lamprecht (1936), but subsequently recognized as an allele of *P*, and given the gene symbol p^{gri} by Bassett (1994).

The *persistent color* (*pc*) gene was named by Dean (1968). The *pc* gene only expresses in the seed in the presence of *p* and has pleiotropic effects on the plant. As the plant approaches senescence, *pc* inhibits loss of green color in leaves, stems, and pods. Mature dry seed is light green in color. Additionally, *pc* snap bean cultivars possess white rather than green cotyledon color during germination and emergence from the soil. Thus, we could classify the pleiotropic effects of *pc* gene as having two opposite effects: leaves, stems, pods and seeds persist in green color; but during germination and emergence phase the cotyledons become bleached (Bassett, 2007).

Persistent color was originally discovered in 19th century French cultivars (such as Flageolet a Grain Vert) (Hedrick et al., 1931). This class is described generally as haricot vert and was also described as *persistent green* before arriving at the name of *persistent color* (Baggett et al., 1999). Haricot vert types are traditionally used as shell-outs and dry beans but Bill Dean (1968)

as a snap bean breeder recognized that the trait might be desirable from commercial standpoint to endow snap beans with dark, uniform green pods.

1.2.d. Stay-Green Trait

The *pc* gene is a member of the stay-green gene family. Stay-green (SGR) is defined as a trait that changes the process of chlorophyll degradation such that compared to a normal genotype, the leaves of stay-green plants show an extended period of green color caused by delayed chlorophyll catabolism (Thomas and Smart, 1993). Chlorophyll is an essential pigment to the photosynthetic process, where it plays a role in gathering and transferring light energy in an orderly manner to various organs in plants (Grossman et al., 1995). Chlorophyll content is positively correlated with photosynthetic activity and subsequent crop yield but also important is the duration of photosynthetic activity, and early chlorophyll degradation during the maturation period may limit yield potential due to leaf senescence (Peng et al., 2008). Thus, improving the photosynthetic productivity in the plants can be achieved through higher chlorophyll content and expression of forms of stay-green where photosynthesis remains active while delaying senescence (Zhao et al., 2019).

Stay-green mutations in the plants have been classified as either functional or cosmetic according to their physiological expression. With functional stay-green, the plant leaves have longer photosynthetic duration than their parental genotypes (Thomas and Smart, 1993) and may have higher specific leaf nitrogen (Borrell et al., 2001; Richards, 2006) . This is related to capture of organic carbon (C), nitrogen (N), and other nutrients which are fundamental to sustaining photosynthesis and plant development (Thomas and Ougham, 2014). Induction of senescence happens with the transition from C capture to N remobilization (Thomas and Ougham, 2014). If

the plant has functional stay-green, C-N transition is delayed or occurs on time but yellowing and N remobilization follow more slowly (Thomas and Howarth, 2000; Yoo et al., 2007). Functional stay-green has been used in field crop cultivars because of the potential to enhance yield and its tolerance to abiotic stresses (Myers et al., 2018). A positive correlation between stay-green and stress tolerance has been found in several studies (Ougham et al., 2008; Vijayalakshmi et al., 2010; Jordan et al., 2012; Emebiri, 2013). Moreover, functional stay-green mutations may play a role in disease resistance (Jordan et al., 2003).

With cosmetic stay-green, the plants maintain their green color during senescence but the plant loses its photosynthetic competence (Thomas and Howarth, 2000). Cosmetic stay-green was first observed in forage/turfgrass species (Thomas and Stoddart, 1975; Thomas, 1987), and rice (Jiang et al., 2007), then in tomato, pepper (Barry et al., 2008), and in common bean (Bachmann, et al., 1994; Davis et al., 2009). Plants with a cosmetic stay-green mutation have been of interest for study of the photosynthetic process, particularly to examine the chloroplast catabolism pathway (Thomas and Howarth, 2000). Furthermore, cosmetic stay-green mutations are associated with desirable quality attributes in a number of specialty crops. Thus, cosmetic stay-green mutations may have considerable economic importance (Myers et al., 2018).

Functional and cosmetic stay-green are further separated into five syndromes based on the characteristic response of chlorophyll function during leaf senescence (Thomas and Howarth, 2000). Types A and B stay-green are functional due to seed filling during sustained photosynthetic activity. Type C, D, and E are regarded as cosmetic due to having a similar photosynthetic state in the phase of senescence compared with non-stay-green plants (Thomas and Howarth, 2000).

Among stay-green types, Type C has been more extensively studied because of what it reveals about the chloroplast catabolism pathway during senescence (Myers et al., 2018). It was

first described as "senescence induced degradation" protein (Thomas and Stoddart, 1975; Thomas, 1987) with subsequent annotation for the protein being *SGR* (stay-green) and NYE (non-yellowing) (Jiang et al., 2007; Park et al., 2007; Balazadeh, 2014). NYE which exhibits persistent green leaves, was originally described in Arabidopsis, and was named NYE before its functional similarity to SGR had been determined (Barry et al., 2008; Sakuraba et al., 2014). *SGR* is a homolog (to SGR1, SGR2 and SGR-Like) of the stay-green gene, which is expressed at the beginning of the chlorophyll catabolism pathway (Armstead et al., 2007; Ougham et al., 2008; Hörtensteiner, 2009; Sakuraba et al., 2012).

The physiological differences between *pc* and normal beans extends to the foliage (Bouwkamp and Honma, 1970; Ronning et al., 1991) and led to observations of similarities between Type C stay-green (Thomas and Smart, 1993) and *pc* (Bachmann et al., 1994). The *pc* trait in common bean behaves as Type C stay-green in the sense that leaf chlorophyll-derivatives persist even though the plant loses its capacity to photosynthesize. Davis et al., (2009) used a SGR sequence from pea (PsSGR) to identify the homologous sequence in common bean and soybean. Sequence length varied depending on the phenotype, with wild type showing a full-length sequence and *pc* being associated with a truncated exon. Subsequently, they verified that *pc* is a member of the stay-green gene family (Davis et al., 2009).

In consideration of all of the points above, the commercially desirable *persistent color* bean types are identifiable as *SGR* mutants that pleiotropically affect several plant traits. An unexpected effect on *pc* snap beans is a reduction in germination and emergence rates in the field, with the effect more noticeable than what is observed for white- vs. colored-seeded genotypes (Davis et al., 2009; Al-Jadi, 2016). Given the characteristics of *pc* reported here, an examination of germination process in common bean with focus on the *pc* trait is warranted.

1.2.e. Bean Germination and Emergence Response

The growing cycle of beans takes generally between 100 and 130 days from germination until the dry seed maturity stage of the crop (Myers and Baggett, 1999). During initial growth phases, having uniform rapid germination and emergence is an essential biological characteristic of bean crops (De Ron et al., 2016). The main critical factors affecting these rates can be classified as genotypic and environmental as well as genotype by environment interactions (Hernández-Nistal et al., 1989; Alonso-Blanco et al., 2003; Schmuths et al., 2006). The soil temperature and water content are considered as the principal environment variables affecting the germination process and early seedling development (Raveneau et al., 2011). For example, the germination process commonly completes itself in a week with suitable conditions (the optimum temperature is 23°-30° C). However, if the soil temperature is less than optimum, the germination process may take as long as a month with reduced emergence rates and weak plants (Myers and Baggett, 1999). The longer the seed is held in the ground, the greater will be the danger of infection by soil borne pathogens increased seedling mortality.

Other factors which may have an effect on germination and emergence rate are seed age, seed shape, mechanical injury, storage conditions, pathogens present, and seed quality (Ferguson et al., 1991). The quality of seeds may be linked to mechanical damage during harvest and handling, and temperature, humidity and length in storage (Bass, 1980), as well as to the effects of seedborne diseases. Snap bean shows even great sensitivity to injury than do dry beans during harvest, seed conditioning, planting as well as during germination. Refraining from dropping seeds over 50 cm and stabilizing them at approximately 12% moisture content is important for reducing mechanical injury (Dickson and Boettger, 1976; Taylor and Dickson, 1987). Doijode's (1990) research showed that high temperature reduces seed longevity in French bean, but seeds stored at

temperatures of 5º or 18º C retained viability for five years for different French bean varieties. Seed size can directly affect germination and seedling growth, which can affect crop performance (Leishman, 2001; Rego et al., 2007). Size can influence not only the germination level (Van Mölken et al., 2005) and speed (Murali, 1997), but also seedling vigor (He et al., 2007). Larger seed size may lead to higher germination rates due to greater food storage to support germination (Missanjo et al., 2013) yet, smaller seeds have been observed to be competitive in early successional stages because they germinate faster (Baskin and Baskin, 1998).

1.2.f. Seed Development

Seed development and physiological activity is significantly associated in bean and the development phases can be organized into a series of events resulting in dry matter accumulation (Coelho and Benedito, 2008). Embryogenesis starts with the formation of a single-cell zygote, then continues through cell divisions until all embryonic structures are complete at the heart stage (Mayer et al., 1991). In the maturation phase, embryo growth is by cell division (Goldberg et al., 1994). Sugar and nitrogen compounds serve as signaling molecules that are generated by the seed coat (Borisjuk et al., 2003), and regulate seed development (Wobus and Weber, 1999) and metabolic exchanges, as well as also mediate communication between seed and parent plant (Borisjuk et al., 2003).

From the early stages of embryo development, the nutritional competition among the embryo, endosperm and suspensor continues (Coelho and Benedito, 2008). Then during the seed maturation, the growth is stopped by the embryo and mitosis decreases (Gutierrez et al., 2007). In addition to these reactions, the storage compounds begin to accumulate, and seeds develop tolerance to desiccation (Gutierrez et al., 2007).

Feeding the embryo during the development period as well as assuring seed germination and plantlet emergence are accomplished by accumulating reserve compounds in the seeds. The main compounds are carbohydrates, proteins, lipids and phytic acid (Coelho and Benedito, 2008). In legume seeds the principal reserve compounds are produced by carbon assimilation by leaf mesophyll cells and converted into sucrose. Sucrose moves though the phloem and into the apoplastic region between mother-plant and seed cells. The seed coat incorporates sucrose (Weber et al., 1997; Patrick and Offler, 2001) which is transported into the seeds during the maturation phase. All reserve compounds in mature seed are transported to the developing seeds as simple molecules such as sucrose, amino acids or mineral ions (Golombek et al., 2001).

1.3. HYPOTHESIS

We know that fungicide treatment of *pc* type seeds increases germination and emergence rates and brings these to a level comparable to fungicide-treated white seed (Al-Jadi, 2016). Thus, it appears that soil borne pathogens may be more attracted to or infect and colonize *pc* genotypes before the bean seedling is established.

o One hypothesis is that the seed coats of *pc* types may be more fragile and may crack more easily than other seed types, either from mechanical damage and/or imbibitional injury.

o Alternatively, *pc* genotypes may have higher seed sugar content which leaks into the surrounding spermosphere in greater abundance than from white- or colored-seeded genotypes.

In either case, leaking solutes into the spermosphere attract microbes to seeds before the seedling can become established.

1.4. OBJECTIVE

Our focus is on understanding why germination and emergence rate of *pc* types is lower than the ones for white- or colored-seeded cultivars. Addressing this problem of germination rate would likely increase the percentage of *pc* gene beans in the production and could give more popularity to *pc* genotypes.

CHAPTER TWO: SEED GERMINATION STUDIES AND FURTHER CHARACTERIZATION OF BEAN CULTIVARS WITH THE *PERSISTENT COLOR* TRAIT

2.1 INTRODUCTION

The first part of this chapter describes production of seed samples grown under uniform conditions from the greenhouse. Then, several experiments using these seeds will be detailed. The first experiment describes analysis of seed germination in the laboratory and field. The second experiment evaluated plant development to document any variation within and among *pc* and white-seeded types during emergence, including variation in cotyledon color during germination. The third experiment reports results from a molecular marker study of *pc* types that vary in cotyledon color to verify that plants with green cotyledons retain the *pc* allele. The last experiment evaluated leaf color of *pc* lines and their paired white-seeded genotypes to determine whether there are color differences among them.

2.2 MATERIAL AND METHODS

2.2.a. Greenhouse Seed Increase and Phenotypic Evaluation

The process of germination and emergence in snap bean crop was assessed in terms of the of seed color. Several factors can influence how seeds germinate and transition into seedlings. Thus, before designing and performing our research studies, we required uniform seed samples produced in the same environment. For our experiments, three type of seeds were used for comparisons of genotypic differences: white-seeded, colored-seeded and *persistent color* (*pc*)

types. Forty cultivars that included these three seed types were planted in the greenhouse on December 13th, 2017 for increase under identical growing conditions.

Twelve seeds for each cultivar were divided among three 2.4L pots containing Sungro Professional Growing Mix. Seeds were placed 1.5-2.0 cm deep in the soil and were fertilized with ~3g per pot Osmocote Classic Controlled Release Fertilizer (14-14-14). All plants were grown in the same greenhouse room. The relative humidity was around 73% and the room temperature was maintained at 18º C (64º F) - 25º C (77º F) daytime and 13.8ºC (57ºF) - 17.2º C (63º F) during nighttime. Plants were watered as needed and the supplemental lighting (400W Hg and Na vapor pressure lamps) provided light from 6:00 am to 11:00 pm. Throughout the growing period, pests (mainly thrips) were controlled using the biocontrols *Amblyseius cucumeris* predatory mites, *Orius insidiosis* minute pirate bugs, and Hypoaspis mites (*Stratiolaelaps scimitus*) as well as Azaguard, Mainspring, and Aria + Overture insecticides as plants matured.

Ten days after planting, cotyledon colors were recorded as white or green in color and individual plants were labelled. Plants were grown to maturity and were harvested at about 100 days after planting, *pc* plants that had shown green cotyledons as seedlings were harvest separately as single plants while the remaining plants were bulked. Hand harvest and cleaning was used to avoid mechanical damage to the seed. At harvest, dry pod and seed color were recorded for each cultivar.

2.2.b. Increasing Seeds in Field

After our greenhouse harvest in 2018, thirty seeds of each genotype were retained as backup and for laboratory experiments. Lines that had atypical *pc* plants in the greenhouse grow-out were planted separately in the field. Seeds were treated with Captan (Bonide) fungicide then

30 seeds were planted into plots 3 m (10 ft) long with 77 cm (30 in) between rows on June 15th, 2018. Plots were not replicated or randomized since they were intended for seed increase.

The field plots were located at the Oregon State University Vegetable Research Farm which is located near Corvallis, Oregon on Chehalis silty clay loam soil (pH 5.5 - 5.8) on the upper bench of the Willamette River. The farm is at 77 masl and is located at latitude N44.571209, and longitude W123.243261. The field at this farm have been used for over 60 years to grow snap beans in rotation with other vegetable crops and hazelnuts. Standard snap bean management practices were used to control weeds and pests (mainly cucumber beetles). The plots were irrigated before planting to provide uniform soil moisture and water was supplied during the growing season via overhead sprinklers supplying 2.5 cm of water at weekly intervals.

A week after planting and at emergence, cotyledon colors for each plant were documented. Those that were atypical of the line were marked with flags. During the second week, the number normal and abnormal plants (based on plant size and leaf condition) was recorded. During plant development, we also examined flower color and as plants senescenced, dry pod and leaf color were recorded as green or yellow and plants were marked if they had an uncharacteristic color.

Some of the *pc* cultivars and breeding lines for greenhouse increase exhibited segregation for seed color (white vs. pale green). Because these were in advanced generations (>F_5) these were recognized as particularly valuable for experimentation because of their isogenic nature for the *pc* locus, and therefore a number of subsequent experiments focused on these genotypes. These included OSU6504, OSU6510-4, OSU6523, Tempest, and Flamata, all originally selected as *pc* types but found to be polymorphic. OSU6523, OSU6510-4 were used extensively in subsequent

experiments because they were consistent in their genotypes. The cultivars used in this research

are documented in **Table 1**.

Table 1: Snap bean breeding lines and cultivars that were increased at the OSU vegetable research farm in 2017 and subsequently used to characterize *persistent color* types.

Accession	Seed Phenotype	Seed Genotype[z]	Accession	Seed Phenotype	Seed Genotype[z]
Booster	White	*p Pc*	OSU6510-4-*p PC*	Green	*p pc*
Bush Romano	Green	*p pc*	OSU6510-4-*p Pc*	White	*p Pc*
Castano	Green	*p pc*	OSU6512	Green	*p pc*
Charon	White	*p Pc*	OSU6516	Green	*p pc*
Clyde	White	*p Pc*	OSU6523-*p pc*	Green	*p pc*
Cruiser	White	*p Pc*	OSU6523-*p Pc*	White	*p Pc*
Embassy	Green	*p pc*	OSU6562-2	Green	*p pc*
Flagrano	Green	*p pc*	OSU6569	White	*p Pc*
Flamata	Green	*p pc*	OSU6929	Green	*p pc*
Flaveol	Green	*p pc*	OSU7025	White	*p Pc*
Freshpick	Green	*p pc*	Ovation	White	*p Pc*
Hercules	Green	*p pc*	Pascal	Green	*p pc*
Hystyle	Green	*p pc*	Profit	White	*p Pc*
Jade	Green	*p pc*	Quest	Green	*p pc*
Medinah	Green	*p pc*	Redon	White	*p Pc*
OR91G	White	*p Pc*	Saratoga	Green	*p pc*
OR91G-1-1-c^u	Colored	*p Pc c^u*	Shade	Green	*p pc*
OR91G-2-1-*djv*	Colored	*p Pc d j v*	Spartacus	Green	*p pc*
OR91G-2-2-*djv*	Colored	*p Pc d j v*	Tempest-*p pc*	Green	*p pc*
OR91G-p^{gri} *Pc*	Colored	p^{gri} *Pc*	Tempest-*p Pc*	White	*p Pc*
OSU6504-*p Pc*	Green	*p pc*	Titan	Green	*p pc*
OSU6504-*p Pc*	White	*p Pc*	Ulysses	White	*p Pc*

[z]Genotype description: **PIGMENT:** with **P**, seeds, flowers and other plant parts pigmented depending on what other genes are expressed; **P^{GRI}** – attenuated pigment expressed; **P** – plant parts not pigmented. **PERSISTENT COLOR**: with **PC**, plant parts shown normal expression, **PC** – leaves and stems remain green at senescence, dry seeds pale green and cotyledons of emerging seedlings bleached white. **C:** color and pattern regulator in seeds; **C^U** – **UNCHANGEABLE** cartridge buff seed color. **D:** hilum ring factor, with **D** and **J**, hilum ring present; **D** – with **J**, hilum ring absent. Now named **Z**. **JOKER**: with **J**, dark seed colors, after darkening with age; **J** – pale colors not after darkening. **VIOLET**: with **V** violet to black seed color and purple flowers; **V** – brown seeds and white flowers. The interaction of **D J & V** produces a near-white seed color. From Bean Genes List (Genetics-Committee, 2020).

2.2.c. Laboratory Germination Tests I- II

Using the greenhouse increased seed, 39 cultivars (without 'Profit') representing the three seed genotypes were tested to determine whether there are differences in germination in the laboratory. The test was conducted with twenty-five seeds for each genotype and was repeated once (**First Rep:** May 11, 2018 – **Second Rep:** June 08, 2018). Paper towels soaked in deionized water were laid on a clean surface and seeds were arranged on the paper towel. The towels were rolled, labeled and placed in closed plastic boxes to maintain humidity, and were incubated at ambient lab temperature (~20°C) for one week. The boxes were checked daily and if needed, water was added.

At around one week, seeds were rated on the same day using a six-point scale as listed in **Table 2**. The percent good seedlings, uninfected seeds, and total germinated seeds were calculated. Data was analyzed using PROC GLM (SAS v9.4) and means were compared using Fisher's Protected Least Significant Difference if there were no missing values. Where missing values were present and/or an unbalanced design was employed, least square means were calculated, and all pair-wise comparisons were made testing the null hypothesis that means were equal. In addition to individual genotype comparisons, least square means by seed groups (*pc* vs. white-seeded vs. colored-seeded) were compared.

Table 2: Rate Scale for snap bean germination test.

Point Scale	
1	The seed has germinated
2	The seed was infected
3	The seed germinated but has a low vigor
4	The seed was infected and has a low vigor
5	The seed did not germinate and dead
6	Hard seed

2.2.d. Evaluation of Pathogens Reducing Germination in Field

Untreated seed of the *pc* genotype Castano were planted in the field on July 11th, 2018 to examine possible pathogen interactions with germinating seeds. Evaluations began five days after germination had commenced but before emergence. The 200 seeds were planted into the soil in 12 m (40 foot) un-replicated plots at a depth of 2.5 cm (one inch). Approximately 3 m of row was dug at a time and the soil was sieved through 0.6 cm wire mesh and all parts of seed samples was collected to a plastic bag. The process was repeated three times at 2 to – 3 day intervals for a total of four collections. Samples were washed gently and placed on paper towels to dry. Samples were then moved to a clean paper towel and graded for infection by pathogens with the percentage of pathogenic lesions calculated. Seedlings with necrotic lesions were sent to the OSU Plant Disease Clinic for pathogen identification.

This experiment was repeated with a set of cultivars to compare *pc*, colored and white-seeded genotypes. The cultivars were Castano (*pc*), Roc d'Or (colored), and OR-5630 (white). Thirty seeds of each genotype were planted on August 13th, 2018 and starting three days after, 1.5 m (5 foot) of row was dug and evaluated. The process was repeated with 1.5 m of row dug three times at three-day intervals. Seeds and seedlings were evaluated as described above.

2.2.e Field Germination and Emergence Tests

Saved seeds from the greenhouse harvest were used in this experiment. The experiment consisted 3 m (10 ft) plots for each replicate. Forty-four cultivars were replicated two to four times, depending on seed availability. Seeds of each genotype were divided into two lots and one was treated with Captan fungicide. Thirty seeds per replicate were planted in a randomized complete block design at a depth of about three cm (1.5 in) using a hand-pushed belt planter. Seeds were

planted on June 15th, 2018 when soil temperatures were relatively warm at around 21° C at 2 inches (~5cm) soil depth.

Shortly after planting and at emergence, data were collected on the number of normal and abnormally-sized seedlings, and once cotyledon colors became visible above the soil, green or white color was recorded for each plant. Plants that had atypical colors were marked with surveyors' tape and were flagged. Similar procedures as described in 2.2b were used to document flower and dry pod and leaf colors. At harvest a single plant with greater pod numbers and without diseases symptoms was saved from each population, along with flagged plants and the remainder of the seed was bulked.

2.2.f.   Single Plants and Molecular Marker Study

The field emergence test revealed variation in cotyledon color which we wanted to evaluate with molecular markers as to whether *pc* was still present in atypical plants that produced green rather than white cotyledons at emergence. Single plants saved from populations showing variation in cotyledon color (see section 2.2.e for further information) were planted in the greenhouse, the cotyledon color noted and two weeks after, small actively growing leaves were collected for DNA isolation and molecular marker analysis. The lines which were examined are provided in **Table 3**.

The DNA extraction procedure used a hexa- decyltrimethyl ammonium bromide (CTAB) protocol customized from Miklas, Stavely, and Kelly (1993). Markers for *pc* were amplified by PCR following the procedure of Davis et al. (2009). Amplified samples were run on 1% agarose gels, then visualized by ethidium bromide staining. The fragment sizes were compared to a 100bp molecular ladder (Promega, Cat.# G8291).

Table 3: Single plants of green beans harvested from the field at the OSU Vegetable Research farm that varied from the expected cotyledon color and which were evaluated for the presence of the *pc* molecular marker.

Single Line ID	Plot number from F.E.T.[1]	Cotyledon Color	Seed No	Plant ID	Cotyledon Color in Greenhouse
Spartacus	127	Green	1	Spartacus	Green
			2	Spartacus	Green
			3	Spartacus	Green
			4	Spartacus	Green
			5	Spartacus	White
Spartacus	223	Green	6	Spartacus	Green
			7	Spartacus	Green
Spartacus	323	Green	8	Spartacus	Green
			9	Spartacus	Green
Spartacus	329	Green	10	Spartacus	White
Medinah	401	Green	11	Medinah	Green
			12	Medinah	White
			13	Medinah	White

[1]**F.E.T.:** Field Emergence Test.

2.2.g. Color Evaluation of *Persistent color,* White- and Colored-seeded Genotypes Leaves

The purpose of this experiment was to determine whether *pc* and normal green beans could be identified by their foliage color while actively growing during the field season. Several of the breeding lines and a commercial cultivar were found to be polymorphic for *pc* in field and greenhouse grow-outs as described above. The lines used in this study were OSU6504 white-seeded and OSU6504 *p pc*; OSU6510-4 *p Pc* (white-seeded) and OSU6510-4 *p pc*; OSU6523 *p Pc* (white-seeded) and OSU6523 *p pc*; and Tempest white-seeded and Tempest *pc* seeds. In the case of the three breeding line pairs, based on phenotypic similarities, these appeared to be the result of continued segregation in the population for *pc*, so that lines were near-isogenic. The Tempest pair seems to have arisen the same way and the type types were phenotypically identical, but we could not rule out the possibility of a chance outcross or spontaneous mutation.

For each of the eight lines the 10th, 15th and 20th plants were selected for evaluation. Readings with a BC-10 colorimeter (Minolta) were taken from three leaves located in the bottom, middle and top of the canopy. Each leaf was read three times to obtain its CIE (Commission Internationale de l'Eclairage), L* a* b* coordinates. L* is a measure of lightness (scale of 0 – 100 where 0 is black and 100 is white), a* the red - green color coordinate, and b* the yellow - blue axis (Joint and ISO, 2008).

2.3. RESULTS

2.3.a Greenhouse and Field Seed Increase

During the greenhouse increase, several lines identified as *persistent color* showed altered expression with regard to seed and cotyledon colors. The lines showing segregation included OSU6504, OSU6523, OSU6510-4 and Tempest, and those with altered expression were maintained separately from the original population to create pairs of near-isogenic lines.

Plants of OSU6523 and Tempest were originally classified as *pc,* but those with altered expression produced green cotyledons as seedlings and had yellow rather than green leaves on senescence, and daughter seeds were white. For OSU6504 and OSU6510-4, no phenotypic differences were observed prior to harvest, but when pods were threshed some plants revealed white seeds (**Table 4)**.

Most other genotypes did not exhibit segregation and produced the expected phenotype (green cotyledons for white- and colored-seeded genotypes, white cotyledons for *pc* genotypes; *pc* foliage remained green during senescence while white-, and colored-seeded forms turned to yellow). Flagrano, Flamata, and Flaveol demonstrated weak expression of *pc* where both green and white dry seed were observed. Moreover, the green color of dry seeds was very faint and sometimes difficult to classify. However, they did have green rather than yellow pods at senescence.

The field grow out included the additional breeding lines and cultivar selections -- OSU6504- *p Pc*, OSU6523-*p Pc*, OSU6510-4- *p Pc*, and Tempest- *p Pc* (white-seeded types) -- to create isogenic pairs with *pc* versions of these genotypes that could be used in future experiments (**Table 4**).

Table 4: The snap bean lines from a greenhouse harvest that showed variation for the *pc* trait. From these, pairs of near isogenic lines were constructed. These are compared here for cotyledon color, dry pod color and seed color after harvest.

CV Pairs	Seed Type	Cotyledon	Dry Pod	Seed Color
OSU6504	*Persistent color*	White	Green	Green
OSU6504	White-seeded	Green	Yellow	White
OSU6510-4	*Persistent color*	White	Green	Green
OSU6510-4	White-seeded	Green	Yellow	White
OSU6523	*Persistent color*	White	Green	Green
OSU6523	White-seeded	Green	Yellow	White
Tempest	*Persistent color*	White	Green	Green
Tempest	White-seeded	Green	Yellow	Pale Green/White

2.3.b. Laboratory Germination Tests I- II

The first and second germination tests were rated according to the scale described in **Table 2.** No significant difference in germination percentage was detected among the seed coat colors for both replicates. The first replicate had a relatively high rate of germination for all genotypes, but the second replicate exhibited higher levels of microbial contamination and lower overall germination rates as documented by a mean of 83.8% for the first replicate and 64.9% for the second replicate. Comparison of least-square means for germination of the three different snap bean seed types under laboratory conditions were not statistically significant (**Table 5**).

Table 5: Least square (LS) means for different snap bean seed types for germination in the laboratory. Probabilities for each pair-wise comparison of LS means pairs is shown for the null hypothesis that means were equal.

Seed Type	Germination (%)	Prob (*pc*)	Prob (wh)
			Probability
Colored-seeded	73.5	0.86	0.99
Persistent color	74.9		0.79
White-seeded	73.4		

2.3.c.   Evaluation of Pathogens Reducing Germination in Field

In the first field emergence experiment, a total of 64 seeds were recovered. They were divided into three categories of good seed; infected and germinated seed; infected, un-germinated or dead seed, and percentages as a proportion of total seed number were calculated.

Castano (a *pc* genotype) had 42.2% normal seedlings while 57.8% of seeds and seedlings showed signs of infection. Analysis by the OSU Plant Disease Diagnostic Clinic found *Fusarium* and/or *Rhizoctonia* spp. to be present on hypocotyl and cotyledon sections as pictured in **Figure 1**. Of the infected seeds and seedlings, 34.4% had lesions and 23.4% were remains of un-germinated seeds that appeared have been killed by the pathogens.

Another noticeable aspect was differences in vigor of pathogen-free seedlings compared to those with *Fusarium* and/or *Rhizoctonia* spp. infection. Pathogen infection reduced seedling length by around 2 cm as shown in **Table 6** which was statistically

Figure 1: Snap bean seedling infected on the cotyledons with *Fusarium* spp. and/or *Rhizoctonia* spp**.**

significant at the 95% probability level (P = 0.0417). The relationship between germination rate and infected seedlings was particularly noticeable in the second week.

Table 6: Comparison of average seedling length for Castano snap beans grown in the field at the OSU Vegetable Research farm in 2018. Values represent the mean of four samples and a t-test of the means revealed that they were significantly different (P = 0.0417).

Parameter	Uninfected seedling length	Germinated & infected seedling length
	cm	
Mean	7.02	4.84
St. Dev.	2.97	1.72

A second field experiment comparing Castano with OSU5630 (white-seeded) and 'Roc d'Or' (colored-seeded) produced similar results for Castano but very few signs of pathogen infection were observed for OSU5630 or Roc d'Or. With Castano, several seeds died prior to seedling development and those that did develop disease showed symptoms mostly on the cotyledons. The recovered seeds per line were 63.3%, 80.0%, 63.3% for Castano, OSU5630 and Roc d'Or, respectively. Germination percentages of normal seedlings were 57.9% for Castano, 95.8% for OSU5630 and 79.0% for Roc d'Or (**Figure 2**). A similar reduction in vigor for Castano was observed in this experiment as shown in **Figure 3**.
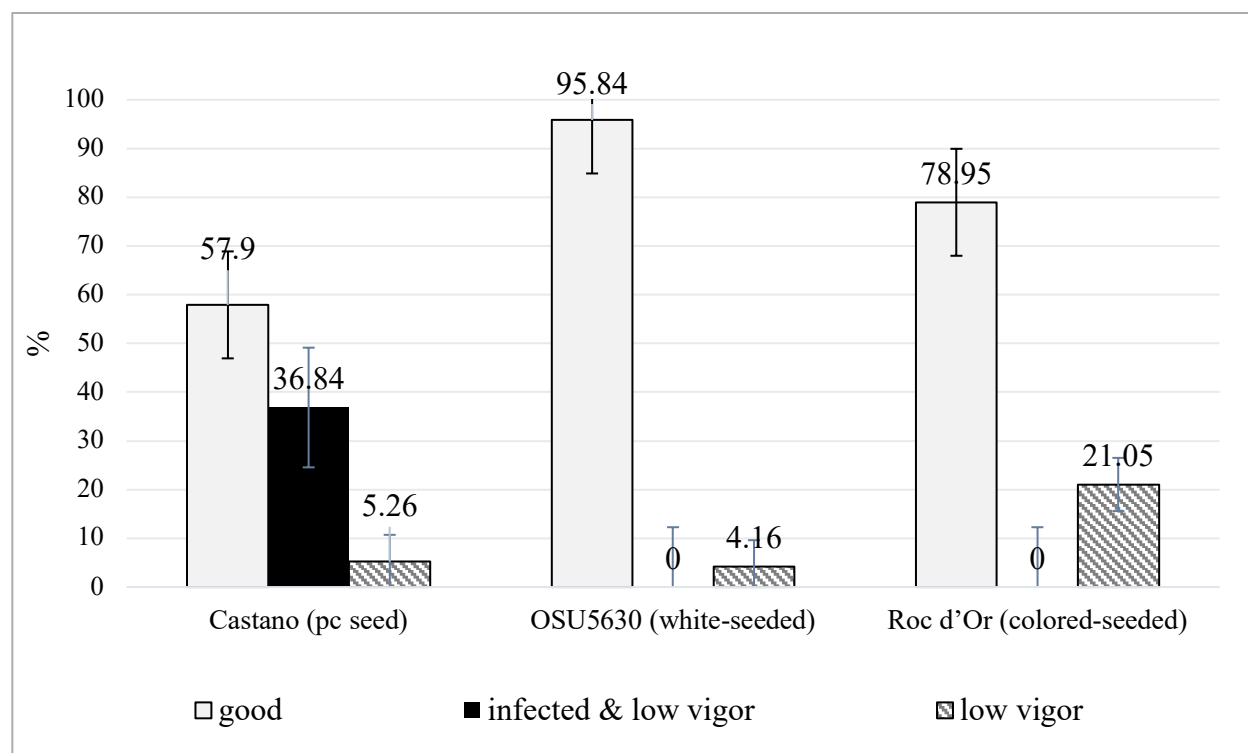
Figure 2: Comparison of germination without fungicide treatment for *pc*, white-seeded and colored-seeded snap bean genotypes grown at the OSU Vegetable Research farm in 2018.

Figure 3: Some examples of seeds from Castano, OSU5630 and Roc D'or snap beans after digging in the field at the OSU Vegetable Research Farm. Left: *pc* type starting to germinate while (middle and right) white and colored-seeded types have already germinated.

2.3.d. Field Emergence Tests

Fungicide treatment of seeds had significant and positive effects on the percentage of normal seedlings (treated: 71.4%, vs. untreated: 64.9%) and emergence (Treated: 80.8%, vs. untreated: 74.9%). A comparison by seed type for percent normal seedling and percent emergence revealed significant differences between white and *persistent color* seeds as well as *pc* and colored-seeded types. There was no significant difference among white-seeded and colored-seeded (**Table 7**).

Table 7: Comparison of percentage of normal seedlings and emergence among snap bean seed types from a field trial grown at the OSU Vegetable Research farm. Means followed by the same letter in a column are not significantly different at P > 0.05.

Seed type	Mean normal	Mean emergence
	%	
White	71.3a	80.2a
Colored	70.4a	80.4a
Persistent color	63.2b	73.8b

When examining the combined effect of seed type and seed treatment on percentage of normal seedlings and emergence, there was around a 10% differential, but the interaction was not significant (**Table 8).**

Table 8: Interaction of seed type and fungicide treatment and its effect on percentage of normal seedlings and emergence of snap bean lines grown in the field at the OSU Vegetable Research farm. Means followed by the same letter in a column are not significantly different at P > 0.05.

Seed Type	Fungicide treatment	% Normal Seedling	% Emergence
Colored-seeded	Treated	75.2a	84.4a
Colored-seeded	Untreated	65.6b	76.3b
Persistent color	Treated	66.8b	78.2b
Persistent color	Untreated	59.5c	69.3c
White-seeded	Treated	73.7a	81.6a
White-seeded	Untreated	68.9b	78.8b

We also compared the treated and untreated percentages by line for normal seedlings and emergence. The differences among treated and nontreated lines were most noticeable for several lines that treated seeds had a greater percentage of emergence; *pc* types: Castano (40%), Flaveol (63.4%), Flagrano (36.5%) Hystyle (23.7%). Additionally, the colored- and white-seeded types showed a differential (around 50%) where germination was higher in colored-seeded genotypes: OR91G1-1-*c^u*, OR91G-2-1-*djv*, and OR91G-2-2-*djv*.

2.3.e. Single Plants and Molecular Marker Study

The all putative *pc* bean lines that showed variation for cotyledon color from what was expected for *pc* types showed the expected molecular markers. The primers SGR1-F (forward) and SGR3-R (reverse) amplified a 1,000 bp fragment from the SGR genomic region, but only for OSU5630 was a 1600bp fragment amplified using the SGR1-F and SGR1-R primers as would be expected for genotypes with an intact SGR sequence (**Figure 4)**. In this image, the numbers 1 to 10 correspond to Spartacus with 5 and 10 having white cotyledons and the others with green

cotyledons. Numbers 11 - 13 are Medinah with 11 representing a green cotyledon plant, while 12 -13 have white cotyledons.
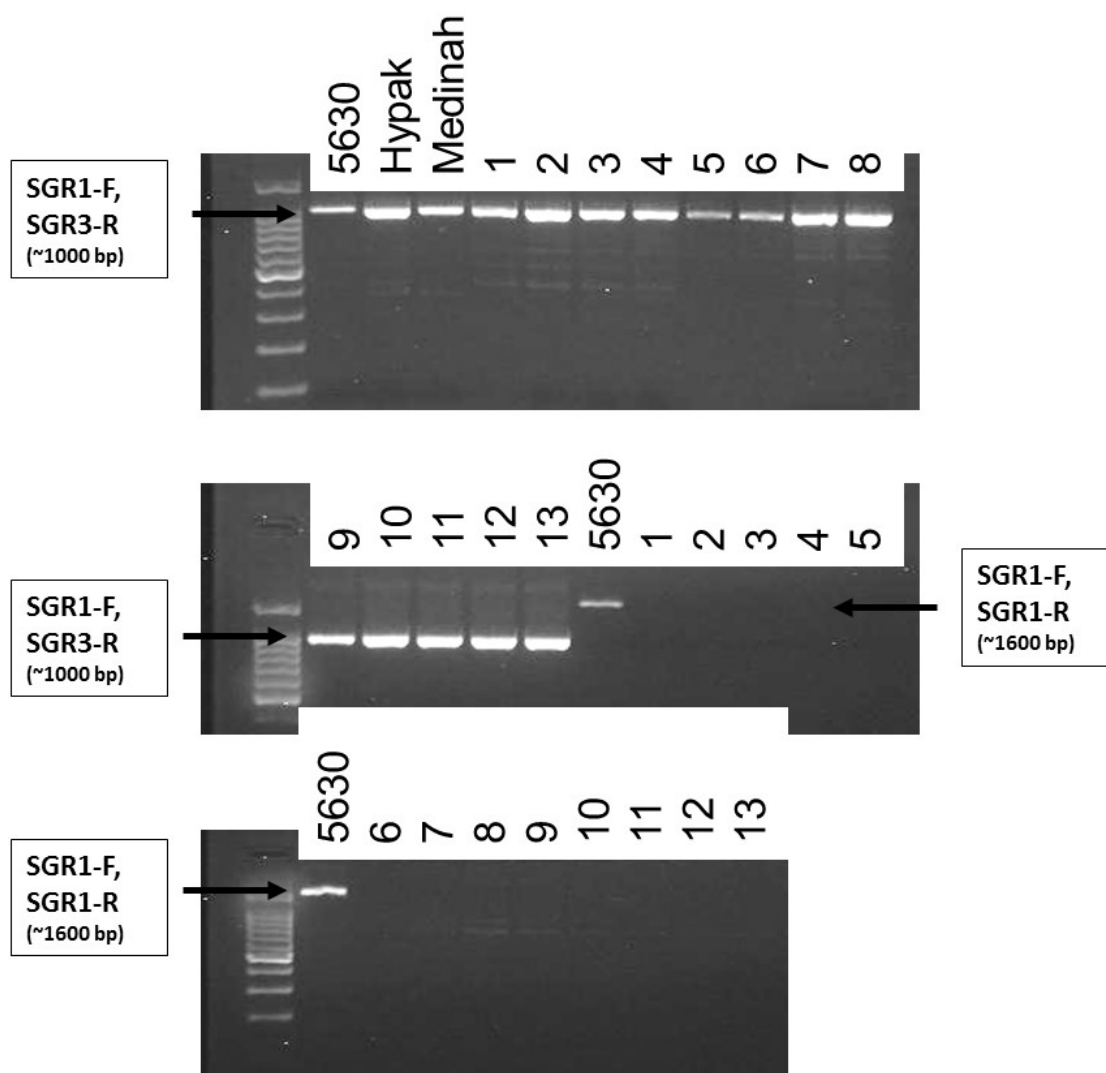
Figure 4: Molecular marker amplification of the SGR locus in *pc* and normal snap beans. Numbers 1 – 10 represent Spartacus with 5 and 10 having white cotyledons and the remainder with green cotyledons. Medinah is shown in 11 -13 with 11 having green cotyledons and 12 -13 with white cotyledons.

2.3.f. Leaf color for Persistent color, White- and Colored-seeded Genotypes

Leaf color was examined to determine whether differences between *pc* and normal genotypes existed. Colorimeter readings were taken on four isogenic seed type pairs at three canopy heights. Analysis of variance (**Table 9**) revealed statistically significant differences for mean squares for most L* a* and b* main effects and interactions.

Table 9: ANOVA of leaf color measurements for white and *pc* snap bean lines grown at the OSU Vegetable Research Farm.

Source	DF	L*[1]	a*	b*
			Mean square[2]	
Paired Genotypes	3	45.45***	66.24***	198.68***
Leaf Height	2	62.48***	34.65***	184.12***
Seed Type	1	0.07	5.80*	42.68*
Cultivar*Leaf Height	6	9.59*	3.25*	34.50***
Seed Type*Leaf Height	2	26.29**	0.29	5.72
Cultivar* Seed Type	3	12.36*	20.31***	66.99***
Cultivar*Seed Type*Leaf Height	6	7.99	7.41***	36.99***
Error	192	3.96	1.49	6.63

[1]CIE L*a*b* indicate color values: L* corresponds to lightness (0 = black and 100 = white), a* progress from green (-) to red (+) colors, b* corresponds to blue (-) to yellow (+) colors.
[2]*, **, *** Significant at $p < 0.05$, 0.01, 0.001, respectively.

The main effects cultivar and leaf height were highly significant for L* but were not significant for seed type. Significant differences were also observed for the interactions cultivar x leaf height, cultivar x seed type and seed type x leaf height.

A similar pattern was observed for a*, except seed type was also significant and the seed type x leaf height interaction was not significant. However, the three-way cultivar x seed type x leaf height interaction was highly significant. The results for b* was essentially identical to that of a*.

The mean L* and b* values were higher for Tempest compared to the other genotypes. In contrast, Tempest had a significantly lower a* value Thus, Tempest's leaves tended to be lighter in color and more yellow-green color than other genotypes. (**Figure 5**)

When seed type main effect was compared (**Figure 6**), L* was not significant but a* had a significantly more negative value and b* was significantly more positive. Thus, *pc* types had the same degree of lightness compared to normal types but were slightly greener and more yellow. For the interaction of cultivar x seed type for L* and b*, the *pc* genotypes were always lighter and yellower than their white seeded counterpart except for OSU 6523 which showed the reverse pattern **(Figure 7)**. For a*, *pc* types for OSU 6504 and OSU 6523 were less green than their white seeded counterpart, whereas the reverse trend was observed for OSU 6510-4 and Tempest.

For both *pc* and normal types, leaf color showed similar patterns (**Figure 8**). The significant seed type x leaf height interaction appeared to be caused for L* by a steeper transition from light top leaves to darker lower leaves for *pc* types compared to normal types. The differences between *pc* and normal types across leaf heights for a* and b* showed no significant differences.
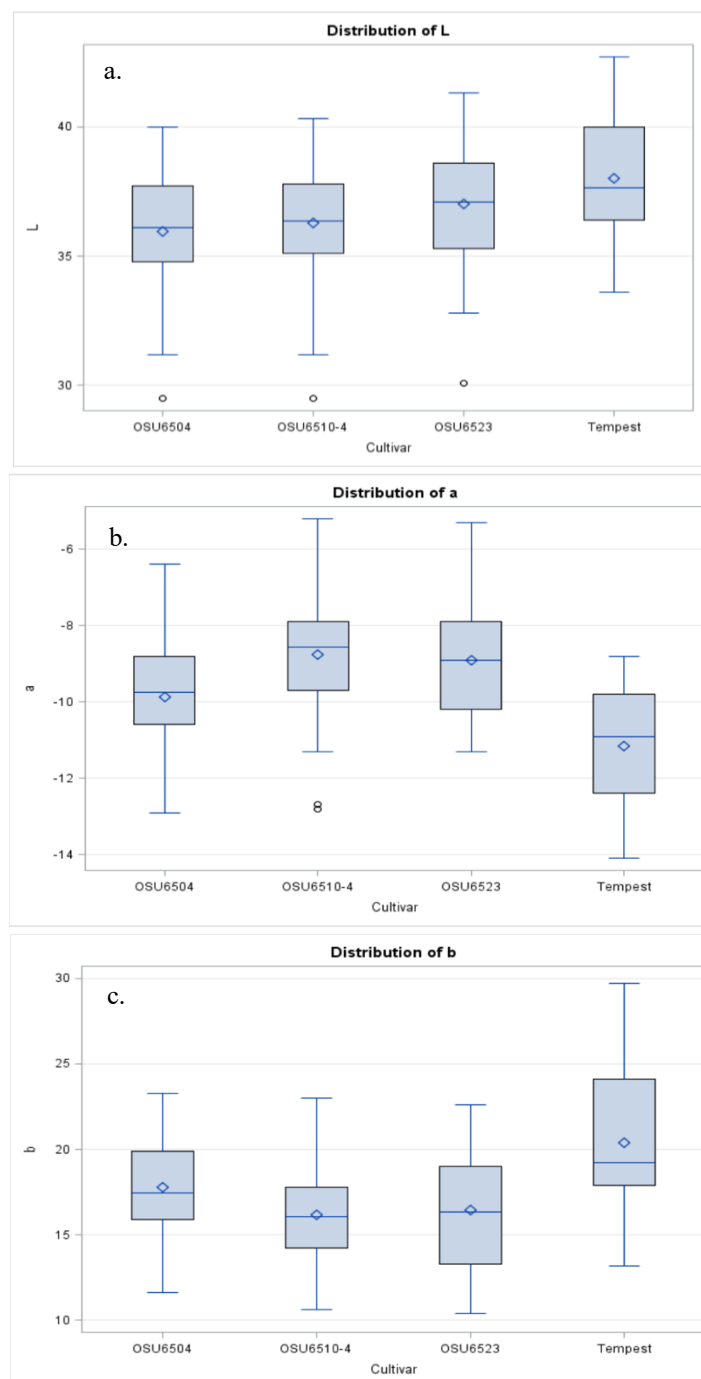
Figure 5: Box and whisker plots for CIE L*a*b* color comparison of four snap bean lines (values averaged across seed types and leaf heights). **a.** Distribution of L* which corresponds to lightness (0 = black and 100 = white). **b.** Distribution of a* progressing from green (-) to red (+) colors. **c.** Distribution of b* corresponding to blue (-) to yellow (+) colors.
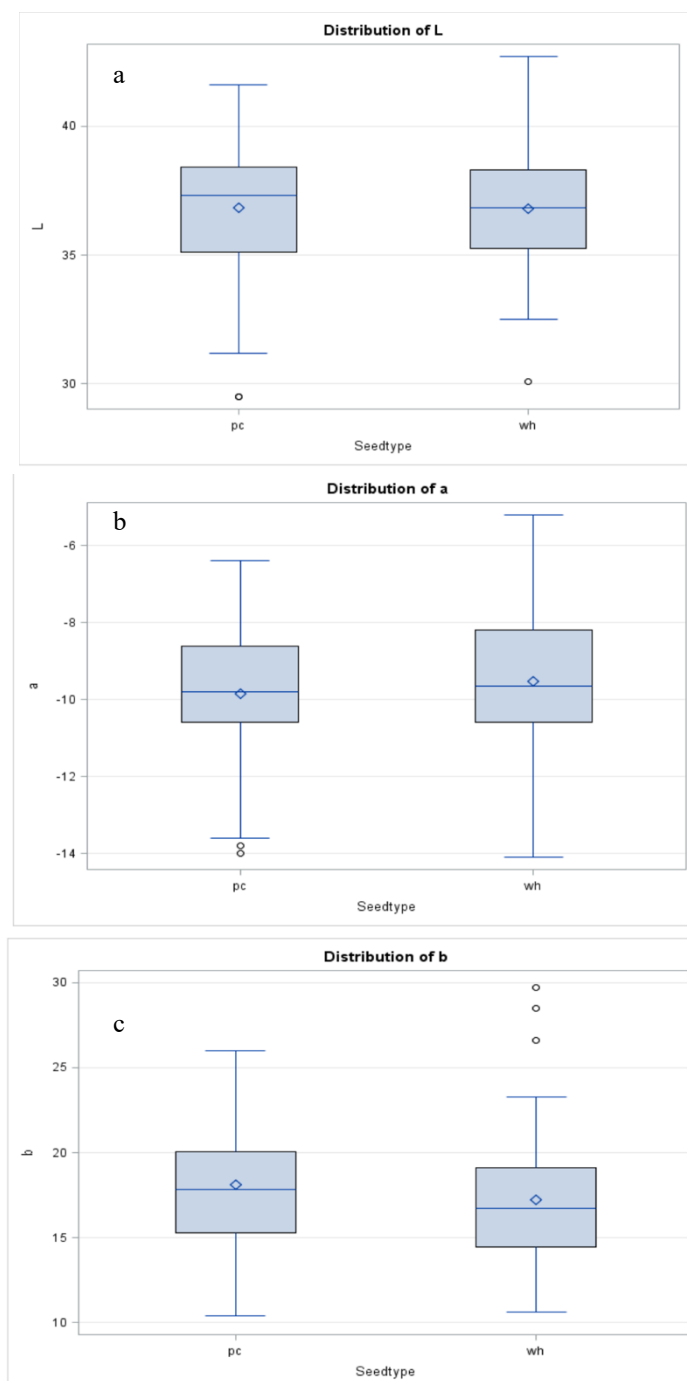
Figure 6: Box and whisker plots for CIE L*a*b* color comparison of *pc* vs. white seeded genotypes (values averaged across cultivars and leaf heights). **a.** Distribution of L* which corresponds to lightness (0 = black and 100 = white). **b.** Distribution of a* progressing from green (-) to red (+) colors. **c.** Distribution of b* corresponding to blue (-) to yellow (+) colors.
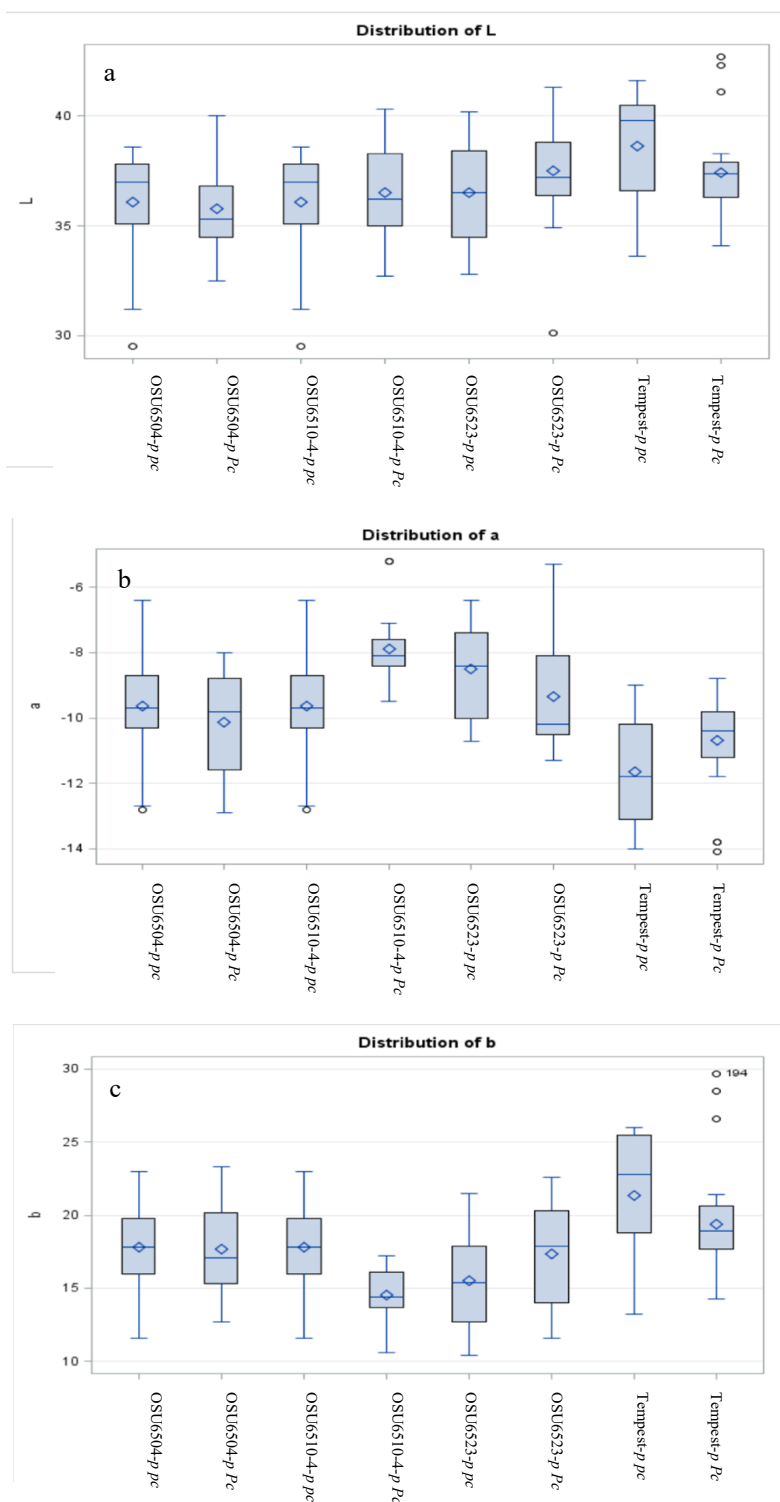
Figure 7: Box and whisker plots for CIE L*a*b* color comparison of the interaction of snap bean genotype by seed type (values averaged across leaf heights). **a.** Distribution of L* which corresponds to lightness (0 = black and 100 = white). **b.** Distribution of a* progressing from green (-) to red (+) colors. **c.** Distribution of b* corresponding to blue (-) to yellow (+) colors.
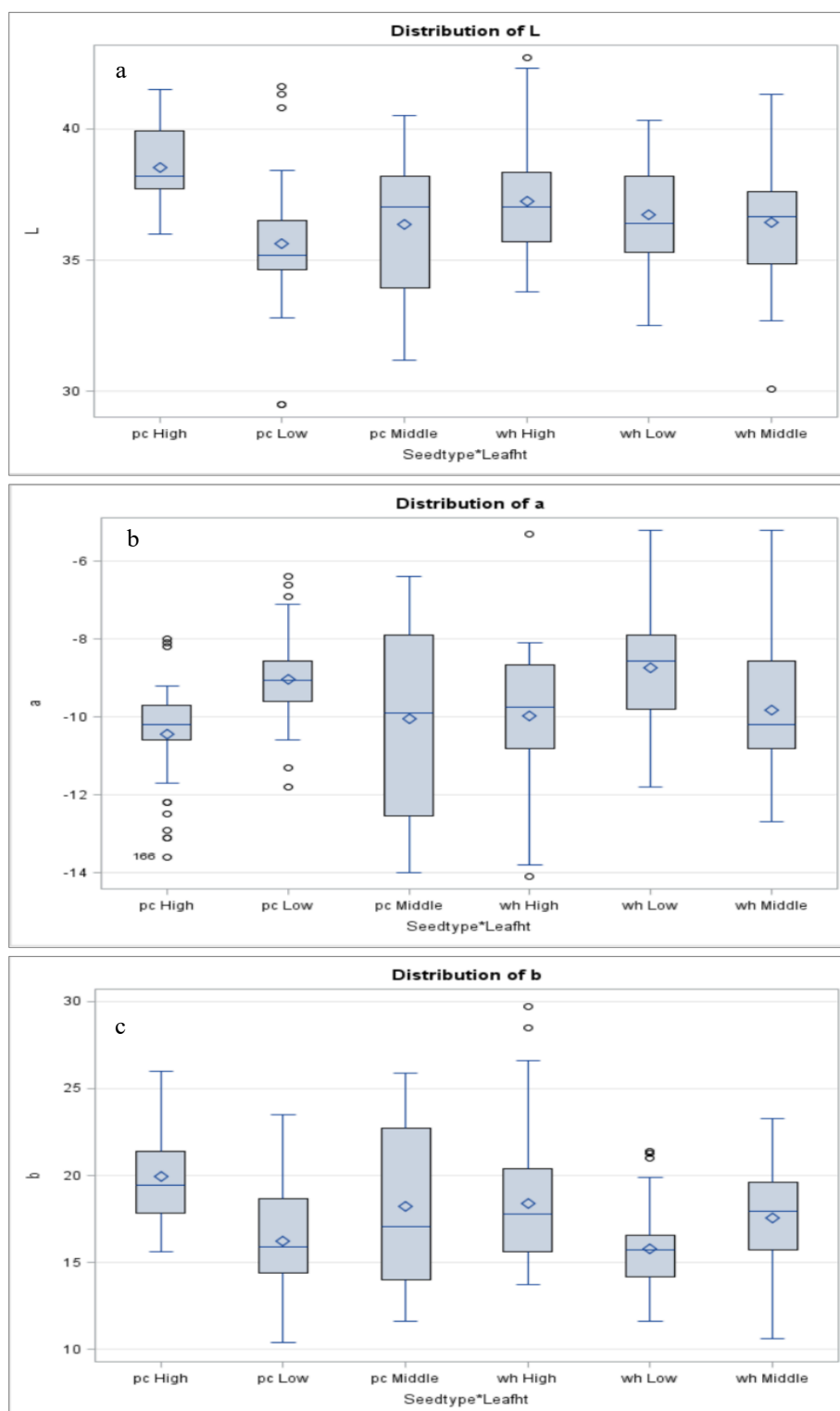
Figure 8: Box and whisker plots for CIE L*a*b* color comparison of seed type (averaged across cultivars) by canopy position. **a.** Distribution of L* which corresponds to lightness (0 = black and 100 = white). **b.** Distribution of a* progressing from green (-) to red (+) colors. **c.** Distribution of b* corresponding to blue (-) to yellow (+) colors.

2.4. DISCUSSION AND CONCLUSION

The higher level of infected seedlings of *pc* relative to other types in the field confirmed that *pc* seeds are more susceptible to pathogens. As we know that a seed consists of an embryo and stored nutrients (Mauseth and Mauseth, 1988), and when seeds begin imbibition into the soil, these nutrients are protected from pathogen infection by the seed coat. The seed coat also provides a barrier to diffusion of nutrients into the soil. When nutrients do diffuse into the soil, it can result in the proliferation of pathogenic fungi immediately around seeds (Mayne et al., 1969; Halloin, 1986; Mohamed-Yasseen, 1991; Mohamed-Yasseen and Splittstoesser, 1991). Scarification of seeds increased electrolyte leakage and increased fungal infection and reduced germination rates of onions (Splittstosser and Mohamed-Yasseen, 1991; Splittstoesser et al., 1994). Thus, if *pc* seeds lose integrity more easily, this may result in transfer of solutes into the surrounding spermosphere whereby pathogens are attracted and colonize the seed. One possibility is that *pc* seeds do not have as strong a testa as white- or colored-seeded types and crack more easily. A negative correlation was reported between cracking seeds coats and seed coat thickness of soybeans (Yasue and Kinomura, 1984). It is also reported that if the seeds coat has a hard structure or an impermeable feature, it protects the embryo from microorganisms by regulating fluctuations in humidity and temperature (Christiansen et al., 1960; Christiansen and Justus, 1963; Mayne et al., 1969; Halloin, 1986).

Data from the field emergence test revealed better emergence for treated seeds for all seed types. When comparing treatment effects by line, several lines had larger differentials between treatments. These lines - Castano, Flaveol, Flagrano, Hystyle - were all *pc* cultivars and these results are similar to those found by Al-Jadi et al., (2016) and confirm previous findings. The differential between types and treatments was relatively small compared to Al-Jadi et al., (2016),

but this may have been due to planting into warmer soils when conditions were most favorable for bean seed germination. Finally, this research showed that there were significant differences between *pc* and other types, but not between white- and colored-seeded types for emergence.

Molecular marker studies verified that abnormal *pc* genotypes which expressed green cotyledon color instead of white color, retain the *pc* markers and presumably the trait as well. Why *pc* type cultivars generally have white cotyledon color instead of the green of normal seed types is not known. A key question is whether there are any differences in chlorophyll content in the first leaves to emerge after germination in *pc* types with differing cotyledon colors. Pollock and Toole (1964) indicated that the loss of chlorophyll was interconnected to more fundamental physiological changes resulting in lowered vigor. A second interesting question is whether the green cotyledon trait in the *pc* background is transmissible. Do daughter plants inherit the trait, or do they revert back to the original phenotype? In this study we observed the same cotyledon color in two generations for Spartacus, but Medinah showed reversion back to white cotyledons in two of three plants. A third question would be whether differing cotyledon color in a *pc* background affects germination rates. In any case, this is an avenue of research on *pc* types that should be further explored.

Near isogenic pairs were examined for differences in leaf color quantified using the CIE L* a* b* coordinate system. While we were able to detect some statistically significant differences among genotypes and seed types, the differences were small and not consistent from genotype to genotype due to cultivar x seed type interaction. While statistically significant, these differences may not be biologically significant. In any case, leaf color cannot be used as a means to differentiate *pc* types from other seed types.

CHAPTER THREE: ANATOMICAL AND PHYSIOLOGICAL STUDIES OF THE

PERSISTENT COLOR TRAIT

3.1.   INTRODUCTION

The previous chapter (chapter-two) detailed the seed germination performance both in the lab and field. In the laboratory, no differences in germination between *pc* types and white or colored seed were observed. In the field, more extreme pathogenic effects were observed on *pc* type seeds resulting in reduced germination. We know that any fungal or bacterial contamination of seed can decrease the germination rate, seed viability as well as a longevity of seed (Mehrotra, 2013). Therefore, the experiments described in this chapter were performed to determine whether the reason for increased pathogenesis were related to alterations in physiological and/or anatomical parameters of *pc* seeds.

In this chapter, we focused on four near-isogenic pairs of genotypes originally identified in chapter 2. Each pair has the same genetic background but differ in seed type. These are OSU6523-*p pc* and OSU6523-*p Pc*, OSU6510-4- *p pc* and OSU6510-4- *p Pc*, and OR91G-*p Pc* (white -seeded) and OR91G-p^{gri} *Pc* (colored-seeded). Two commercial cultivars that are sister lines and near isogenic are Ulysses (white-seeded) and Spartacus (*persistent color*) (Seminis pair). The white-seeded lines have recessive alleles at the *p* (*pigment*) locus which suppresses anthocyanin production throughout the plant. Persistent color lines are recessive at both the *pc* and *p* loci. The colored line OR91G-p^{gri} is homozygous for the p^{gri} allele at the *p* locus, which allows weak expression of underlying color genes.

The experiments performed for this chapter consist of 1) a tetrazolium test, 2) water uptake rates, 3) drop test, 4) electrical conductivity, 5) anatomical study of the seed coat and 6) comparison of seed sugars in *pc* and white seeded genotypes. The results from chapter 2 led to the

hypothesis that *pc* seed was more susceptible to imbibitional cracking and greater solute leakage, which rapidly attracted pathogens to the seed and initiated the infection process earlier in the germination process than normal seed. An alternative hypothesis is that *pc* types have inherently higher sugar levels in the seed, which leach into the surrounding spermosphere and attract pathogens. The tetrazolium test established the baseline for seed viability for these experiments. Seeds with a fragile testa would be expected to take up water more rapidly and to leak solutes more readily, and these ideas were tested via the water uptake experiment and measuring electrical conductivity. Direct examination of the testa anatomy might also reveal clues about *pc* seed as would measurement of sugars in the seeds. The seed durability and impact response from a drop test potentially damaged the seeds which was evaluated by a comparison of water uptake among seed types.

The biochemical tetrazolium (TZ) test provides additional information about seed vigor before germination and the percentage of potentially dormant seed in samples. The TZ test is a way to rapidly evaluate viability and vigor and estimate the maximum percentage capacity for normal seedlings regardless of whether the seeds are dormant (Franca Neto, 1999; Tunes et al., 2009). A tetrazolium chloride solution is used to measure dehydrogenase enzymes activity, a hallmark of living tissue. Dehydrogenases catalyze tetrazolium chloride to formazan which is visible as a red pigment in living tissues (Franca Neto, 1999). Dead, non-viable tissues are blurred, greyish, unspotted, pale red/pink or with chalky white color (Patil and Dadlani, 2009).

Testa thickness may affect rate of water uptake and can influence how easily the seed coat will crack during handling or upon imbibition. If *pc* types had thinner testas, this might induce more rapid water uptake, which could increase testa cracking. Such was the case for soybean where increase in cracked seed coats was associated with the thinner seed coat thickness (Yasue and

Kinomura, 1984). Additionally, seed coat microstructure was identified as a factor in water imbibition in legume seeds (Swanson et al., 1985).

The seed coat or testa is the primary defense against deterioration of seeds caused by pathogens (Mohamed-Yasseen et al., 1994). The legume testa consists of three layers: the outer macrosclereid, middle osteosclereid and inner parenchyma layers. The macrosclereid cells are thought to control the water permeability (Wolf et al., 1981; Yaklich et al., 1986; Ma et al., 2004) and include a cuticle-covered surface that is generally characteristic of legumes (Hartmann et al., 2019). The middle layer is the osteosclereid layer, also termed bone or hourglass cell layer based on cell shapes that are similar to macrosclereid cells, except larger (Smýkal et al., 2014). The osteosclereid layer has conspicuous air-filled intercellular spaces that during testa differentiation engage in massive cell wall deposition in the middle spaces (Harris, 1983; Miller et al., 2010). This layer is described as aerenchymatous which means it has air channels, and cannot provide a water-tight seal (Ma et al., 2004). The function of osteosclereids may be more related to imbibition and solute movement via cell expansion during imbibition (Harris, 1984). It was reported that the first major cells formed during testa differentiation were identified as osteosclereids, followed by parenchyma and macrosclereids (Ranathunge et al., 2010). The parenchyma with thick-walled or star-shaped cells, is located beneath the osteosclereid layer. The function of parenchyma layer is related to embryo development and is accepted by some authors as a "nutrient layer" (Van Dongen et al., 2003).

Bean seeds contain sugars with the main types being monosaccharides, glucose and fructose, the disaccharide sucrose and the oligosaccharides raffinose and stachyose. In snap bean, the sugar fraction ranges from 0.6% to 5.2% of fresh weight, and the main ones are glucose, fructose and sucrose (Lee et al., 1970; U. S. Department of Agriculture, 2010). The leakage of

sugars into the surrounding spermosphere may aid soil borne pathogens in locating and colonizing seed. We were interested in whether *pc* types had sugar content in the normal range or whether these were elevated.

3.2.   MATERIAL AND METHODS

3.2.a.   Seed Germination and Viability Test in Tetrazolium I- II

The TZ test was conducted with ten cultivars of 100 seeds each divided into two replicates. These ten lines represented the five near-isogenic pairs for white and *persistent color* genotypes: OSU6523-*p pc*; OSU6523-*p Pc*, OSU6510-4-*p pc*; OSU6510-4-*p Pc*, Flamata- *p pc*; Flamata-*p Pc*, Spartacus (*p pc*); Ulysses (*p Pc*) and white-seeded and colored-seeded pair, OR91G-*p Pc* (white) and OR1G-*p^{gri} Pc* (colored). Fifty seeds were placed into labeled beakers filled with tap water and were left overnight in the laboratory at 22° C to imbibe. The following morning, seeds were cut in half with a scalpel to expose the embryo. In this experiment, three hard seeds were encountered even after imbibition and that could not be cut. In this case, the radicle of the embryo was gently abraded. Henceforward, seeds were soaked again in water for imbibition, then, were all processed for the TZ assay.

The seed halves possessing the embryo were transferred to new labeled glass beakers filled with one ppm tetrazolium chloride solution and were incubated in a growth chamber at 30°C overnight. The half seeds were removed from the TZ solution, and without washing were directly viewed under magnifying desk light for evaluation. For seeds where the results were not well defined, these were observed under a binocular stereo microscope.

The seeds were separated into viable or nonviable categories. If the seeds were colored red particularly in the embryonic region or the closest part of the cotyledon to the embryo, they were categorized as viable. If the seeds were not colored; or the cotyledons were colored but not the embryo, they were rated as nonviable and non-germinable.

3.2.b. Water Uptake Experiment I- II- III- IV

Water uptake was compared between three isogenic pairs. For OSU6523-*p Pc* (white) and

OSU6523-*p pc* (*pc*), and the Seminis pair Ulysses (*p Pc*, white-seeded) and Spartacus (*p pc*). For

OR91G-*p Pc* (white) and OR1G-*p^{gri} Pc* (colored) the comparison was between white and brown

seeds. The OSU6523 pair have round, medium-sized seeds, the Ulysses - Spartacus pair seeds also

were round but larger sized and the OR91G pair had round, large sized seeds similar to the Ulysses

– Spartacus pair.

Forty dry seeds, which were controlled for physical quality (did not have any visible

damage) were divided into four replicates for each line and were weighed separately. Lines were

placed in labeled glass beakers, to which 25 ml distilled water at room temperature (22°C) was

added. The data for water uptake was collected by weighing seeds at 90 minutes intervals six times.

Before weighing after imbibition had begun, the seeds were drained and blotted for a minute with

dry paper toweling. After weighing, the seeds were placed back in the beakers and additional water

was added.

Cracked Seed Observation: Following water uptake experiments, the fully imbibed seeds

were dried and the testa of each was evaluated for cracks. Observations were assigned to one of

two classes based on visual appearance: 1) cracks, 2) hard seed. The sum of cracked seeds from

each line was calculated as a percentage of total seed number over the four reps per genotype.

Then, each proportion of cracked seeds between isogenic pairs was compared.

3.2.c. Drop Test I- II

The seeds were subjected to the drop test to assess the fragility of the seed testa during dry seed handling and conditioning for the different genotypes. Nine genotypes, as indicated in **Table 10**, with seed from the field increase were tested. The lines selected were sister lines or near-isogenic pairs differing for seed traits

Seeds were carefully examined, and any cracked, moldy or abnormal seeds were discarded. Twenty seeds per cultivar divided in two replicates were dropped down a metal tube 1.8 m (six ft) in length onto a metal plate. This step was repeated 4 times sequentially. They were then visually inspected for mechanical damage and were evaluated for water uptake rates as described below.

<u>Water uptake examination</u>: The interaction between dropped/treatment seed and non-dropped/control seeds were tested by water uptake comparisons. Seeds were soaked in 25 ml distilled water at room temperature (22°C). The seeds were assessed at three stages. Initially, after 10 minutes the seeds were inspected for degree of wrinkling as opposed to smooth and uniform imbibition. Secondly, seeds were weighted at 90 minutes intervals 6 times. Before seeds were weighed, they were drained and blotted for about a minute with dry paper towels. Amount of uptake was quantified by comparison of drop treated seeds and control seeds by taking the difference between initial seed weight and soaked seed weights. The statistical analysis was conducted as described above in section 2.2c.

Table 10: Snap bean genotypes used in a drop test and water uptake experiment.

Cultivar[1]	Seed Type
OSU6523-*p pc*	*Persistent Color*
OSU6523-*p Pc*	White-seeded
OSU6510-4-*p pc*	*Persistent Color*
OSU6510-4-*p Pc*	White-seeded
Spartacus (*p pc*)	*Persistent Color*
Ulysses (*p Pc*)	White-seeded
OR91G-*p Pc*	White-seeded
OR91G-*p^{gri} Pc*	Colored-seeded
Pascal (*p pc*)	*Persistent Color*

[1]Spartacus and Ulysses are sister lines and Pascal is an unpaired flageolet type. The remainder are near isogenic pairs.

3.2.d. Electrical Conductivity I-II and Moisture Content Evolution I-II

Seeds of four isogenic pairs of genotypes were tested for electrical conductivity. For three pairs, the comparison was between *pc* and white-seeded genotypes OSU6523-*p pc vs.* OSU6523-*p Pc*; OSU6510-4-*p pc vs.* OSU6510-4-*p Pc* and Spartacus (*p pc*) *vs.* Ulysses (*p Pc*). In addition, OR91G-*p Pc vs.* OR1G-*p^{gri} Pc* provided a comparison of white- and colored-seeded genotypes.

Prior to the electrical conductivity test, the moisture content of seeds was evaluated to assess uniformity according to AOSA protocol (Smýkal et al., 2014). Seeds were ground into a fine powder and weighed, and vials were tared to 5 grams. The powdered seeds were dried in an oven at 130° C for an hour and reweighted. Moisture content was calculated as the initial minus the dry seed weight divided by the initial weight and expressed as a percentage. Spartacus, Ulysses and OSU6523-*p pc* were recorded at 8.1, 9.3 and 9.3% percent, respectively, and were at relatively lower moisture content than other lines. Therefore, these lines were moisturized to equilibrate

moisture content to the others. Those seeds at 8.1% were held between slices of moistened germination paper for 60 minutes while seeds at 9.3% were moisturized for 30 minutes.

Following moisture equilibration, the electrical conductivity was measured. Seeds were carefully selected to avoid those with obvious damage (no scars, mold or broken seeds). One-hundred seeds divided into two reps per genotype were weighed and placed in clean, labelled beakers, 250 ml distilled water was added, and seeds were left for 24 hours at room temperature (22°C). Seeds were gently shaken for 10 seconds and conductivity measurements were taken using a Thermo Scientific Orion VersaStar, Benchtop conductivity meter. The experiment was repeated to obtain data for the second replicate.

The electrical conductivity is measured in one millionth of a Siemen per centimeter (micro-Siemens per centimeter or µS/c) per gram of seed weight. The method was conducted according to ISDA protocols (ISTA, 2005) and the formula used in calculation was $\mu S\ cm^{-1}\ g^{-1}$ = (Electrical conductivity reading $\mu S\ cm^{-1}$ – Background reading) / Weight of replicate (g).

Following calculations, the average electrical conductivity across replicates was obtained and compared both between isogenic pairs and among seed color types averaged across genotypes by the variables in the data set.

3.2.e. Seed Anatomical Structure I- II

The anatomical comparison of seed coat thickness was measured in the three outer testa layers of the snap bean seeds; these being from outside to inside the macrosclereid, osteosclereid and parenchyma layers. In the first experiment, three genotypes were compared. 'Pascal' represented a *persistent color* type, OR91G-*p Pc* a white-seeded type and OR91G-*p^{gri} Pc* as a

colored-seeded line. OR91G-p^{gri} *Pc* is near-isogenic to OR91G-*p Pc* snap bean while Pascal is genetically distinct.

To prepare for sectioning and microscopic observation, fragments of seeds from near the embryo were fixed, dehydrated, and embedded in plastic. Sections were cut with a microtome and mounted on slides and stained. Each step was as follows: Fixation: The seeds were fixed under vacuum in a formalin acetic alcohol solution for 24 hours at room temperature. Then samples were dried in a vacuum desiccator for a week. Dehydration: Samples were removed from the vacuum and dehydrated in a 50%, 70% and 95% graded ethanol series for 24, 24 and 72 hours, respectively. Infiltration: Seeds were pre-infiltrated under the vacuum in a 2:1 solution of 95% ethanol:plastic infiltration mixture (Technovit 7100). The process was repeated under vacuum with 1:1 and 1:2 95% ethanol: plastic infiltration solution for 48 and 72 hours, respectively.

After infiltration, seeds were embedded in Technovit 7100 glycol methacrylate plastic (electron microscopy services) and sectioned with a steel knife at 5-7 µm on a rotary microtome. Around 3-5 sections depending on size were placed and aligned onto droplets of water on clean microscope slides and at least 4 slides were collected per genotype. Then, the slides were gently moved to the slide warmer (pre-set to 37°C) and dried for 2 hours to prepare them for staining.

For staining, slides were placed in Coplin jars containing 0.5% Toluidine Blue O in Citrate Buffer (pH 4.2). After 4-5 minutes, each slide was taken from the jar and was rinsed under running tap water to remove excessive stain from the slide then washed in distilled water. Coverslips were then applied to each stained slide.

The seed testa layers were examined under a light microscope at 400X and were visualized as shown in **Figure 9**. The macrosclereid, osteosclereid and parenchyma layers were identified in

ten sections for each layer per genotype, and were measured using an ocular micrometer (each unit 3μ).

The anatomical structure analysis was repeated with six additional genotypes subjected to the same protocol. The additional genotypes were three paired genotypes previously used in water uptake examination: isogenic pairs OSU6523-*p pc*: OSU6523-*p Pc*, OSU6510-4-*p pc*: OSU6510-4-*p Pc* and Seminis pair Spartacus (*p pc*): Ulysses (*p Pc*). In this experiment, the *pc* and non-*pc* types was compared among isogenic pairs as well as among unrelated genotypes.
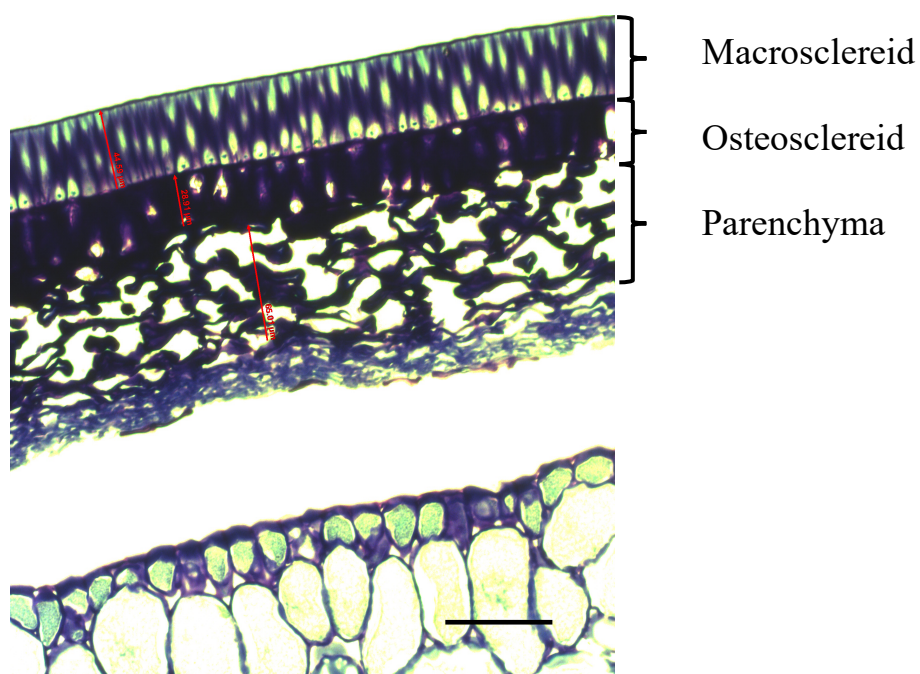
Figure 9: Transversal section of the seed coat of OSU6510-*p pc*; showing the three layers of the testa that were measured: Top: outer macrosclereid layer, Middle: osteosclereids forming the hypodermal layer, Bottom: inner parenchyma layer. Bar = 100 μm.

3.2.f.   Sugar Analysis I- II- III- IV

The concentration of sugars in *pc*, white-seeded and colored-seeded snap bean varieties were assessed by gas chromatography-mass spectrometry (GC-MS). The seeds were screened

mainly for the soluble sugars including fructose, sucrose, raffinose. The experiment was repeated four times.

The first replicate was performed using six isogenic pairs: OSU6523-*p pc* and OSU6523-*p Pc*; OSU6510-4- *p pc* and OSU6510-4-*p Pc* and Seminis pair of Spartacus (*p pc*) and Ulysses (*p Pc*). Other replicates used the same set with the addition of one more isogenic pair (Flamata- *p pc*, Flamata-*p Pc*).

Extraction protocol: After each seed was separated from their seed coats, the cotyledons and embryos were ground in a mortar and pestle until they could be solublized in liquid. Then ~10mg of powdered seeds were placed in tubes and were freeze-dried for 48 hours. Upon removal from the freeze-dryer, each sample was weighed, and approximately 10 ± 5 mg was placed in a new 1.5mL microfuge tube and exact weight was recorded. Metabolites in the seed powder were extracted with 1000 µL of a solution containing chloroform:water:methanol in a 1:3:1 ratio. After the addition of the extraction solution, the seed powder samples were thoroughly mixed using an analog vortex mixer, and subsequently sonicated in a waterbath sonicator. The samples were then centrifuged at 21,130 rcf for ten minutes and the supernatant was transferred into new centrifuge tubes. To isolate the extracted polar compounds, 400 µL of 10mg/L xylitol dissolved in water was added to the tubes and the tubes were then vortexed and centrifuged for ten minutes at 21,130 rcf. The addition of the water causes a phase separation between an upper aqueous phase and a lower organic phase while the xylitol serves as an internal standard. The upper aqueous phase was transferred and split into two portions of 100 µL and 200 µL, put into new centrifuge tubes and freeze-dried. Subsequently, they were stored at -80º C until GC-MS analysis.

Derivatization Protocol for the GC-MS analysis: To prepare samples for GC-MS analysis, samples were derivatized in a two-step process. The freeze-dried extracts were first derivatized

with the addition of 40uL of 30 mg/mL methoxyamine HCL in pyridine followed by incubation at 37º C for 90 minutes on a shaker. In the second step, 60 μL of of N-Methyl-N-(trimethylsilyl) trifluoroacemide (MSTFA) with 1% trimethylchorosilane (TMCS) were added to each sample. The samples were incubated again at 37º C for 30 minutes with vigorous shaking.

The derivatized samples were injected in a 10:1 split ratio into a 250 °C inlet in an Agilent 7890B gas chromatograph equipped with a 30m DB-5MS+DG column with a 10m DuraGuard guard column using helium as the carrier gas. The oven was initially held at 60 °C for 1 minute, then ramped 10 °C/min to a final temperature of 300 °C, which was held for 10 minutes for a total run time of 35 minutes. The analytes were ionized via electron ionization with a 230 °C source in an Agilent 5977B mass spectrum detector and the m/z ratios were detected by a single quadrupole detector at 150 °C.

Chromatographs were deconvoluted using the Automated Mass Spectral Deconvolution and Identification System (AMDIS) and the mass spectrum and retention times of the components were searched against the mass spectra library "FiehnLib" developed by the Fiehn lab. The identified compounds and their peak areas were then exported to a CVS file and processed with an in-house program. Protocol was designed according to Kind et al., (2009). Values were adjusted using xylitol as an internal standard and expressed on peak area basis.

3.3. RESULTS

3.3.a. Seed Germination and Viability Test in Tetrazolium I- II

Based on color intensity from the TZ test, most genotypes showed high percentages of viability. OSU6523-*p pc* exhibited three hard seeds out of one hundred seeds with two viable and one nonviable. Because of this, the first replication which included the hard seeds was repeated. The second time, no hard seed were encountered and nearly all were viable. As a result, the additional test brought the viability level from 98 to 99 %, for OSU6523-*p pc*. Overall, the viability percentages were 99% for *pc* types, 98.8% for white-seeded and 98% for colored-seeded types.

When we compared genotypes within their isogenic pair, no significant differences among them were observed.
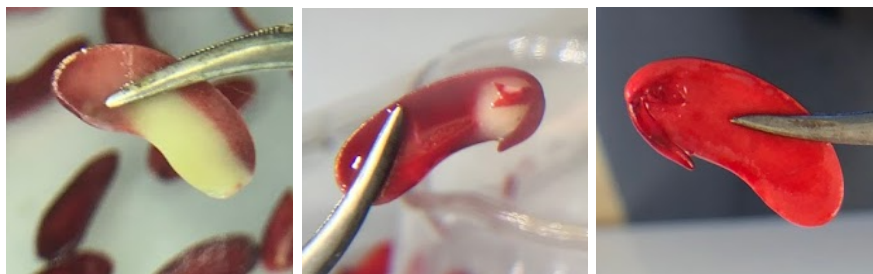
Figure 10: Determination of seed viability in snap beans. Left and middle images show examples of nonviable seeds; Right shows fully viable seed. In the middle image, even though the embryo is red, the connection to the cotyledon is not viable, so embryo would lack the ability to take up nutrients from the cotyledons.

3.3.b. Water Uptake Experiment I- II- III- IV

The water uptake experiment was performed with three pairs of genotypes to describe the rate of water uptake for *pc*, white- and colored-seeded genotypes. The pair OR91G-*p Pc* and OR91G-*p^{gri} Pc* corresponding to white- and colored-seeded genotypes did not exhibit significant differences in accumulation of water (**Figure 11**). The seeds of OR91G-*p^{gri} Pc* are about 0.2 g

heavier than OR91G-*p Pc* for initial weight, and this difference carried through the experiment. However, the slopes of the lines between time points was essentially the same.

In contrast, the *persistent color* and white-seeded paired lines exhibited differences in water uptake curves. This trend was evident for Spartacus and Ulysses, where the former had greater water uptake particularly at time points 180 – 360 minutes (**Figure 12**). Both cultivars had similar initial seed weight, and seed weight again converged as seeds achieved full expansion at 540 minutes.

OSU6523-*p pc* and OSU6523-*p Pc* paired comparison was similar to the Spartacus-Ulysses pair in that the *pc* type imbibed more rapidly (**Figure 13**). However, water uptake curves differed from that of the previous pair. Initial seed weight for OS6523-*p pc* was about 0.1g heavier than OSU6523- *p Pc*; significant differences became apparent in the first 90 minutes and the weight of the lines had not converged even by the end of the experiment.
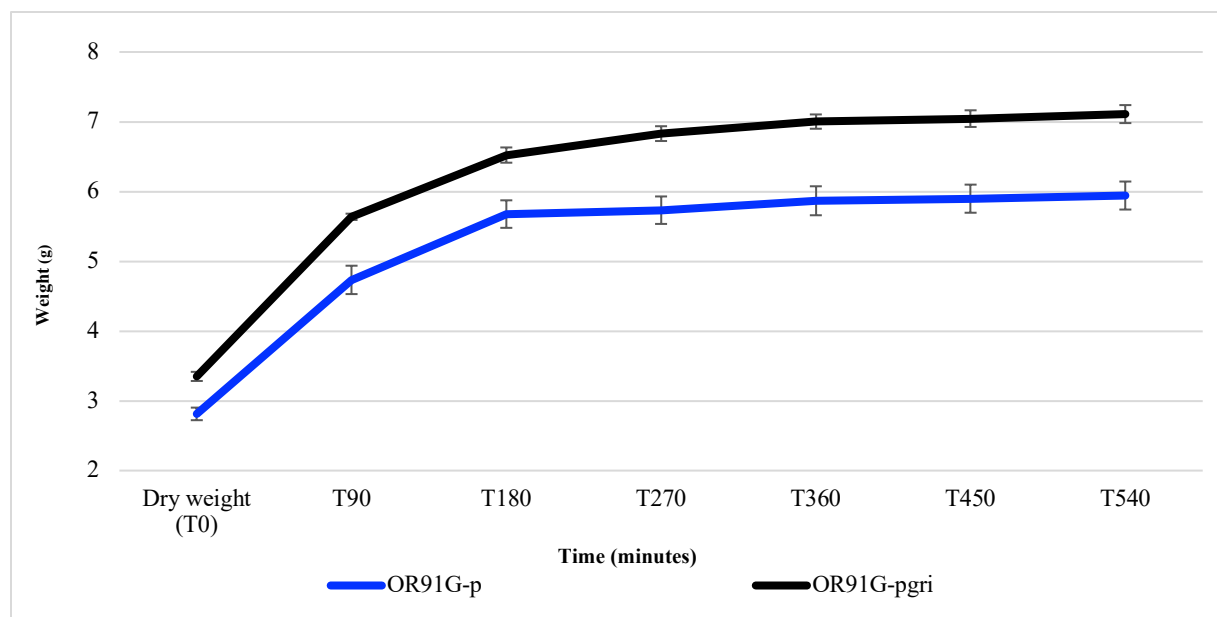
Figure 11: Comparison of OR91G white- and colored-seeded snap bean isolines for amount of water uptake in seeds over 540 minutes. Error bars represent standard error. OR91G-*p* (white-seeded), OR91G-*p^{gri}* (colored-seeded).
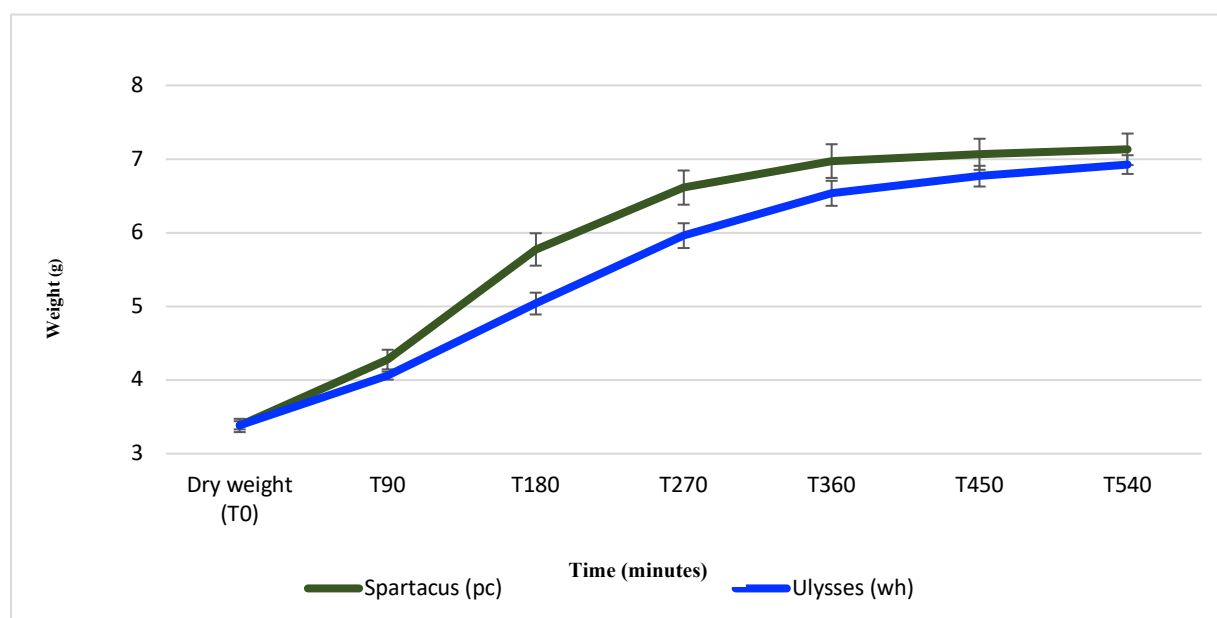
Figure 12: Comparison of Spartacus (*p pc*) and Ulysses (*p Pc*, white-seeded) snap bean sister lines for amount of water uptake in seeds over 540 minutes. Error bars represent standard error.
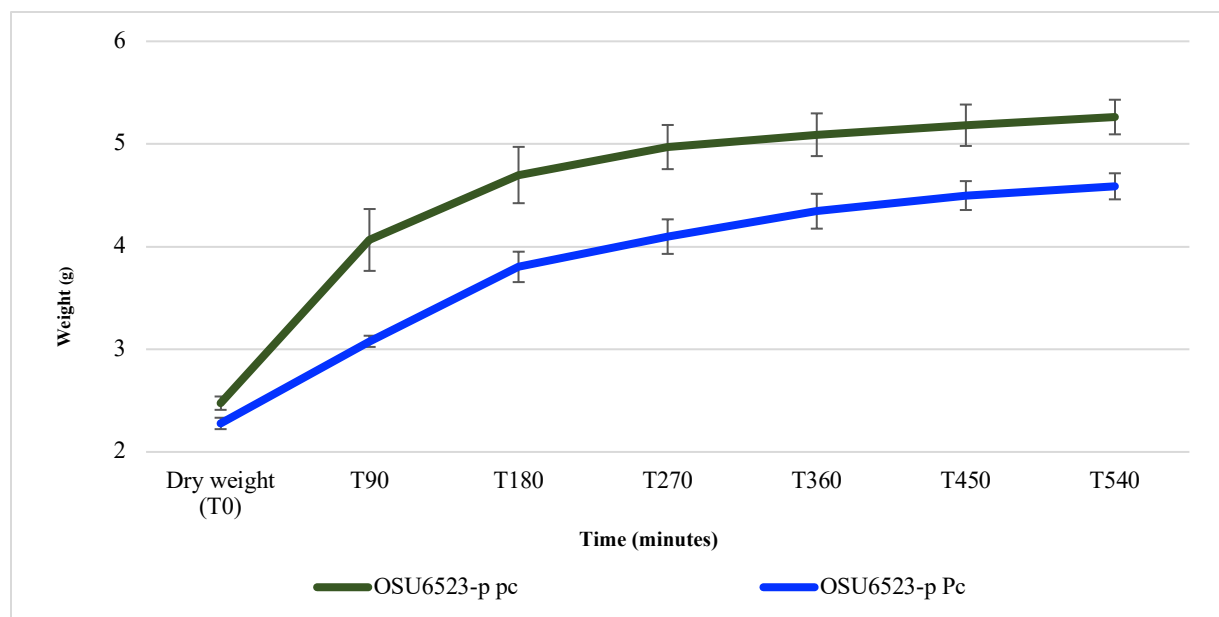
Figure 13: Comparison of OSU6523-*p pc* (persistent color) and OSU6523-*p Pc* (white-seeded) snap bean isogenic lines for amount of water uptake in seeds over 540 minutes. Error bars represent standard error.

3.3.c.   Seed Cracking following Water Uptake Experiment I- II- III- IV

Soaking seeds in water as was done in the water uptake experiment is a worst-case scenario in terms of the stresses placed on the testa by the rapid expansion of the seed, and the water uptake experiment resulted in some seed coat cracking following imbibition (**Figure 14**). After full imbibition, each seed was dried and examined for lesions in the testa. The white seeded cultivar OR91G-*p Pc* manifested significantly more cracked seeds (42.5%) compared to its colored seed counterpart OR91G-*p^{gri} Pc* (7.5 %).

The *persistent color* types exhibited about twice as many cracked seeds compared to their white-seeded counterparts for the Seminis pair. 70% cracked seeds for Spartacus (*p pc*) and 37.5% for Ulysses (*p Pc*). The isogenic pair OSU6523-*p pc* and OSU6523-*p Pc* had 17.5% cracked seeds for the *pc* isoline and 10% for its white-seeded counterpart. To summarize, *pc* types had the highest percentage of cracked seeds following a stressful imbibition treatment followed by white-seeded types and then colored-seeded types.

Figure 14: Crack seeds observation. Exhibits an example from the class of cracked seeds.

2.3.d. Drop test I- II

Treated (dropped) and nontreated seed were compared for amount of water uptake. The observations of seed testa wrinkling in first 10 minutes revealed greater wrinkling in the treated seed compared to the control. The difference was highly statistically significant when means were compared by a t-test. The mean for treated seeds was 35.0%, and for control seeds 3.9%. (SE = 9.75).

Analysis of variance revealed significant differences for genotypes and treatments over the seven time points of water uptake by seeds (**Table 11).** Genotypes showed significant differences among seed types and dropped seeds had significantly more rapid water uptake compared with the undamaged seeds as shown in **Table 11**. However, the genotype by treatment interaction was not significant. More rapid water uptake was noticeable during the first and middle periods of the time curves (**Figure 15**). When seed types were compared, more rapid water uptake was observed for *pc* types compared to their non-*pc* counterparts in the control treatment for the Seminis pair and the OSU6523 isogenic pair. In the period between 90 - 270 minutes, the means for the Seminis pair transitioned from 3.49g to 5.27g for Spartacus, and 3.47g to 4.20g for Ulysses (**Figure 15-B)**. In the first 90 minutes the means of OSU6523 transitioned from 2.47g to 2.86g for *pc* and from 2.24g to 2.33g for the non-*pc* genotype (**Figure 15- C)**. Such was not the case for the OSU6510 pair where both the control and treatment water uptake curves were nearly identical for the isogenic pairs (**Figure 15- E)**. Although differing in seed weight the OR91G isogenic pair showed near identical curves for both treated and untreated seeds (**Figure 15-D)**.

The isogenic pairs OSU6523 subjected to the drop treatment also showed significant differences for the first and middle range of the time curves. The mean seed weight was 2.39g to 4.28g for *pc*, 2.37g to 3.98g for non *pc* in the first 180 minutes (**Figure 15-C)**. For the Seminis

pair in the damaged seed treatment, Spartacus (*pc*) showed significantly more water uptake for the first 90 minutes than white-seeded Ulysses (**Figure 15-B**). Then, the differences in water uptake among seed types mainly disappeared.

Table 11: ANOVA: analysis of variance for water uptake over time for treated (dropped) and control seeds of isogenic pairs of snap beans.

Source	DF	t0[1]	t90	t180	t270	t360	t480	t570
					Mean square[2]			
Genotype	8	0.84***	1.41***	2.23***	2.43***	2.57***	2.62***	2.92***
Rep	1	0.08	0.15	0.08	0.04	0.16	0.31	0.28
Treatment	1	0.38**	20.34***	22.69***	15.83***	11.02***	8.62***	8.78***
Genotype x Treatment	8	0.07	0.36	0.55**	0.44*	0.32	0.28	0.25
Error	17	0.33	0.20	0.11	0.13	0.14	0.12	0.14

[1]**T[no.]:** Indicates the time interval between weighing. [2]Significant at probability levels: *, **, *** Significant at $p < 0.05$, 0.01, and 0.001, respectively.
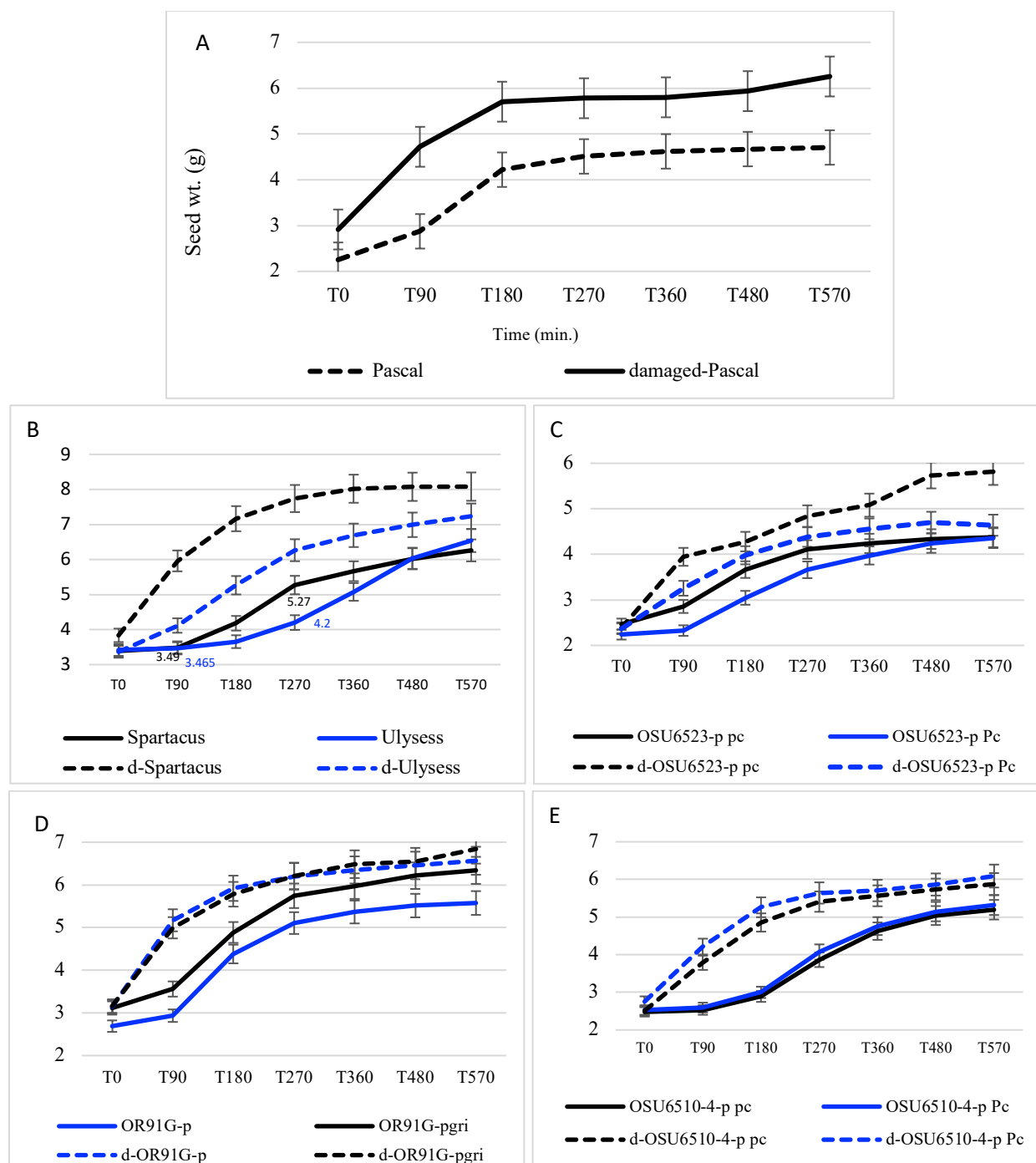
Figure 15: Water uptake of isogenic pairs of snap beans differing in seed types when treated by dropping seeds vs. those that were not dropped. 'd-' represents damaged seed treatment. Error bars represent standard error. **A.** Pascal treated and untreated water uptake curves. **B.** Comparison of Spartacus (*p pc*) and Ulysses (*p Pc*); **C.** Comparison of the isogenic pair OSU6523; **D.** Comparison of the isogenic pair OR91G [OR91G-*p*] and [OR91G-*p^gri^*] (colored) and **E.** Comparison of the isogenic pair OSU6510.

3.3.e. Moisture Content Evaluation I- II and Electrical Conductivity I- II

Moisture Content Evaluation: The seed moisture content was relatively uniform among cultivars (**Table 12)** with the exception of Spartacus which was lower at 8.1%. Most others were at 9.5% percent except OSU6523-*pc* and Ulysses which were at 9.3%. These moisture levels were equilibrated with that of other beans in the study before electrical conductivity testing.

Electrical Conductivity: The seed electrical conductivity readings were summarized by averaging across similar seed types (**Figure 16**). The *pc* genotypes had the greatest electrical conductivity with 37.7 $\mu Scm^{-1}g^{-1}$, followed by white-seeded genotypes with 28.0 $\mu Scm^{-1}g^{-1}$. The colored-seeded genotype had the lowest with 18.7 $\mu Scm^{-1}g^{-1}$. Least Squares Means were calculated and tested for statistically significant differences. The *persistent color* LS mean was highly significantly different from white- (P = 0.0167) and colored-seeded (P = 0.0093) means. White and colored seeded means were not significantly different (P = 0. 0762).

When the electrical conductivity was compared for genotypes individually, for each isogenic pair, the *pc* genotype always had higher electrical conductivity compared to its white seeded counterpart (**Figure 17).** For the OR91G white- and colored-seeded pair, white seed had higher electrical conductivity. Among pairs, the greatest dissimilarity was for OSU6523-*p pc*, and OSU6523-*p Pc*.

Table 12: Seed moisture content of paired snap bean genotypes that differ in seed type.

Treatment	Seed Type	Moisture Content (%)
Spartacus	*Persistent Color*	8.1
Ulysses	White-Seeded	9.3
OSU6523-*p pc*	*Persistent Color*	9.3
OSU6523-*p Pc*	White-Seeded	9.5
OSU6510-4- *p pc*	*Persistent Color*	9.8
OSU6510-4- *p Pc*	White-Seeded	9.8
OR91G-*p^{gri}*	Colored-seeded	9.9
OR91G-*p*	White-Seeded	9.5
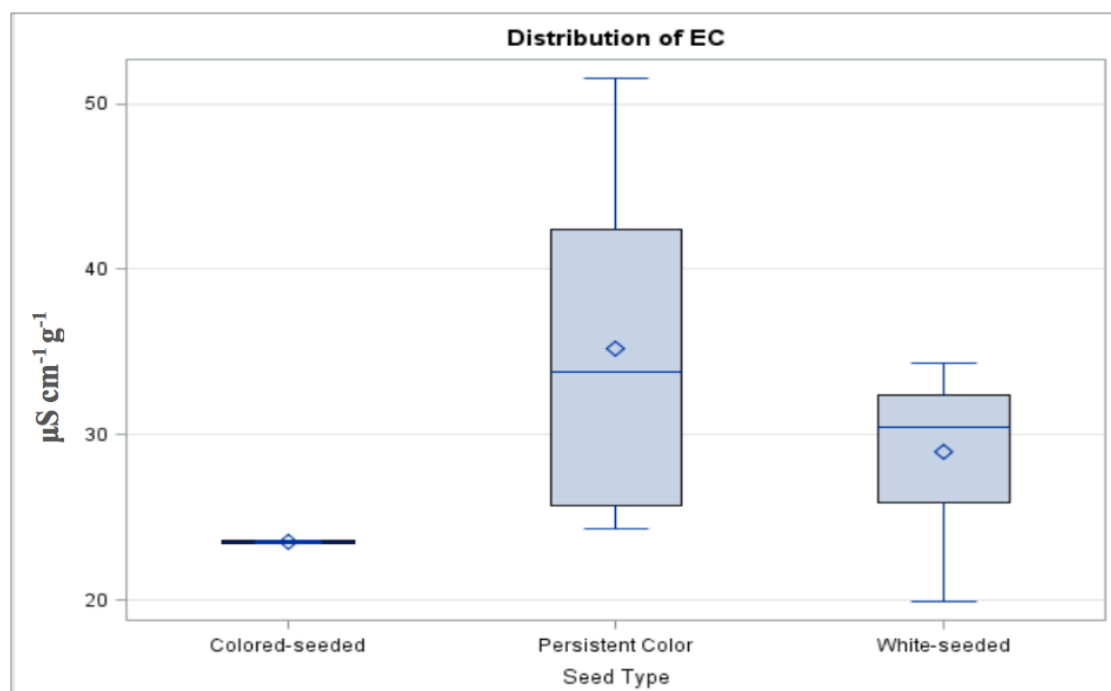
Figure 16: Box and whisker plots for seed solute electrical conductivity grouped by seed type for a set of snap bean paired genotypes.
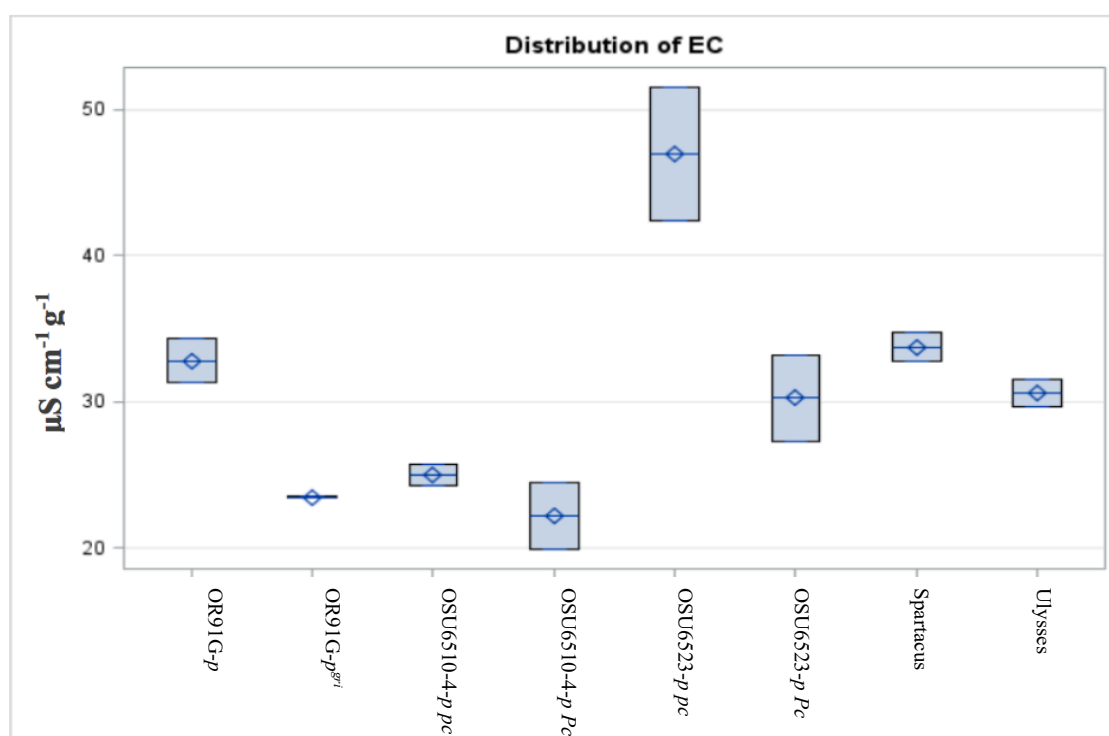
Figure 17: Box and whisker plots of seed solute electrical conductivity on an individual genotype basis for a set of paired snap bean genotypes.

3.3.f. Seed Anatomical Structure I - II

Examination I: Comparison of seed testa thickness revealed a significantly thinner osteosclereid layer in Pascal (*pc*) than the white- and colored-seeded genotypes (**Figure 18**). Other testa layers were also thinner in the *pc* type but the difference with white- and colored-seeded genotypes was not as pronounced. The total testa thickness (sum of the three layers) was 31.6 µm in the *pc* type while others were 69 and 54.7 µm for white- and colored-seeded genotypes, respectively.

Examination II: One concern from the first experiment was that the variation in testa thickness might be influenced by other genetic factors independent of *pc*. Therefore, a set of isogenic pairs was examined. The same difference in thinner osteosclereid layer in *pc* types was again observed for these genotypes. The average thickness of the osteosclereid layer in the *pc* seeds was 8.5 µm while non *pc* types were over 12 µm (**Figure 19**). A similar result to the first experiment for the macrosclereid and parenchyma layers was again observed. Although the differences were not as pronounced as for the osteosclereid layer, the thinner macrosclereid and parenchyma layers on *pc* type seeds were perceivable. Comparing the individual genotypes to their counterparts, there were clear differences in the thickness of the osteosclereid layer (**Figure 20**). This difference was 4.9 µm for the OSU6523 pair and 8.0 µm for the OSU6510-4 pair.
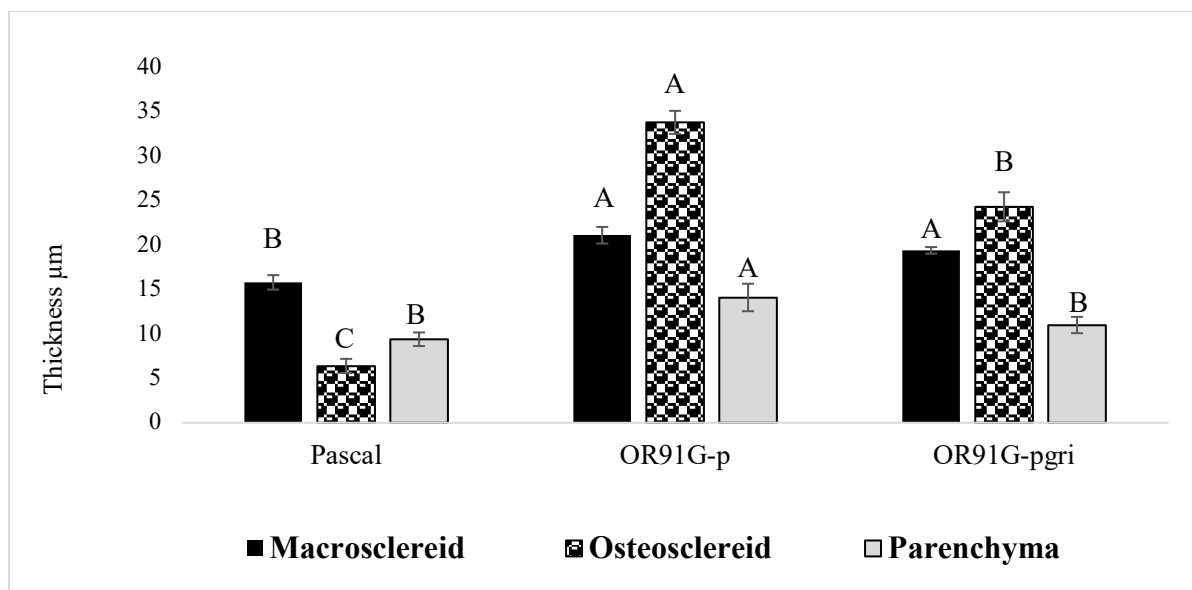
Figure 18: Thickness of testa layers of Pascal (*pc*), OR91G-*p Pc* (white-seeded) and OR91G-*p^{gri} Pc* (colored-seeded) common bean lines. Error bars represent standard error. Within a testa layer, bars with the same letter are not significantly different at P > 0.05.
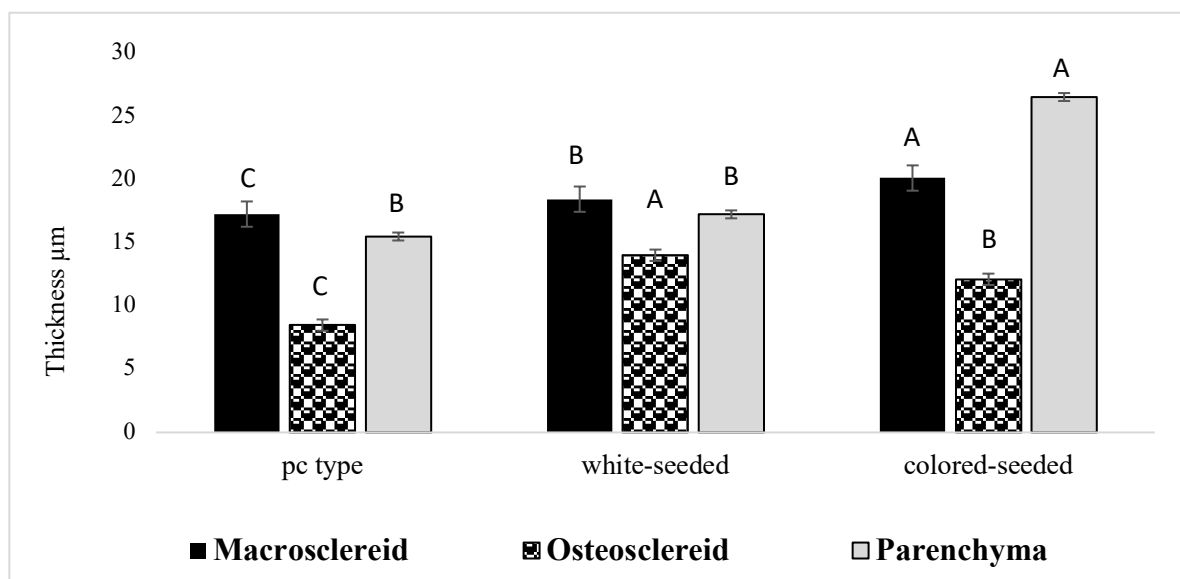
Figure 19: Thickness of outer testa layers of persistent color, white-seeded and colored-seeded types for nine snap bean lines. Error bars represent standard error. Within a testa layer, bars with the same letter are not significantly different at P > 0.05.
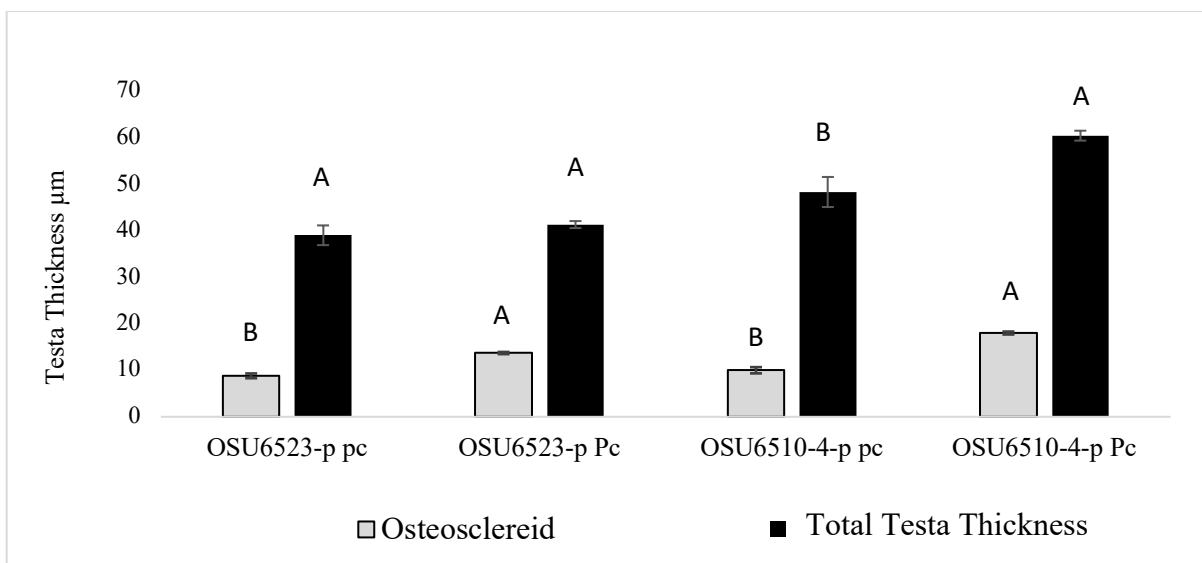
Figure 20: Osteosclereid and total thickness (sum of three outer layers) of two *pc*, and white-seeded isogenic snap bean pairs Error bars represent standard error. Within a testa layer, bars with the same letter are not significantly different at P > 0.05.

3.3.g. Sugar Analysis I - II - III - IV

Individual sugar compounds were separated, quantified and identified by GC-MS. A total of 47 complex carbohydrate compounds were observed but most in minute amounts. One compound (glucose) that we were particularly interested in and expected to find were not observed. The other major sugars and oligosaccharides that were identified included fructose, sucrose and raffinose. We also did not observe the oligosaccharide stachyose, which is usually found in conjunction with raffinose in beans.

In general, most compounds did not demonstrate significant differences between seed types (**Table 13**); just small differences among cultivars were seen for some compounds. Sugar concentrations were first examined in a comparison of seed types as a mean across genotypes with either *pc* or white seed. For sucrose, *pc* seeds showed greater variability than white seeds, but the means were not significantly different. Raffinose showed less variation but was not statistically significant between seed types. Fructose was higher in *pc* seeds, but this difference was also not significant although very close (P = 0.07) (**Figure 21**).

Significant differences among cultivars were observed for sucrose, raffinose and fructose (**Figure 22**). Sucrose was significantly lower in OSU6510-4 than most other genotypes. For raffinose, Flamata had significantly higher levels. The genotype by seed type interaction was not significant for any compound.

Table 13: ANOVA of peak areas for sugar compounds found in snap bean seed detected by GC-MS.

		Mean Square[1]		
Source	**DF**	**Sucrose**	**Fructose**	**Raffinose**
Genotype Pairs	3	7.22*	0.000000436	0.01069***
Seed Type	1	0.61	0.000001902	0.00098
Pairs*Seed type	3	3.64	0.000001299	0.00036
Error	14	27.53	0.00000698	0.00900

[1]*, **, *** Significant at $p < 0.05$, 0.01, 0.001, respectively.
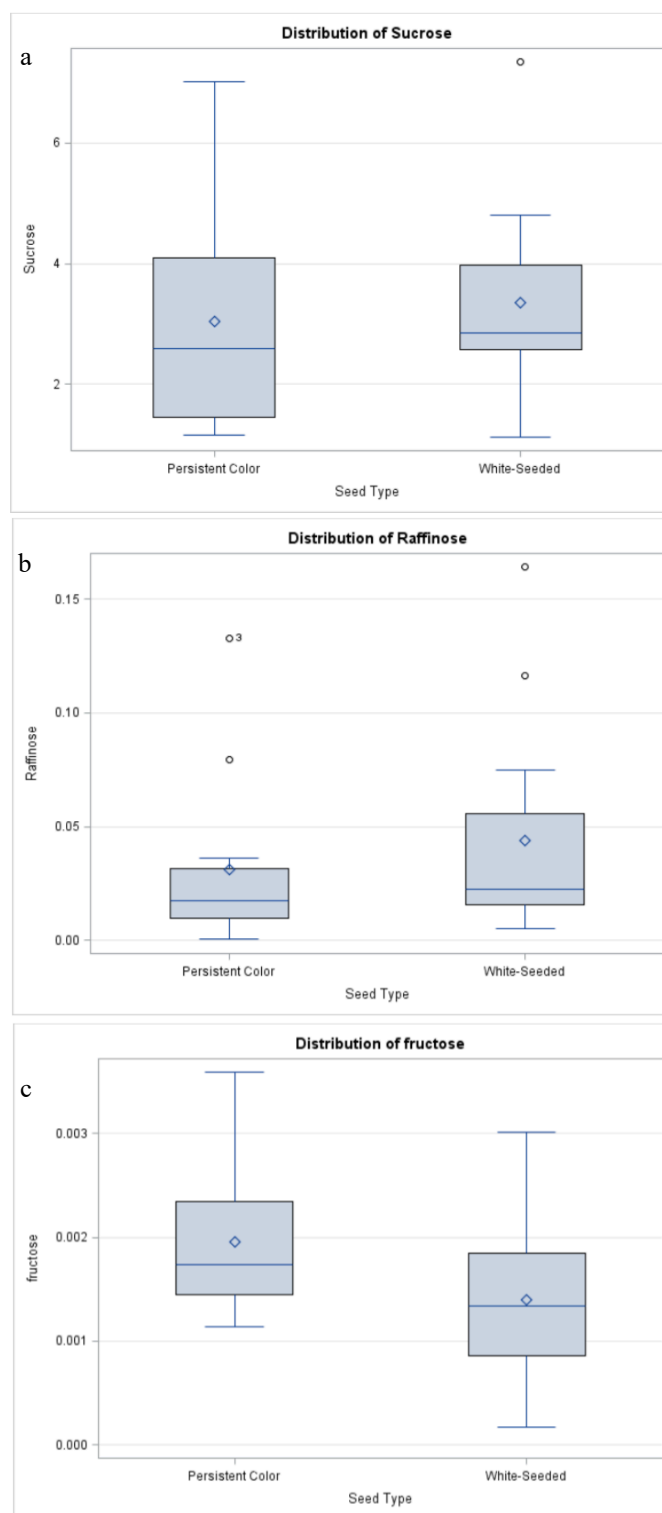
Figure 21: Comparison of white-seeded and *pc* genotypes of snap bean for levels of (a) sucrose, (b) raffinose and (c) fructose in dry seed. Peak area adjusted by the internal standard and sample weight.
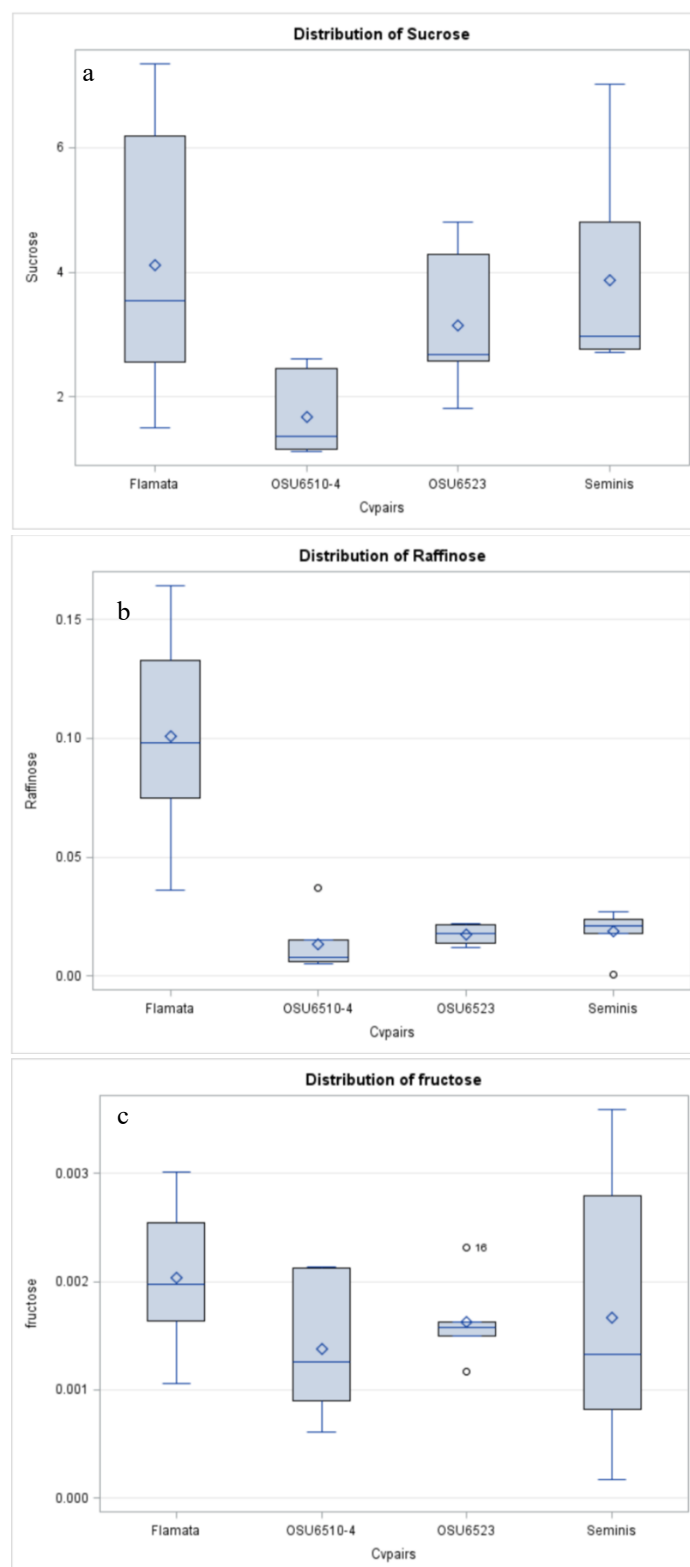
Figure 22: Comparison of snap bean genotypes for dry seed sugars. Peak area adjusted by the internal standard and sample weight for (a) Sucrose, (b) Raffinose and (c) Fructose. Seminis: Spartacus (*pc*), Ulysses (white-seeded).

3.4. DISCUSSION AND CONCLUSIONS

The percentage of seed viability as measured by a tetrazolium (TZ) test confirmed high levels of viability for our seed samples. An average of 98% for physiological performance was observed for *pc*, white-seeded and colored-seeded genotypes, and all had a similar vigor and would be expected to germinate in equal numbers under suitable conditions. The TZ test also revealed that the OSU6523-*p pc* type had a tendency towards hard seeds although this was not observed in an additional replication. Also, this phenomenon was not observed in the OSU6523-*p Pc* genotype of the isogenic pair. This is an interesting finding because we would expect the hard seed phenomena to be related to the composition of the testa and independent of seed color type. *Persistent color* types do affect testa thickness and perhaps composition, and because of their thinner testa, might be expected to be less prone to hard seed. In beans, a semi-hard seed characteristic was described by Dickson and Boettger (1982) and was defined as seed that could not fully imbibe water in first 24 hours with initial moisture of 8% or less, but once the seed moisture level was increased to 10 % or more, semi-hard seed would hydrate normally. This could be the trait present in OSU5623, but this avenue of research was not pursued. In any case, *pc* types compared to their white-seeded counterpart might be used to shed light on the hard seed phenomenon in beans.

We know that *pc* types are more susceptible to pathogens. During the initial germination process, pathogens are kept at bay by the seed coat, and any loss of integrity of this structure will increase the risk of infection. The seed coat has an important role in regulating the imbibition rate and affects susceptibility to injuries and subsequent germinability (Taylori et al., 1992). Similarly a relationship between the percentage of seed coat damage and hydration rate was reported by Powell and Matthews (1979), and Wolk (1988) indicated that there is reduction in germination

rate with increased hydration rate in snap beans. These outcomes are compatible with observations from our water uptake analysis, where *pc* seeds had more rapid water uptake than other forms of seeds and had greater numbers of cracked seeds in *pc* types. More numerous cracked seeds in *pc* types may be a reflection of their fragility and the cause of increased pathogenesis in seeds that reduces germination under field conditions.

Subjecting different seed types to the drop test resulted in no visual differences among seeds in terms of damage. Damage was apparent however, with water uptake and appeared within the first 10 minutes where seeds wrinkled with increased rapidity of water absorption, instead of a smooth absorption as observed with non-dropped seeds. In addition, more rapid water uptake was observed for damaged seeds of all genotypes over the longer time course of the water uptake experiment (Figure 15).

The electrical conductivity results were also congruent with the water uptake results. The *pc* seeds leaked more solutes as measured by the higher electrical conductivity. The combined results of the water uptake and electrical conductivity tests show that solutes move more readily both into and out of *pc* seed compared to other types, even when the seed is of highest quality.

These experiments all suggested that the testa might play a role and that it would be useful to look at the physical structure of the testa. Measurements of the physical structure of the testa of *pc* genotypes revealed thinner layers compared to white- and colored- seeded lines. In particular, the osteosclereid layer was significantly thinner in *pc* types. Although osteosclereid function has not been wholly elucidated, it is involved in regulating intercellular spaces, and as such, may be related to cell expansion and maintaining membrane integrity during water absorption. Thinner seed coats of *pc* seeds may have expedited imbibition, which led to increased disruption of the

seed coat through cracking, which in turn could lead to greater leakage of solutes into the spermosphere which would attract pathogens as has been documented by (Spaeth, 1986).

We also compared the sugar concentrations in *pc* and white-seeded genotypes since another possible mechanism is that *pc* seeds might have higher inherent soluble sugar contents which could lead to pathogens colonizing seeds. Although few statistically significant differences were found, some sugar compounds were generally higher in white-seeded genotypes compared with *pc* genotypes. Sugars may serve as signaling compounds to regulate seed development (Wobus and Weber, 1999) as well as being involved in metabolic exchanges (Borisjuk et al., 2003), and as such, there might be a positive correspondence between the higher sugar levels of white-seeded types and better germination rates. There is also a possibility that the oligosaccharides raffinose and stachyose may play a role in stabilizing cell membranes during imbibition, especially under cold conditions where these membranes might be particularly brittle. One cultivar pair (Flamata) had significantly higher raffinose levels and comparison of the *pc* vs white genotypes suggested that higher raffinose levels were observed in the white-seeded form, although the differences was not significant.

There could be additional reasons as to why *pc* seeds are more susceptible to pathogens, but the seed coat effect appears to substantially influence the process through allowing more solute leakage from *pc* seeds. The anatomical analysis of additional isogenic pairs helped confirm the differences that we initially found. In general, the use of isogenic pairs was a powerful tool in many experiments because it reduced if not eliminated any background genotype effect.

A question yet to be answered is whether all *pc* cultivars have these deleterious features. It is possible that some *pc* types have a thicker seed coat, although in breeding nurseries where germination percentages have been observed over many generations, no *pc* types with increased

germination have ever been found (Myers, personal communication). Identifying *pc* lines having thicker seed coats or breeding for a thicker testa in a *pc* background might also be a way to overcome deleterious properties. The search for thicker seed coats need not be restricted to *pc* types. One could find genotypes with thicker seed coats in other seed type backgrounds then transfer the trait either with backcrossing or CRISPR-CAS9 to create thicker, more durable seed coats.

CHAPTER FOUR: GENERAL CONCLUSIONS

The lower germination rate of *pc* seeds compared with the white- or colored-seeded genotypes in the field, was related to higher numbers of infected seedlings. The question was why is there a tendency for pathogens to preferentially colonize *pc* seeds? One hypothesis was that the syndrome was correlated with the physical and chemical properties of seeds that made the seed coats more fragile and susceptible to disruption of the seed coat either by mechanical or imbibitional injury, leading to solutes leaking into the *pc* seed spermosphere thereby providing a nutrient base for pathogens to sense and colonize the seed and developing seedling.

Our hypothesis was supported by quantifying the anatomical and physical structure of *pc* genotypes. Compared to other seed types, *pc* seeds crack more easily, which was associated with a rapid water uptake. Another issue which we did not investigate in the present study is that increased water uptake from *pc* seeds might trigger cell damage which would depress germination (Larson, 1968; Bewley and Black, 1978; Powell and Matthews, 1978; Hahalis et al., 1996). The seed coat is critical to mitigate the inrush of water into the seeds during the germination and may modulate imbibition by its thickness or due to other structures in the testa (Powell and Matthews, 1978; Powell et al., 1986; Powell et al., 1986). Our anatomical studies revealed a thinner seed coat on *pc* type beans which was associated with more rapid water uptake. Additionally, this thinner condition suggested that the seed coats could be more fragile in *pc* genotypes.

The thinner seed coat may allow solute leakage even in the absence of physical disruption of the seed coat. Of particular interest is that the main thinning of the testa in *pc* types comes about from a thinner osteosclereid layer. The function of this cell layer is not well understood but does appear to be involved in water and solute transport. Fundamental studies of *pc* seeds may be useful in generating new knowledge about the imbibition and germination process.

In any case, pathogens may respond to leaking solutes and grow towards the germinating *pc* seeds. From the electrical conductivity analysis, elevated levels of leaking solutes were observed for *pc* seeds. The comparisons of isogenic pairs were consistent in that the *pc* lines always had greater electrical conductivity than their white -seeded counterparts. One curious point is that comparing the plot distributions of OSU6510-4 and OR91G, OSU6510-4 isogenic pair had an overall lower electrical conductivity compared to the OR91G pair, although the latter had better emergence percentage in the field. This may be related seed shape and/or size as OSU6510-4 has flat and slightly smaller seed than do OR91G genotypes. Paul and Ramaswamy (1979) found a positive correlation between increasing seed size and increasing electrical conductivity from leachate in cowpea. Another aspect is that even if there is a higher solute leakage from OR91G-p^{gri} (colored-seeded) anthocyanins and other water-soluble phenolics may inhibit microbial growth (Cowan, 1999).

We rejected the hypothesis that inherently higher sugar compounds in *pc* types might result in greater solute leakage and pathogen colonization. Instead, white-seeded types had trends towards larger amounts of sugar compounds, excluding fructose. It would have been instructive to examine two other sources of organic molecules that might be attractive to pathogens.

During seed maturation, sucrose is transported into the seed and converted to starches, which are stored in the cotyledons until germination. Starches are then converted back to sugars as germination commences to serve as a carbon source for the growing seedling. It would have been useful to analyze the breakdown of starches to sugars during germination to determine whether these were associated with increases in sugar solute leakage (Frias et al., 2000). The *pc* types might show increased starch conversion and higher amounts of sugars that could potentially move through a more permeable seed coat.

Secondly, nitrogen from proteins might be even more attractive to pathogens than sugars. It has been documented that soil borne pathogens may sense and migrate towards seeds for their nitrogen based resources, which are important to survival and to reproduction (Sterner and Elser, 2002). A potential nitrogen source in *pc* bean seeds could come from the loss of chloroplasts in bleached cotyledons characteristic of *pc* types. Chloroplasts hold about three-quarters of the nitrogen found in a plant leaf (Myers et al., 2018). Normal seeds have green cotyledons during germination and emergence and presumably, the green color is related to chlorophyll and the ability to photosynthesize once cotyledons are exposed to light. It is unclear when *pc* cotyledons lose their green color, but at the time of germination, they are usually white. Within chloroplasts, chlorophyll catabolism is usually an orderly process because some catabolites are strongly reactive and may damage other cellular reactions if not compartmentalized. The bleaching in *pc* cotyledons may not be an orderly process, and may result in cell injury as well as uncontrolled release of nitrogenous compounds. The bleaching process might also affect starch to sugar conversion during germination because chloroplasts accumulate substantial stores of starch during biosynthesis.

A third possibility for why soil borne infections rates are higher in *pc* seed is that they may have a seed microbiome that is different from other seed types. The OSU Clinic reported the *pc* seeds are infected with *Fusarium* and/or *Rhizoctonia* spp. and symptoms were mainly seen on cotyledons and hypocotyl sections, as well as on ungerminated seeds. Investigating the role of endogenous and exogenous microbes associated with bean seeds and their vertical transmission in *pc* types might reveal different communities and differences in ability to compete with pathogens (Shahzad et al., 2018).

The use of a molecular marker confirmed that lines which differed in cotyledon color in a *pc* background remained *pc* genotypes. We do not know what causes cotyledon bleaching of *pc*

lines, but these materials can provide a tool for answering that question, as well as studying whether *pc* genotypes with green cotyledons have better germination and emergence rates. Interestingly, one of the cultivars that is used commercially (Medinah) has been observed to regularly have mixed cotyledon color (Myers, personal communication), and this mix might provide better field performance. Thus, a further genetic examination of the *pc* allele combined with examination of cotyledon color might be a way to capture some additional information.

We observed macro cracking during water uptake experiments, and by observation of seed wrinkling during early stages of imbibition in the seed drop experiment, were able to infer that minute cracks were present. It would have been helpful to use existing chemical analyses (Kaupp and Kaupp, 2007) to quantify seed coat integrity. Future investigation into initial pressure and changes in pressure once seed starts to imbibe water could be instructive. In addition, such tests using OSU6523-*p pc* might shed light on the hard seed phenomenon.

All in all, the cosmetic stay-green trait in snap bean and the event cascade that reduces seed germination and emergence rate may be modified by increasing seed coat thickness of *pc* genotypes. We do not know of any *pc* types that have thicker seed coats, but we have not made a systematic examination of the trait. This is something that could be done using USDA-NPGS *Phaseolus* germplasm collection, with particular emphasis on screening the dry bean flageolet and/or haricot vert types. Interestingly, there are no reports on the reduced germination of these types. Overcoming this problem will make the *pc* seeds more desirable to breeders and facilitate their incorporation into improved snap bean cultivars.

Beyond genetic modification of *pc* snap beans is changing cultural practices to prevent seed injury. Processes for harvesting and conditioning seeds could be altered to minimize damage such as using a belt thresher during harvest. Seed moisture content both at harvest and in storage

is another issue. Harvesting the seeds at approximately 12% moisture content is important for reducing mechanical injury (Dickson and Boettger, 1976; Taylor and Dickson, 1987). This can be a problem in Idaho where the majority of snap bean seed is produced because seed moistures may drop into the single digits at harvest. Some growers with particularly sensitive cultivars may wait to combine seed until evening when diurnal humidity increases (Myers, personal communication). Storage conditions are another issue associated with seed moisture content and seed quality. For instance, using cold airflow cabinets at 20°C and 35% relative humidity will keep the seed optimum moisture content (Salcedo, 2008). Reducing seed cracking during the water imbibition could be accomplished by pre-irrigating the field before planting so that the seeds will not be watered up in dry soil. Another potential solution to reducing rate of water uptake and cracking could be the pelleting of the seeds. Currently, coatings on bean seeds are not used commercially, but these can impose a physical barrier to water and oxygen diffusion and regulate the rate of imbibition (Sachs et al., 1981; Sachs et al., 1982). One other possible option could be the use of primed or pre-germinated seed. Priming of seeds can reduce the exposure to field pathogens improving overall survival rates (Parera and Cantliffe, 1991).

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APPENDIX:

A. BACKCROSS BREEDING TO CREATE AN ISOGENIC PERSISTENT COLOR BEAN LINE

1. INTRODUCTION

Understanding the effects of *persistent color* (*pc*) in different genetic backgrounds is important in characterizing this trait. It is controlled by a single recessive gene *pc.* The objective of this project was to transfer the trait to white- and colored-seeded type plants that carry the dominant allele at the *pc* locus. The transfer was implemented through backcrossing to colored-seeded and white-seeded cultivars in the greenhouse.

Snap bean has a perfect flower and has an annual flowering habit. It is highly self-pollinated crops due to cleistogamy because the enclosure of the stigma and stamens within the closed keel. Thus, the emasculation was necessary procedure to guarantee backcrosses.

2. MATERIAL AND METHODS

The donor plant to transfer the *pcpc* allele was 'Pascal', a haricot vert or flageolet cultivar and the recurrent plants were OR-91G-p for white-seed and OR91g-p^{gri} for a colored-seed background. Nine seeds per genotype were divided into three pots, then planted approximately 1-2 inches deep in soil at greenhouse on November 04, 2017. Afterwards, each pot was fertilized and watered. An additional 9 seeds of the recurrent parent were planted a week before and after this date to provide a longer crossing period.

After seeding and when cotyledons became observable above the soil, the cotyledon color for each plant were observed and recorded. After a couple of weeks, once the plants reached climbing ability, staking using trellis has been supported to each plant.

Once the recurrent plants began flowering, the flowers of the donor parent were emasculated using fine tipped forceps pollinated with the pollen from recurrent plants. Each cross was tagged. To increase the crossing the retention of rate of the bean hybrids, crossing in the mornings (7am-11am) was preferred.

Once the pods are mature, plants were no longer watered, and plants were maintained in greenhouse until pods senesced. Dry pods were harvested by hand and placed in labelled paper bags. Soon after, pods were split by hand and seeds were transferred to labeled seed envelopes. All harvest procedures were conducted by hand to make sure the seeds did not receive any mechanical damage.

During these experiments, seeds untreated with fungicides were used and the *pc* type cultivar, '*pcpc*' gene, was crossed with OR91G-p^{gri} (colored-seeded) and OR91G-p (white-seeded) plants to obtain F1 crosses, which are heterozygous (*Pcpc*), then according the method of "transferring recessive gene by backcrosses" which were presented in **Figure 23**, the backcross procedure was followed by the diagram.

Once the BC3F1 is obtained, the plants leaf will be examined by the molecular marker by the similar procedure which was used in Single Plant Molecular Marker Study (section, 2.3.e).
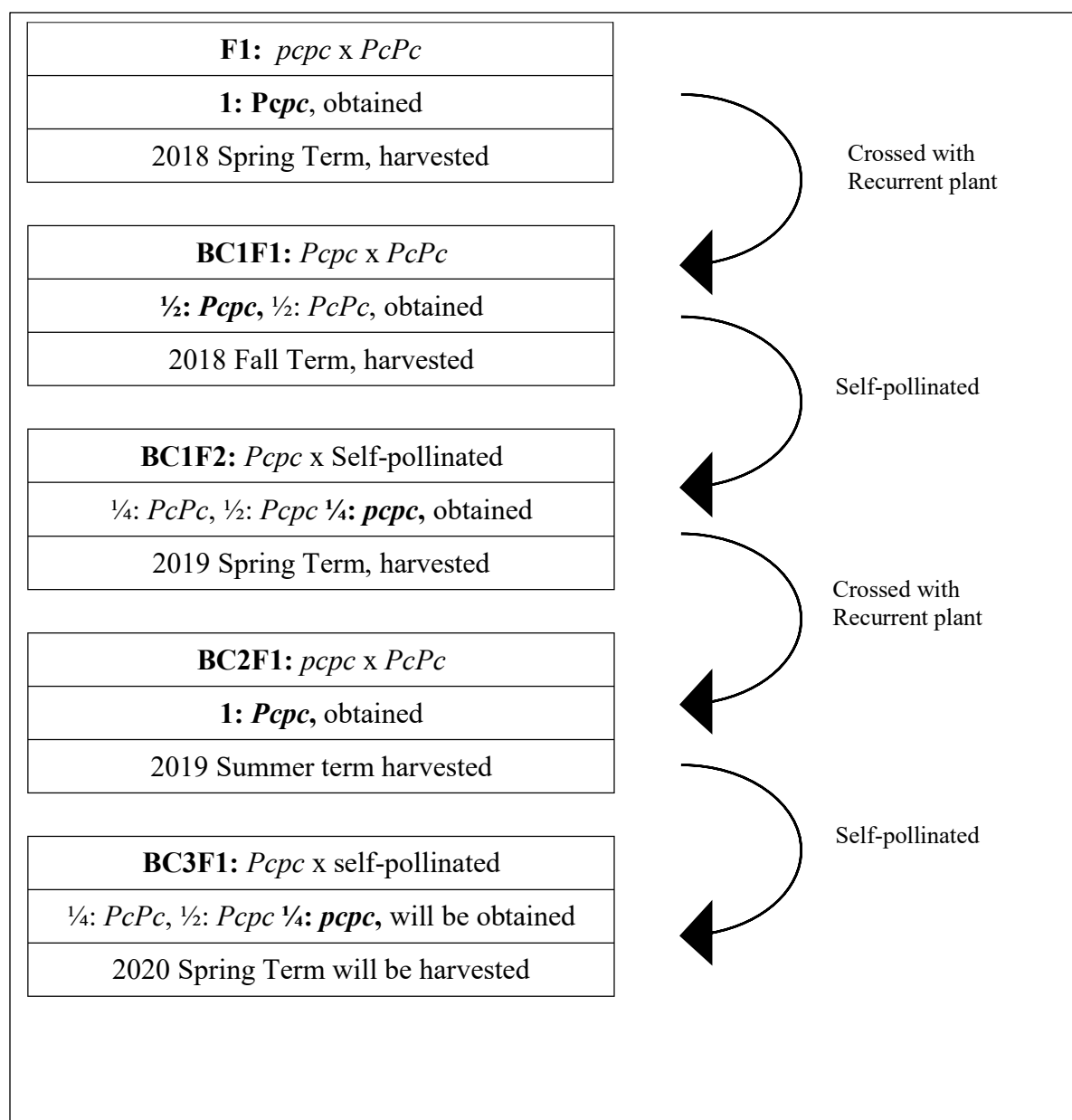
F1: *pcpc* x *PcPc*
1: Pc*pc*, obtained
2018 Spring Term, harvested

Crossed with
Recurrent plant

BC1F1: *Pcpc* x *PcPc*
½: ***Pcpc*,** ½: *PcPc*, obtained
2018 Fall Term, harvested

Self-pollinated

BC1F2: *Pcpc* x Self-pollinated
¼: *PcPc*, ½: *Pcpc* **¼: *pcpc*,** obtained
2019 Spring Term, harvested

Crossed with
Recurrent plant

BC2F1: *pcpc* x *PcPc*
1: *Pcpc*, obtained
2019 Summer term harvested

Self-pollinated

BC3F1: *Pcpc* x self-pollinated
¼: *PcPc*, ½: *Pcpc* **¼: *pcpc*,** will be obtained
2020 Spring Term will be harvested

Figure 23: Diagram showing steps involved in the backcross method of self-pollinated bean plants. The dominant *Pc* trait is transferred from variety *PcPc* which is the recurrent plant, to an otherwise improved variety will have *pcpc* trait.