Light Effect on Seed Chlorophyll Content and Germination Performance of Tomato and Muskmelon Seeds

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Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science

In

Horticulture

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June 20th, 2008

Blacksburg, Virginia

Keywords: (muskmelon, tomato, chlorophyll, phytochrome, light, germination, vigor)

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ABSTRACT

The stage of maturity of seeds at harvest is an important factor that determines seed vigor. Separating seeds from a seed lot composed of many different stages of development can be difficult especially after maximum dry mass is attained. Separating seeds based on their physiological maturity is more challenging than sorting seeds based on their physical properties. Seeds may be non-destructively sorted using chlorophyll fluorescence (CF) as a marker of seed maturity. This study was conducted to test whether CF could be used to remove low vigor immature seeds from muskmelon (*Cucumis melo* L.'Top Mark') and tomato (*Lycopersicum esculentum*) seed lots. Light treatments were applied to determine whether the light environment during seed harvesting and processing could affect chlorophyll content and seed vigor. Seeds from nine stages of development were collected from 'TopMark'. Seeds from three stages of fruit development (red ripe, breaker, and mature green) were harvested from tomato cultivar Money Maker and two phytochrome mutants: phytochrome A mutant, *fri*$^{-1}$ and phytochrome B mutant, *tri*$^{-1}$. The SeedMaster Analyzer (Satake USA Inc., Houston Texas) was used to measure CF and to sort individual seeds according to CF levels. Immature tomato seeds and muskmelon, harvested from green fruits, had the highest CF

($p>0.001$). Contrary to the results obtained with the other tomato genotypes, the vigor of *tri*$^{-1}$ did not change inversely with changing CF levels, rather, seeds with low CF had the same vigor as seeds with high CF. This result may suggest that the presence of phytochrome B exerts an inhibitory influence on vigor in tomato seeds, and that the persistent presence of chlorophyll during seed development does not affect vigor. The light treatments had no consistent effect on seed chlorophyll content or on vigor in either tomato or muskmelon.

Table of Contents

List of Figures

List of Tables

Chapter 1: LITERATURE REVIEW

1.1 Seed Development and Seed Performance (germination and vigor)

Seeds are very important for agriculture, since most food and fiber crops are grown from seeds. To a seed biologist, germination is complete at radicle emergence; but, to a seed technologist, germination tests determine whether a seed is able to produce a normal seedling (Desai et al., 1997). The Association of Official Seed Analysts (AOSA) develops seed testing standards in the United States, and the seeds sold in the US must meet standards for labeling, trueness to type, purity, and germination (McDonald and Copeland, 1997). The government sets germination standards, and seeds sold in the US must meet the germination standards specified in the Federal Seed Act (AOSA, 2008).

Germination can be defined as "the emergence and development from the seed embryo of those essential structures, which for the kinds of seeds in question, are indicative of the ability to produce a normal plant under favorable conditions" (AOSA, 2008). By AOSA standards, seeds are scored as normal, abnormal or dead after a predetermined incubation period under optimum moisture and light. Most AOSA tests are laboratory-based using artificial media such as paper towels or special germination blotter paper. Seeds are tested at ideal temperatures that favor seed germination and hardly resemble the harsh field conditions in terms of both biotic and abiotic stresses that seeds often encounter. The AOSA germination tests are conducted in accordance with the guidelines established in the AOSA Germination Testing Handbook. It outlines not only the duration of the test, temperature range, and the light cycle, but also the moisture level of the germination media. The standardization of testing procedures aids the uniformity

of results and reproducibility of experiments (AOSA, 2008). Germinability of seeds is measured in terms of percentage only, and the vigor or "quality" of germination is not further evaluated among normal seedlings.

Seed vigor test information is important to seed companies in a variety of ways. Agriculturalists today want seeds that germinate rapidly under stressful conditions; that is the main reason why there are so many kinds of vigor testing. When faced with choosing between a seed lot of a cultivar that germinates in 10 days and a seed lot that requires 15 days, most growers want seeds that germinate faster to shorten production and to limit seedling predation and disease. However, seed vigor testing is not mandated under the Federal Seed Act and thus not required by law.

Seeds may be germinated in polyethylene glycol (PEG) solutions to simulate drought conditions, or they may be germinated under high or low temperature extremes to simulate thermal stress. Vigor tests can be used to monitor seed quality through every seed production phase from harvesting through, conditioning, bagging, storage, and planting. They enable adverse practices to be readily detected and corrective action taken. In some cases, such information can identify additional measures needed to improve germination performance, such as seed treatments (e.g., fungicides or seed enhancements), since seed vigor generally declines before viability is lost (Welbaum, 1999). Seed vigor tests also are used in inventory management. Vigor tests, such as accelerated aging or controlled deterioration, can identify which seed lots are most likely to retain quality during long-term storage. They also assist seed companies in establishing minimal vigor levels for marketed seeds. These important attributes have resulted in rapid

acceptance of seed vigor tests as essential quality control measures by the seed industry (AOSA, 2008).

The AOSA tests are primarily destructive tests, in which seeds are grown out during germination tests to determine the germinability and/or vigor of the seed. These grow-out tests are time consuming, labor intensive, and expensive to conduct. The mean germinability and vigor of the seed lot is often not an accurate indication of the range of seed performance in a lot. This is because each seed is a unique member of a diverse population of individuals with wide ranging germination performance (AOSA, 2008).

1.2 Factors affecting seed germination

Seeds do not usually germinate precociously during development on the mother plant, but undergo a process of maturation, generally including drying, before being dispersed from the mother plant. This drying period is not required for germination in many species, as the embryo is capable of germinating during an early phase of seed maturation. In most species, the time required for germination is far longer for very young embryos, than those that are fully matured. Seeds of muskmelon and tomato, which develop in a fully hydrated environment inside fleshy fruits, are able to germinate without maturation drying. These seeds will germinate when incubated in water after removal from developing fruit, even if the seed has not reached its maximum dry weight (Bewley and Black, 1985). Welbaum and Bradford (1989) reported that, at 25 to 30 days after anthesis (DAA), only 40% of epicotyls and roots from isolated muskmelon embryos of 'Top Mark' were competent to grow on water. By 35 DAA, at the time of maximum dry weight accumulation, all embryonic tissues grew and normal seedlings developed but

only when the seed coat and endosperm were removed. At this stage of development, the germination percent of intact seeds was less then 50%. Germination percentages greater than 95% were not observed until 45 DAA. Seeds remained 95% viable until 60 DAA when germination percentages declined (Welbaum, 1999). Maximum vigor in muskmelon seeds developed more slowly than germinability but remained high until 65 DAA when the germination percent declined (Welbaum, 1999).

There are numerous theories about how seed maturity affects germination performance in seeds. Two major hypothesis have been proposed by Nonogaki (2006) as mechanisms responsible for the initiation of radicle emergence. The first hypothesis states that the physical barrier composed of the testa and the endosperm restrict radicle growth and thus germination. In this hypothesis, the embryo is unable to germinate in an intact seed because it is not developed enough to overcome the barriers such as the testa and the endosperm. However when the barriers are removed, the young embryo is able to germinate. Gibberellic acid (GA) increases growth potential of the embryo by inducing production of cellulases that cause cell wall hydrolysis, which triggers radicle emergence (Desai, 2004). GA synthesis is under phytochrome control. Yamaguchi et al. (1998) reported that for *Arabidopsis thaliana* and lettuce seeds, gene expression of GA 3β-hydroxylase, an enzyme that catalyzes the final step of GA biosynthesis, is controlled by phytochrome. GA-deficient mutants of 'MoneyMaker' do not germinate in any environment, demonstrating the importance of GA in promoting tomato seed germination (Nonogaki, 2006). Germination also occurs due to the weakening of covering tissue such as endosperm (Nonogaki, 2006). Endosperm weakening is a well-known phenomenon in lettuce, pepper, tomato and tobacco seeds, and one of the enzymes involved in endosperm

rupture is endo-ß-mannanase. Nonogaki et al. (1995, 1998, 1996, 2000) have reported that endo-ß-mannanase levels increased in the micropylar endosperm of tomato seeds prior to radicle emergence. The increase in endo-ß-mannanase is strongly related to the increased vigor and viability in tomato (Nonogaki et al., 1995, 1998, 1996, 2000) and muskmelon (Welbaum, 1999) as the seeds mature. The existence of cross-talk between phytochrome and endo-ß-mannanase is still an open question.

The second hypothesis states that germinability of a seed is a factor of the maturity of the seeds. Immature seeds have low germinability because they have something lacking in the germination mechanism that facilitates the process. The maturation processes occurring in various seed tissues -seed coat, endosperm and embryo- contribute to seed germinability and vigor (Welbaum et al., 1998). During the maturation phase of seed development the accumulation of various compounds such as oil, storage proteins and starch in the various tissues of the seed occurs (Ruuska et al., 2002). Genetic analysis in Arabidopsis have demonstrated that factors such as accumulation of seed storage proteins, chlorophyll degradation in mature seeds, and desiccation tolerance are transcriptionally regulated. LEAFY COTYLEDON genes (LEC1, LEC2 and FUS 3) and ABSCISIC ACID INSENSITIVE genes (ABI3 and ABI5) are a few of the known key transcriptional regulators of seed maturation (Giraudat et al., 1992; Lotan et al., 1998; Meinke et al., 1994 and Baumlein et al., 1994).

1.3 Factors Affecting Seed Development

The response to abiotic factors during seed development is diverse and complex. Stresses such as water deficits, low or high temperature, nutrient deprivation, and shading

can occur at any time during seed development. Water stress during grain development in cereals and maize decreased the number of inflorescences, reducing in the total number of grains formed (Desai et al., 1997). Cell division and enlargement of the embryo after fertilization determined the size and storage capacity of the grain and both can be adversely affected by water and heat stress (Bewley and Black, 1985). Water stress during the early stages of soybean seed development decreased the number of pods per plant and increased the incidence of abortion and abscission (Desai et al., 1997).

The intensity of dormancy in some species is regulated by light. Some *Brassica* crops exposed to long photoperiods produce seeds that require greater light intensity for germination. In some legumes, seeds matured under long day conditions remain in the pod longer and develop thicker and more impermeable seed coats (McDonald and Copeland, 1997). The amount and quality of light exposed to the parent plant has been known to affect the size and the germination performance of the seed (Copeland and McDonald, 2001). Reduced light to the parent plant resulted in smaller seeds in carrot, pea, and soybean due to a reduced rate of photosynthesis (Janick, 1992). The quality and amount of light (red light vs. far-red light) intercepted by the leaf nearest the fruit are known to affect the amount of phytochrome in seeds, which in turn affects the germination percent of the seed (Oluoch and Welbaum, 1996; Casal and Sanchez, 1998).

There are many factors that appear to affect seed-to-seed variability: stage of maturity at seed harvest, pollen load, position of the seed in the fruit, health of the mother plant, fruit position on the mother plant and numerous environmental factors. Variation in performance of commercial lots of muskmelon seeds has been attributed to combining fruits from different stages of development (Oluoch and Welbaum, 1996). Commercial

muskmelon seeds grown primarily in California are open pollinated and harvested in bulk by large harvesters to minimize labor.

Seed quality has been linked to, among things, developmental factors on the maternal plant. Stage of maturity at harvest is an important factor associated with seed quality. The optimal time, or maturity stage, for fruit harvest and seed processing depends on the species and on environmental conditions. Therefore, combining seeds from different fruits increases seed-to-seed variability, even if they are all from the same stage of development. Due to these seed harvesting practices, separating seeds from different stages of development and performance is often difficult, since low vigor seeds may have the same physical characteristics and weight as mature seeds (Oluoch and Welbaum, 1996).

1.4 Seed Sorting Methods

There are two general methods for separating seeds according to seed performance: destructive methods in which seeds are germinated to determine germination percent and vigor, and non-destructive methods in which poorer seeds are sorted out of the lot according to some physical property. The most common non-destructive method is air-screening, which removes the light weight seeds and separates seeds based on their density (McDonald and Copeland, 1997). Abortions often occur in tomatoes and muskmelons, but these seeds are relatively easy to separate by conventional conditioning technologies like air screening. However the air-screen machine can only separate seeds based on their physical characteristics and not on germination performance (McDonald and Copeland, 1997).

Seed lots are often sized and separated according to their length. This technique is valuable for removing weed seeds, cross-broken crop seeds that are shorter than the desirable crop seed, and inert materials longer then the crop seed. Length sizing is done to create a more uniform seed lot to facilitate singulation during planting.

Density grading is used to separate undesirable seeds or inert material that are of similar size, shape, and seed coat characteristics compared to the crop seed. Empty seeds, insect-infected or moldy seeds have the same dimensions as the desired crop seed, however they are lighter in weight (McDonald and Copeland, 1997). These lightweight seeds are low in quality and thus must be removed from the desired seed lot. There are indications that germination rate, or the speed at which the seeds germinate, is related to seed size and weight (Whittington and Fierlinger, 1972; Nieuwhof et al., 1989). Mature seeds generally have greater weight, germination percentage, and vigor compared to immature seeds (Bewley and Black, 1985).

A fractionating aspirator, is one of the most common machines used to separate crop seeds according to weight/density. Seed is metered into the aspirator and then into a rising column of air; the heaviest seeds fall against the air flow, and the remaining mixture of light material is separated gradually by reducing the velocity of the air (McDonald and Copeland, 1997). Other non-destructive sorting methods include electrostatic separation, which is sometimes used to sort wheat seeds. The seeds are charged by ion bombardment using a corona discharge in the separator. The broken and complete seeds are charged selectively according to their surface area. The mass of the broken seeds is relatively smaller than that of intact seeds. Therefore, separation of

broken seeds from intact ones is achievable because of differences in their charge-to-mass ratios (M. Abdel-Salam et al., 2004).

1.5 Chlorophyll and Seed Vigor

Another effective non-destructive method used to remove low vigor seeds from a mixed seed lot is chlorophyll fluorescence -which measures the chlorophyll accumulation in seed coat- as a measure of seed maturity. Steckel et al. (1988) have studied the negative correlation between seed maturity and chlorophyll content. They reported that the percent germination of carrot seeds ('Chantenay' and 'Amsterdam') were negatively and linearly related to seed moisture content, chlorophyll A and B content in the seed, and seed distortion (Steckel et al., 1988). The relation between the amount of chlorophyll and maturity has been well studied in seeds of oilseed (*Brassica napus* L.) and turnip rape (*Brassica rapa* L.) (Ward et al., 1992, 1995), where the 'green-seed problem' causes major damage to seed oil quality. Low temperature during the growing season can halt maturation at an early developmental stage, preventing the seeds from reaching full maturity. This causes a green appearance of the seeds due to high chlorophyll content. Green seeds increase the amount of chlorophyll in the extracted oil, which is undesirable not only because of the color but also because of the possible promotion of off-flavor by rancidness (Ward et al. 1992, 1994).

Jalink et al. (1996) developed a non-invasive and non-destructive seed sorting technique that measures the relative chlorophyll content in the outer layers of seeds using chlorophyll fluorescence (CF). A Laser Induced Fluorescence (LIF) technique was introduced as a high-speed sorting method to detect differences in quality by evaluating

the magnitude of the CF signal in individual seeds (Jalink et al., 1999). The study, done with *Brassica oleracea*, showed that the magnitude of the CF signal was inversely related to the quality of the seeds as measured by germination percentage, rate, and other indices of germination performance. For example, the germinability of a tomato seed lot was improved from 90 to 97% normal seedlings, by sorting out 13% of the seeds with very high CF (Jalink et al., 1998).

In tomato, seed position within the fruit along with fruit position on the plant affects the germinability of seeds (Heuvelink, 2005; Jalink et al., 1999). Studies by Suhartanto (2003) showed that higher tomato seed quality, as measured by percent germination, percentage of normal seedlings, germination rate, and uniformity, was achieved when the CF in developing seeds leveled-off. Large standard deviations in seed CF existed among seeds within the same fruit. The standard deviation of CF became smaller when seeds attained maximum quality. This explains the seed-to-seed variation in germination performance often observed among seeds within a single fruit. Using fluorescence microscopy, Suhartanto (2003) showed that, in tomato seeds, most of the chlorophyll was located in the seed coat, and smaller amounts were detected in the embryo as well. The study also demonstrated that chlorophyll in tomato seeds is photosynthetically active. Laboratory analysis showed photosynthetic oxygen evolution and Rubisco activity in young tomato seeds (Suhartanto, 2003). The photosynthate produced by seed chlorophyll accounted for almost 40% of biomass accumulated during rapeseed development. Assimilate accumulation was stimulated by light, as higher light intensity increased photosynthetic rates. Light not only improved the efficiency of carbon assimilation, but also increased the growth rate, and fixation of CO_2 into seed storage

reserves (Goffman et al., 2005). Chlorophyll plays an important role in the accumulation of biomass in seeds containing a high amount of fatty acid such as oilseed and canola seeds (Goffman et al., 2005).

1.6 Chlorophyll Biosynthesis and Phytochrome

For many years it was believed that in angiosperms, chlorophyll biosynthesis was under strict photocontrol and no chlorophyll could be formed in the dark (Suty, 1983). However, recent studies by Raskin and Schwartz (2003) proposed a light-independent pathway for chlorophyll biosynthesis.

The reduction of protochlorophyllide to chlorophyllide is a key step in chlorophyll biosynthesis. In the light-dependent pathway, active complex of NADPH and protochlorophyllide oxidoreductase catalyzes protochlorophyllide reduction to chlorophyllide (Beer and Griffiths, 1981). Light is critical to excite the protochlorophyllide to trigger the reaction sequence of protochlorophyllide reduction (Raskin and Schwartz, 2001).

In the new light-independent pathway proposed by Raskin and Schwartz (2003), the protochlorophyllide reduction occurs by an "enzymatically generated electronically excited state". In the absence of light, protochlorophyllide reduction is achieved via protochlorophyllide oxidoreductase by using chemical energy generated in the enzymatic ascorbate peroxidase system (Raskin and Schwartz, 2003). This allows the plant to bypass the light requirement for chlorophyll biosynthesis and allows for the protochlorophyllide reduction to occur even in the absence of light.

In higher plants, accumulation of chlorophyll after this phototransformation starts only after a lag phase, indicating the requirement for regeneration of protochlorophyllide. In mustard seedlings, physiologically active phytochrome (Pfr) increases the capacity for the formation of protochlorophyllide. Moreover, illumination of seedlings with far-red light eliminates the lag phase for the regeneration of protochlorophyllide, increasing the rate of chlorophyll accumulation (Rau and Schrott, 1979; Mohr et al., 1974).

Subsequent investigation with mustard seedlings provided evidence that Pfr regulates the synthesis of δ-aminolevulinic acid (ALA), a very early precursor of chlorophyll synthesis (Masoner and Kasemir, 1975). Since there is no evidence that subsequent enzyme activity in the pathway is correlated with light, light-mediated chlorophyll accumulation is thought to be under the regulation of the level of phytochrome-controlled formation of ALA (Masoner and Kasemir, 1975; Ilag, 1994). The *aurea* (au) and *yellow-green-2* (yg-2) mutants of tomato are unable to synthesize the linear tetrapyrrole chromophore of phytochrome, resulting in plants with a pale, yellow phenotype. Dark grown seedlings of both mutants have reduced levels of protochlorophyllide (chlorophyll precursor) due to an inhibition of protochlorophyllide synthesis at the ALA synthesis level (Terry and Kendrick, 1999). Chlorophyll synthesis is closely associated with phytochrome synthesis, since ALA, a chlorophyll precursor, is regulated by the photoreception of phytochrome. The phenotype of phytochrome-deficient mutants such as *au* and *yg-2* is chlorophyll-deficiency (Terry and Kendrick, 1999).

Phytochrome controls seed germination at many levels, not only by its influence on chlorophyll biosynthesis but also by its function as a photoreceptor. The control of

seed germination by red and far-red light is one of the earliest documented phytochrome-mediated processes. Phytochrome is one of the most studied photoreceptors, a small family of chromoproteins with molecular mass of 120-130 kDa (Furuya, 1993). The chromophore is a linear tetrapyrrole, which is common to all phytochrome apoproteins. A different gene encodes the apoprotein of each phytochrome. At the moment there are five different phytochrome genes known in *Arabidopsis thaliana* and in tomato. The total amount of phytochrome at any given time depends on the balance between synthesis and degradation, and the rates are different for different phytochromes. The P_f form is synthesized de novo in the dark and accumulated in plant cells. When dark-grown plants are exposed to red light, P_r is converted to P_{fr}, which in turn leads to physiological actions such as flower induction and seed germination (Furuya, 1993; Bewley and Black, 1985).

The absorption of the two photo-interconvertible forms of phytochrome, red-light-absorbing form (P_r) and far-red-light-absorbing form (P_{fr}), are dependent upon the presence of both wavelengths as long as the signal is not distorted by pigments that absorb in the same spectral region (Casal and Sanchez, 1998). Absorption maxima for P_r and P_{fr} are 660 and 730 nm, respectively; but the considerable overlap between the absorption spectra for the two forms produces a mixture of P_r and P_{fr}.

Phytochrome A (phyA) is the most abundant phytochrome in etiolated seedlings but undergoes rapid degradation when exposed to light (Casal and Sanchez, 1998). Phytochrome B (phyB) and C (phyC) are synthesized at lower rates then phyA; however, they are more stable in the P_{fr} form (Somers et al., 1991). Based on the stability in the P_{fr} form, phytochromes are grouped in type I (phyA) and type II (phyB, phyC, phytochrome D, phytochrome E). Both phyA and phyB can dark revert to Pr (Kunkel et al., 1996).

Phytochrome B is present in dry seeds and affects germination of dark-imbibed seeds (Casal and Sanchez, 1998). Dry seeds show little photoreversible absorption changes attributable to phytochrome. During imbibition there is a rapid increase in phytochrome signal due to rehydration of inactive phytochrome (P_r). It is widely believed that phytochrome is synthesized in developing seeds; and the form in which these phytochromes are found is largely dependent on the growing environment of the mother plant (Casal and Sanchez, 1998). Phytochromes in seeds can dark revert from P_{fr} to P_r, and this process reduces the P_{fr} levels in buried seeds. Exposure of seeds to far-red light where phytochrome was largely in the P_r form can be followed by the re-appearance of P_{fr} in the dark. This re-appearance of Pf_r is known to be due to the formation of P_{fr} from intermediates (Casal and Sanchez, 1998).

The effect of light on seed quality is tightly correlated with dormancy induction in some species. Seeds of *Arabidopsis thaliana* maturing in white fluorescent light have little dormancy when harvested, whereas those that have been exposed to incandescent light remain deeply dormant for at least several months. The difference in dormancy level is due to the difference in composition of light between white fluorescent light, which contains high level of red light and incandescent light, which contains high level of far-red light. Seeds with high P_{fr} content can germinate in darkness while those with low P_{fr} concentration remains dormant. Treatment with far-red light alone has the same effect, where some Cucurbitaceae seeds are made dormant when seeds are irradiated with far-red light (Bewley and Black, 1985). This kind of phenomenon occurs in nature, when the source of far-red light is filtered though green tissues. Chlorophyll absorbs red light (660 nm) but does not absorb light with wavelengths longer then 710 nm, the far-red light. The

transmitted light contains a high level of far-red light, which serves to lower the amount of P_{fr} in the seed. Seeds of many species mature and dry while the surrounding maternal tissues are still green, and thus the embryos are in far-red rich environment. Such seeds are more dormant compared to those whose surrounding tissue has a low amount of chlorophyll (Bewley and Black, 1985).

Many seeds germinate when incubated in absolute darkness, including muskmelon seeds. This is due to the presence of P_{fr} in seeds (Bewley and Black, 1985). Cresswell and Grime (1981) in a report on various vegetations prevalent in Britain, showed a negative relationship between germination in darkness and chlorophyll content of extra-embryonic tissues. Seeds that developed within tissues that retained chlorophyll had most of their phytochrome in the P_r form, which is opposite to what was believed prior to the study. Phytochrome B present in the seeds in the P_{fr} form is important for germination of dark-imbibed seeds, allowing for phytochrome B overexpressing mutants to germinate well in darkness. However if the mother plant is grown under low red/far-red ratios the amount of phytochrome B P_{fr} was reduced (McCormac et al., 1993).

Phytochrome affects germination through its signaling action on the two light absorbing forms (P_f and P_{fr}). Phytochrome also affects the chlorophyll biosynthesis pathway as it controls the formation of the chlorophyll precursor, ALA. Chlorophyll is important in young seeds due to its function in biomass accumulation. However as the seeds reach physiological maturity and enter into dormancy, chlorophyll was degraded from the seed. Seed physiologists believed for many years that the photosynthetic apparatus had to be disassembled, and chlorophyll degraded, to prevent oxidative stress caused by free radicals.

1.7 Chlorophyll degradation

Light intensity and day length influence seed chlorophyll content and thus the CF. Suhartanto (2003) showed that chlorophyll degradation in tomato seeds harvested from small-fruit tomato cultivars was faster than in seeds harvested from large-fruit tomato cultivars. Smaller fruits have thinner pericarps than larger fruits, allowing light to penetrate to the seeds and accelerate chlorophyll degradation (Suhartanto, 2003). Indeed light along with high temperature accelerates degreening in oilseeds (Ward et al., 1994).

The biochemical pathway of chlorophyll degradation during senescence and fruit ripening leads to an accumulation of colorless products within the vacuole. Chlorophyll degradation removes pigments within the vacuoles in order to allow nitrogen remobilization from chlorophyll-binding proteins to proceed during fruit ripening and senescence (Hortensteiner, 2006).

The chlorophyll degradation pathway can be divided into early steps that are common to all plants, followed by species-specific modification of chlorophyll breakdown products. Four enzymes are required for the degradation of chlorophyll to a colorless, intermediate nonfluorescent chlorophyll catabolite (Krautler et al., 1991). Chlorophyll is first dephytylated to chlorophyllide by chlorophyllase (Hostensteiner, 2006). The loss of green color in canola and mustard seeds has been attributed to the chlorophyllase activity (Jonhson-Flanagan and Spencer 1994, 1996). After the dephytylation by chlorophyllase, a metal chelating substance removes the core Mg. The product, pheophorbide A is then converted to pFCC in a two-step reaction by pheide A oxygenase and red chlorophyll catabolite reductase. The pFCC undergoes several

modifications before it is stored inside the vacuole as a nonfluorescent chlorophyll catabolite (Hortensteiner, 2006).

1.8 The Objectives of this Study

One objective of this study was to investigate if chlorophyll and CF vary with seed maturity in muskmelon seeds, as suggested by Jalink et al. (1998, 1999). Another objective was to assess the use of CF-assisted seed sorting as a tool to improve the overall vigor and germination percent of seed lots by removing poor performing, immature seeds that contain high chlorophyll. Another goal was to determine if chlorophyll developed within the dark fruit or formed during exposure to light during harvesting and processing. Another objective was to determine if chlorophyll synthesis and accumulation in developing seeds is mediated by phytochrome. The applied goal of this project was to determine if post-harvest light environment could be used to increase seed vigor and germination percent in muskmelon and tomato seeds.

Four hypotheses were tested.

1. Laser-induced fluorescence, which measures CF, allows sorting out of immature, high chlorophyll seeds from muskmelon seed lots and thus improves the overall performance of the seed lot.

2. Dark-harvested muskmelon and tomato seeds that were not exposed to light have lower chlorophyll content, and a higher vigor and germination percent compared to those exposed to light.

3. Chlorophyll accumulation in developing tomato seeds is mediated by seed phytochrome B. Tomato mutants that lack phytochrome B contain less chlorophyll in their seeds and these seeds have higher vigor and germinability.

4. Post-harvest light environment alters the amount of P_{fr} in seeds, lowers the amount of chlorophyll accumulation in seeds, and increases seed vigor.

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Chapter 2: The affect of post-harvest light treatment on muskmelon seed performance and CF.

2.1 INTRODUCTION:

Seed qualities such as viability and vigor have been linked to developmental factors of the maternal plant. The stage of seed maturity at harvest and seed processing after harvest varies with species and environmental conditions. The effects of maturity on seed quality have been extensively studied in muskmelon (*Cucumis melo* L.). The best time for seed harvest lies anywhere from 35 to 60 days after anthesis (DAA) (Edelstein and Nerson, 2005). However, for the cultivar 'Top Mark' grown in California, the optimum stage for seed vigor was 50 to 60 DAA. At these stages of development, seeds germinated more rapidly, at lower water potentials and lower temperatures, and the storage life and vigor after storage was better than at earlier stages of development (Oluoch and Welbaum, 1996). Harvesting muskmelon fruits earlier than 35 DAA is detrimental to seed quality, as the germination percentage of intact seeds was less than 50%, while at 45 DAA, germination percent rapidly increased to greater than 95% (Welbaum, 1999).

Seeds produced in fleshy fruits such as pepper, tomato, and melon, remain hydrated after reaching maximum dry weight, and achieve physiological maturity before fruit development is complete. (Berry and Bewley, 1992; Welbaum, 1999). In some species such as muskmelon, seeds develop in a large fruit and are not exposed to direct sunlight, especially during the later stages of development. Light is required for phytochrome biosynthesis, and phytochrome regulates chlorophyll biosynthesis (Terry and Kendrick, 1999). Chlorophyll levels, forming early in seed development but

degraded as seeds mature, have been shown in oilseed crops to be correlated with immature and poorer quality seeds (Ward et al., 1992). In carrots (Steckel et al., 1989), canola (Cenkowski et al., 1989), and oilseed rape (Ward et al., 1992), strong negative correlations between seed germinability and chlorophyll content have been reported. However, it is still uncertain whether chlorophyll is the cause for the low germination percent, or just an indication of immaturity. Chlorophyll degrades quickly as seeds mature, and by the time seeds reach physiological maturity most of the chlorophyll has been completely degraded (Suhartanto, 2003). High light intensity (Suhartanto, 2003) and high temperatures (Ward et al., 1994) increase the rate of chlorophyll degradation in some seeds.

Seed-to-seed variation in vigor within a seed lot is a common problem in many crops. Variation in performance of cucurbit seeds has been attributed to combining fruits from different stages of development from open-pollinated production (Oluoch and Welbaum, 1996). There are many other factors associated with seed-to-seed variability that are known to affect the amount of phytochrome pooled in the seeds: stage of maturity at seed harvest, pollen load, position of the seed inside the fruit, health of the maternal plant, fruit position on the maternal plant, and the quality and amount of light (red light versus far red light) intercepted by the leaf nearest to the fruit (Oluoch and Welbaum, 1996; Casal and Sanchez, 1998).

Light quality and intensity following the imbibition of seeds can influence germination (Copeland and McDonald, 2001). For so-called photoblastic seeds, the greatest promotion of germination occurs in the red area (660-700 nm) followed by an inhibition zone in the far-red area above 700 nm (Flint and McAlister, 1937). This

germination response to the action spectrum of light was first discovered in the mid-1930s with lettuce seed (Flint, 1934; Flint and McAlister, 1937). Red light exposure converts phytochrome to the far-red absorbing form, which induces germination. Exposure to far-red light reconverts phytochrome to the red absorbing form, and germination is inhibited (Copeland and McDonald, 2001). This is apparently an adaptive mechanism that certain plants have evolved so that they will not germinate under a foliage canopy or within the fruit, where there is insufficient light to grow and develop properly. Seeds from the family Cucurbitaceae are generally made dormant when fruits are irradiated with far-red light (Flint and McAlister, 1937). This phenomenon occurs in nature when the source of far-red light is filtered though green tissues. Chlorophyll absorbs red light (660 nm) but does not absorb light with wavelengths longer than 710 nm, the far-red light. The transmitted light contains high level of far-red light, which serves to lower the amount of P_{fr} in the seed. Seeds of many species mature while the surrounding maternal tissues are still green, and thus the embryos are in a far-red rich environment. Such seeds are more dormant compared to those whose surrounding tissues have low amounts of chlorophyll (Bewley and Black, 1985).

Separating seeds that were harvested in different stages of development – and therefore differing in performance – is difficult, since low vigor seeds often have the same physical characteristics and weight as mature seeds (Oluoch and Welbaum, 1996). Using the correlation between seed maturity and chlorophyll content in seed coats, Jalink et al., (1998) has created a non-destructive seed sorting technique that measures the relative chlorophyll content in the outer layers of seeds using chlorophyll fluorescence (CF) in *Brassica oleracea*. CF was introduced as a non-destructive and instantaneous

method to measure the difference in quality by evaluating the "magnitude of CF signal of individual seeds" (Jalink et al., 1999). The results showed that the magnitude of the CF was inversely related to the quality of the seeds. One seed lot was improved from 90 to 97% normal seedlings by sorting out 13% of the seeds with very high CF (Jalink et al., 1999).

The objective of this study was to investigate whether chlorophyll and CF vary with seed maturity, as suggested by Jalink et al., (1998, 1999) for muskmelon. Another objective was to assess the use of CF sorting as a tool to improve the overall vigor and germination of seed lots by removing the putatively poor performing, immature seeds that contained high chlorophyll. A third goal was to determine if chlorophyll developed within the dark fruit, or if it formed during exposure to light during harvesting and processing. An additional objective in this study was to assess possible post-harvest light treatments, and light treatments during seed harvest that could be used commercially to increase seed performance for muskmelon.

Two hypotheses were tested.

1. Laser-Induced Fluorescence, which measures the CF signal, would allow immature, high chlorophyll seeds from the muskmelon seed lot to be sorted out and thus increase the overall performance of the seed lot.

2. The dark-harvested muskmelon seeds (not exposed to light during separation from the fruit) would have lower chlorophyll content, and thus have higher vigor and germinability compared to seeds exposed to light.

3. Post harvest light exposure would alter the P_{fr} level in seeds, lower the amount of chlorophyll accumulation in seeds and increase seed vigor.

2.2 MATERIALS AND METHODS

2.2.1 Plant Material

Melon plants (*Cucumis melo* 'TopMark' seed harvested in Blacksburg from original stock bought from Asgrow Inc, Gonzales, CA) were grown in Blacksburg, VA, in 1995, and the fruits were harvested and seeds separated from the fruits at nine stages of development (20, 25, 30, 35, 40, 45, 55, 60, and 65 DAA). The fruits were transported to the laboratory, and placed in a cooler at 5°C. The seeds were separated from the fruit the same day they were harvested. Seeds were removed from fruit and washed for 5 min under running water in darkness. A subset of the hydrated seeds was subjected to natural sunlight (120 W m^{-2}) for 45 min. The seeds were then dried in the dark to a WC of 6.0% until germination experiments were performed.

Subsets of the hydrated seeds were subjected to one of four light treatments for 45 min each: sunlight (2000 µmol m^{-2} s^{-1}), red light (between 625 nm and 675 nm, 11.0 µmol m^{-2} s^{-1}), far-red light (between 700 nm and 750 nm, 18.00 µmol m^{-2} s^{-}), or dark (no light treatment). The intensity of treatments was measured using the Li-250 light meter from LI-COR Biosciences (LI-COR Biosciences, Lincoln, NE). The seeds were then dried in the dark to a WC of 6.0% and held at 20°C until germination experiments were performed.

2.2.2 Germination and Vigor Tests

A germination test was performed on each stage of development except between 20 and 25 DAA (AOSA, 1983). Three or four replicates of 50 seeds each were placed on two wet germination paper towels (Anchor Paper Co., Minneapolis, MN) with a dry

towel in the middle and covered with another moist towel (AOSA, 1983). The paper towels were rolled, secured with two rubber bands, placed in self-sealing plastic bags, and incubated upright in a dark growth chamber at alternating 20ºC for 16 hr and 30ºC for 8 hr according to AOSA standards for muskmelon germination (AOSA, 1983). On days 4 and 10, germinated seeds were counted and their root lengths were measured.

A germination rate test was performed for each development stage and light treatment. Three replicates of 25 seeds each were placed in a 90-mm Petri dish on two germination blotter papers (Anchor Paper Co., Minneapolis, MN) moistened with 9 ml of distilled water. Petri dishes were placed in self-sealing plastic bags to reduce moisture loss and incubated at alternating 20ºC for 16 hr and 30ºC for 8 hr in the dark (AOSA, 1983). For 14 days, the germinated seeds were counted every 12 hr and removed from the Petri dish. The seeds were considered germinated if 2 mm of the radical was visible.

2.2.3 Statistical Analysis

The mean log time germination ($\log \bar{t}$) was calculated according to $t=\Sigma n_i t_i/\Sigma n_i$, where n_i is the number of newly germinated seeds at time t_i. The time values were $\log_{10}$ transformed to produce log-normal distributions from positively skewed cumulative germination time courses (Scott et al., 1984). The germination percentage was calculated according to germination % = (germinants/total seed)*100. The data for germination percent and vigor were statistically analyzed by ANOVA to determine if the data were statistically significant at $\alpha = 0.10$.

2.2.4 Chlorophyll Fluorescence

Approximately 1000 seeds from each stage of development were sent to Satake USA Inc. (Houston, Texas), and their chlorophyll fluorescence was quantified using a SeedMaster Analyzer, where the fluorescence was reported in pA units. For this analysis, the seeds were isolated on a rotary vibrator and individually fed to the optical CF measuring unit and ejector. The CF unit utilized a laser diode and a lens system, which contained an interference filter and a photodiode. The laser diode produced radiation at 670 nm, the absorption peak of chlorophyll a, and the chlorophyll fluorescence signal was captured by the lens system. The CF signal was fed into a computer to obtain either a frequency histogram or to control the ejector for sorting seeds (Jalink et al., 1999).

2.2.5 Chlorophyll Fluorescence from Different Seed Tissues

The CF of five intact 'Top Mark' muskmelon seeds was measured at 55 DAA. The testa was then removed, and the CF of the de-coated seeds and testa were measured separately in order to determine tissue-specific chlorophyll content.

2.3 RESULTS

2.3.1 Germination Performance

The germination of ‘Top Mark’ seeds was measured from fruits harvested 20 to 65 days after anthesis (DAA) using the Association of Official Seed Analyst (AOSA) standards for normal, abnormal, and dead seedlings (AOSA, 1983). At 20 DAA, no radicle emergence was observed; however, by 35 DAA, when maximum seed dry mass was achieved (data not shown), the germination percent for intact dry seed were <50%. Edible maturity of ‘Top Mark’ muskmelon corresponds to 45 to 50 DAA depending on

environmental conditions. A maximum germination percent of 100% was obtained by 50 DAA and remained high until 65 DAA (Fig. 1). The germination percentage followed an asymptotic curve as it gradually increased until reaching a plateau at 50 DAA and remained high until 60 DAA.

Vigor, determined by the mean root length of the germinating seeds after 4 and 10 days, showed a different developmental pattern than germination percentage (Fig. 2). Seed vigor increased until 40 DAA. Maximum vigor was obtained at 55 DAA, about the same time that the maximum germination percent was attained. While the increase in vigor followed an asymptotic curve, the increase was slower than germination percent. Unlike germination percent, vigor quickly declined after 60 DAA, while germination percent remained high (Fig. 2).

Changes in chlorophyll fluorescence (CF) during 'Top Mark' seed development were measured using a SeedMaster Analyzer in pA units. The blank instrument measurement was 120 pA. Maximum CF was achieved at 25 DAA, when the seeds were not germinable (Fig. 3). CF values gradually decreased from 25 to 40 DAA and remained consistently low, before leveling off just prior to 55 DAA.

2.3.2 Linear Correlation between CF and Germination/Vigor

A linear regression of CF versus germination percentage produced a negative correlation. In other words, when CF was high in immature seeds, germination percent and vigor were low. CF values declined as seeds developed the ability to germinate and vigor increased (Fig. 4).

2.3.3 CF Histogram

The CF of individual seeds in seed lots harvested at the same stage of fruit development was compared. The variation in CF values within a population of 'Top Mark' seeds from the same stage of development decreased with maturity. At 35 DAA, when the seeds reached maximum dry mass, the distribution of CF values was greater than in more mature seeds (Fig. 5, Panel A). When fruits reached edible maturity at 45 DAA, the variation among seeds in terms of CF was less than at 35 DAA, and the mean CF value for the population was lower than in less mature seeds (Fig. 5, Panel B). At 55 DAA when seeds reached both maximum germination percent and vigor, the signal distribution was the lowest of any stage of development (Fig. 5, Panel C). However, in seeds harvested by 65 DAA the variability and the mean of CF values increased (Fig. 5 panel D).

2.3.4 Seed Vigor in Relative to Maturity and Light Treatment

The germination percent and T_{50} of 40 DAA seeds harvested in the dark- and in light was the same (Table 1). At 55 DAA, when seeds exhibited maximum germination performance (percent and vigor), there was a distinct difference in the T_{50} of seeds kept in the dark after harvest and seeds subjected to 45 min sunlight (120 W m^{-2}). The T_{50} for seeds exposed to sunlight was lower compared to seeds harvested in the dark. With seeds at 55 DAA for cultivar 'Athena', exposure to sunlight decreased the T_{50} (Fig. 6). Unlike 'Top Mark', 'Athena' 55 DAA seeds harvested in the dark had a higher T_{50} (Table 2). The T_{50} of 55 DAA 'Athena' seeds exposed to sunlight was lower than those harvested in the dark, but higher than those seeds treated with red or far-red light (Fig. 6).

2.3.5 CF measured for different seed tissues

The CF values of individual seed tissues from 55 DAA 'Top Mark' seeds were determined using the SeedMaster Analyzer to measure the specific seed tissues after hand dissection (Table 3). De-coated seeds had the highest mean CF values. The CF of the seed coat alone was lower than intact or de-coated seeds, since the blank reference for CF was 120 pA. CF decreased by over 50% in one hour after de-coating (Table 3).

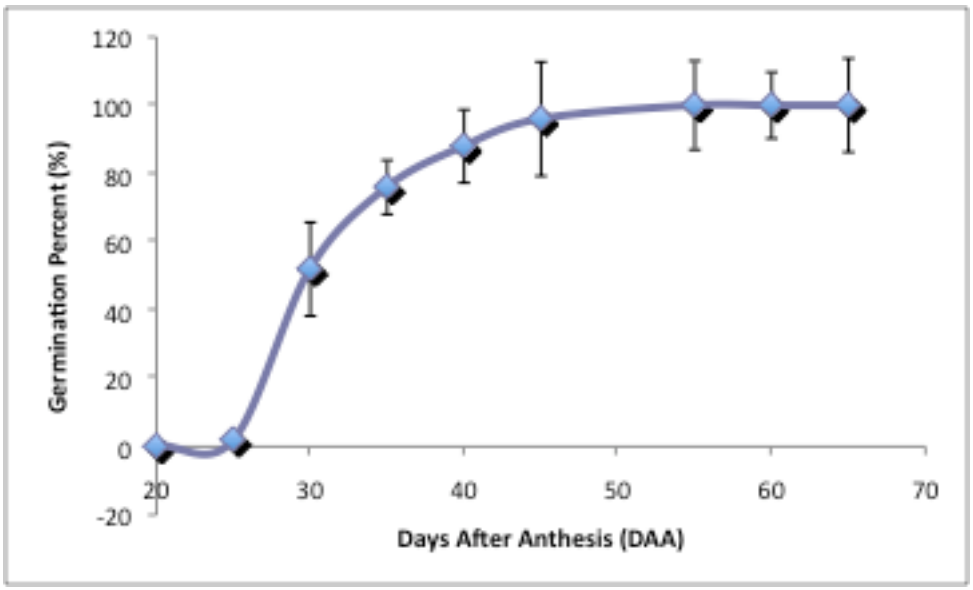

Fig. 1: Changes in germination percentage of 'Top Mark' seeds during fruit development. Germination percentage was measured via an AOSA standard test for *C. melo*. Means represent three to four replicates of 50 seeds each.

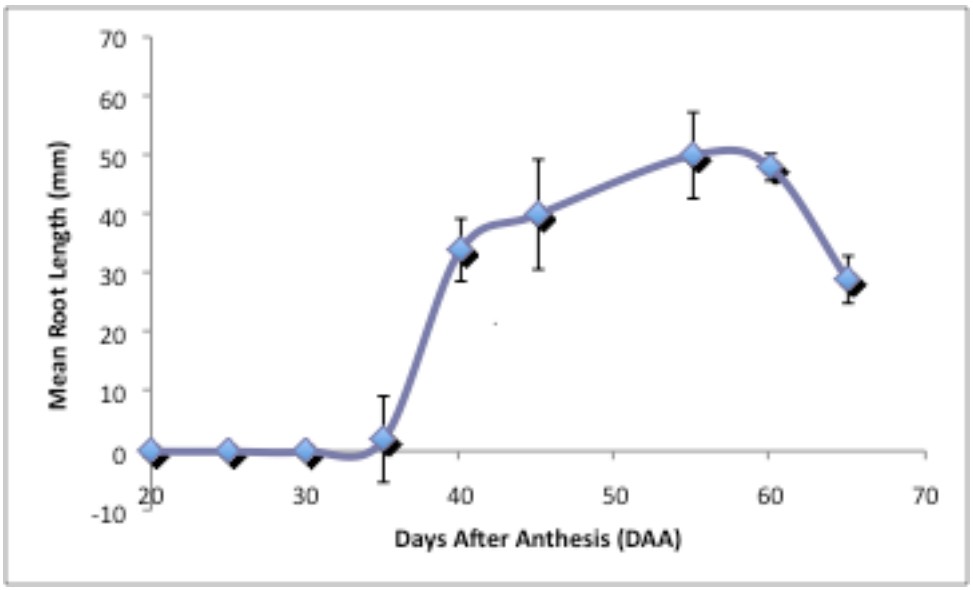

Figure 2: Changes in vigor of 'Top Mark' seeds during development. Mean root length was determined after 4 days' germination. Means represent three or four replicates of 50 seeds each.

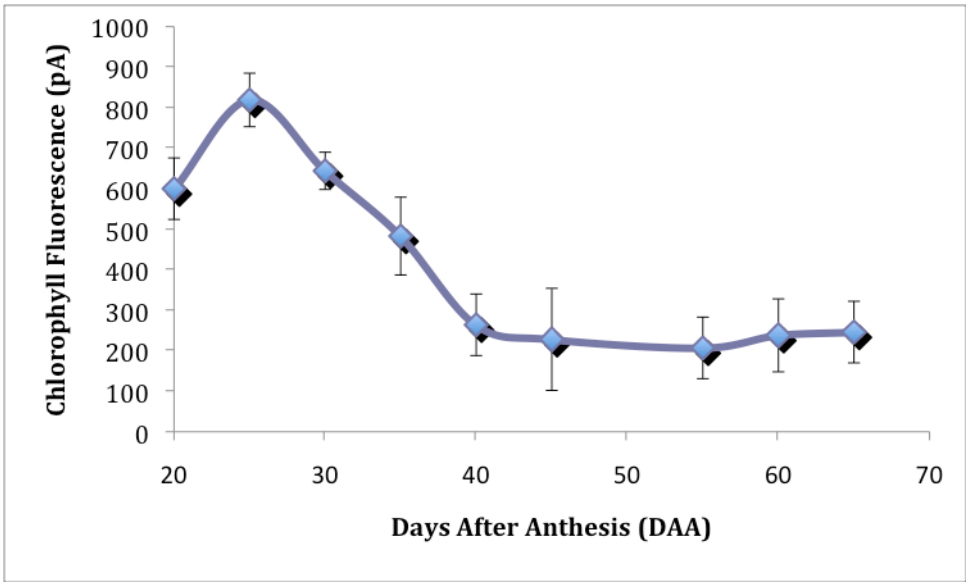

Figure 3: Changes in CF of 'Top Mark' seeds during development with a SeedMaster Analyzer. CF mean values were reported in pA and the average of approximately 50 seed measurements.

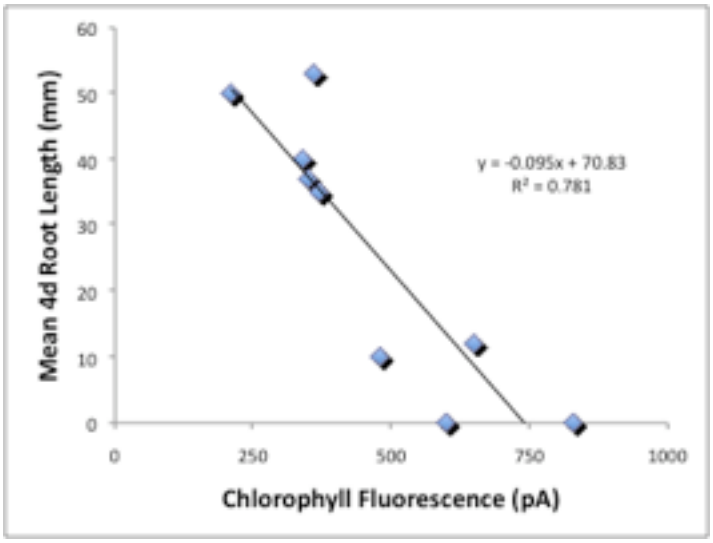

Figure 4: A linear regression of CF measured with a SeedMaster Analyzer from Fig. 3 versus mean 4 d root length in Fig. 2.

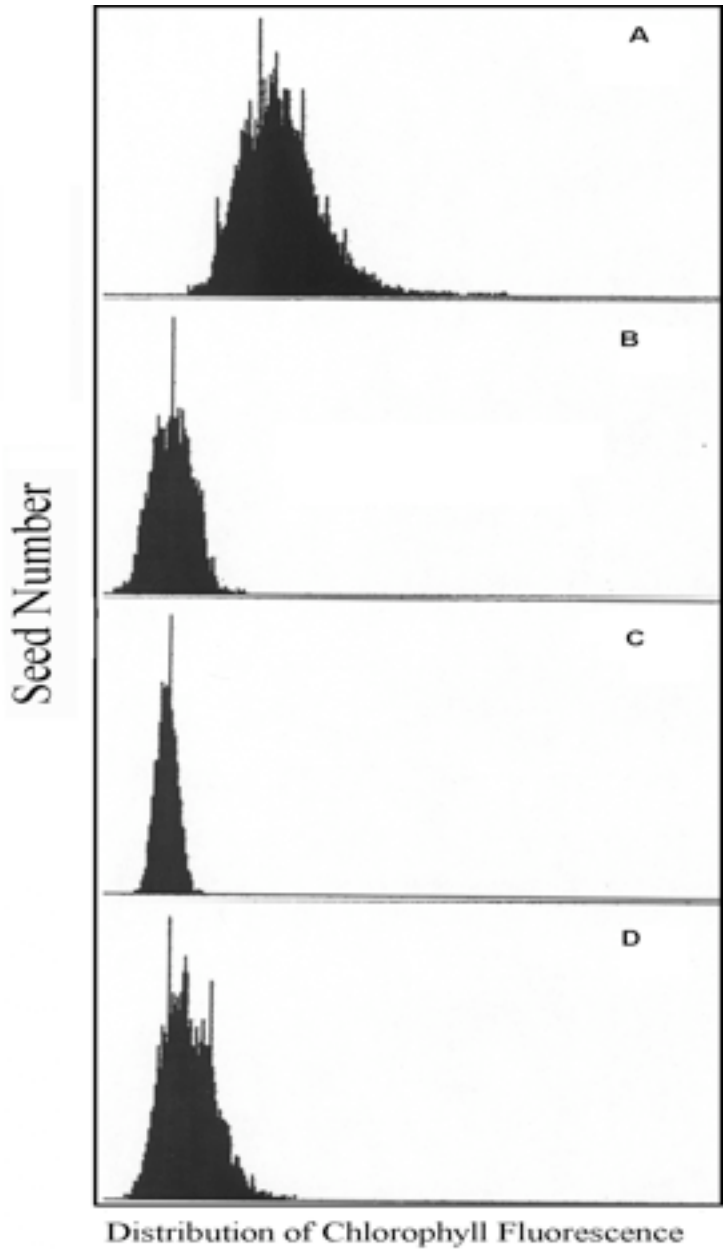

Figure 5: CF of 'TopMark' seeds harvested at different developmental stage, measured by a SeedMaster Analyzer. The relative scale of 0-1000 pA is the same for all three panels, and larger values represent greater fluorescence. (A) 35 DAA, (B) 45 DAA, (C) 55 DAA and (D) 65 DAA. Approximately 1000 'Top Mark' seeds were measured at each stage of development. Amplitude is the number of seeds per CF frequency.

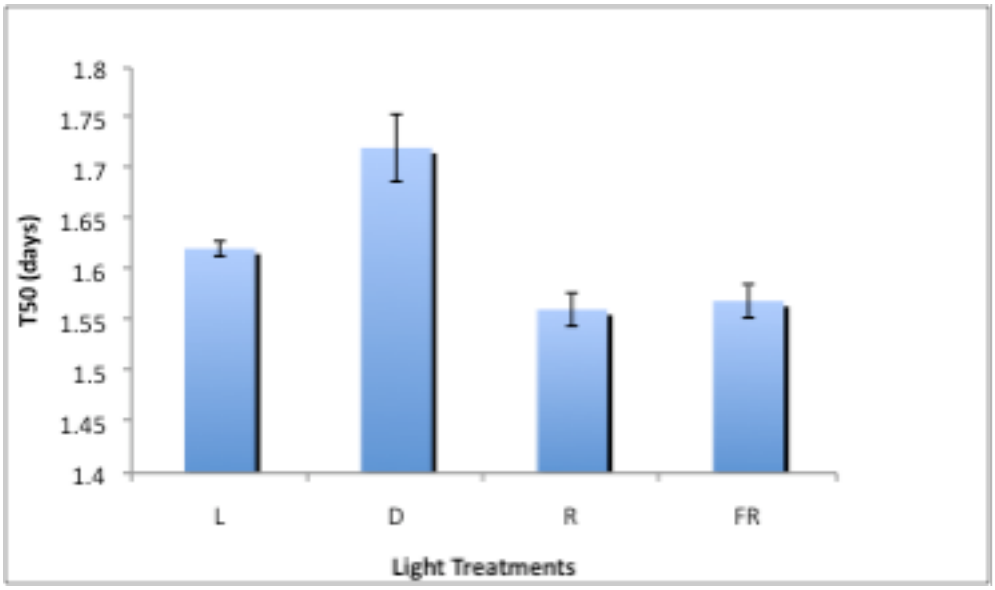

Figure 6: T_{50} of 55 DAA 'Athena' muskmelon seeds subjected to different light (L= 45 min of sunlight; D = dark; R = 45-min exposure to red light; FR = 45-min exposure to far-red light). Means represent three replicates of 25 seeds each. Error bars are L± 0.0073; D± 0.033; R± 0.0163; and FR±0.0165.

Table 1: Seed germination percent and mean time to germination of developing 'Top Mark' seeds. Light treatments (L= light; D = dark). T_{50} is the log mean time to germination with (antilog values). ANOVA was performed to determine if the results were statistically significant. There was significance in the germination percent between the maturity of the seeds (P>0.04). The T_{50} was not significantly different for seeds harvested at 40 DAA that were subjected to different light treatments (P>0.50). However, there was a significant difference within the seeds harvested at 55 DAA that were subjected to different light treatment (P>0.09).

Seed development	40 DAA		55 DAA	
Treatment	Light	Dark	Light	Dark
Germination percent (%)	80[b]	86[b]	97[c]	98[c]
T_{50} (days)	1.83[b] (0.546)	1.86[b] (0.538)	1.71[b] (0.585)	1.50[c] (0.667)

[z]Means represent the average of three replications of 25 seeds with separation by $LSD_{0.10}$.

Table 2. Seed germination percentage and mean time of two cultivars of muskmelon at 55 DAA in response to light or dark. Light treatments (L= light; D = dark). T_{50} is the log mean time to germination with (antilog values). ANOVA was performed to determine if the results were statistically significant. The germination percent of all treatments for the two cultivars was not statistically significant (P>1.00). The T_{50} was significantly different within the genotypes, with the different light treatments received by the seed (P>0.06)..

Cultivar	Athena		Top Mark	
Treatment	Light	Dark	Light	Dark
Germination percent (%)	98[az]	97[a]	97[a]	98[a]
T_{50} (days)	1.62[b] (0.617)	1.72[c] (0.581)	1.71[c] (0.585)	1.50[a] (0.667)

[z]Means represent the average of three replications of 25 seeds with separation by $LSD_{0.10}$

Table 3: Chlorophyll content of seed tissues dissected by hand and measured using a SeedMaster Analyzer. Tissues were selected from 55 DAA 'Top Mark' seeds.

Tissue Analyzed	**Mean CF**
	pA
Intact Seed (side A)	549[b z]
Intact Seed (side B)	546[b]
Decoated Seed	1295[a]
One hour after decoating	622[b]
Seed Coat	175[d]
Blank instrument control	120[d]

[z]Means represent the average of at least 10 seeds with separation by $LSD_{0.05}$.

2.4 DISCUSSION

Muskmelon cultivar 'Top Mark' seeds germinate weakly before the accumulation of maximum dry weight at 35 DAA (Welbaum and Bradford 1988). The vigor of immature seeds is extremely low, which becomes a problem during field planting under adverse conditions, when high-vigor seeds are needed for successful stand establishment. The germination percentage of intact seeds was <50% at mass maturity 35 DAA. Germination percent increased to greater then 95% but not until 45 DAA (Welbaum and Bradford 1988).

According to Welbaum et al., (1998), maternal tissues surrounding muskmelon seed embryos help maintain developmental metabolism through a combination of physical barriers to expansive growth and endogenous plant growth substances. The endosperm of muskmelon completely encloses and creates tension on the fully hydrated embryo before germination; thus, full hydration required for germination can only occur after the endosperm envelope is broken. Once the endosperm envelope is broken, the tension is released; creating an osmotic gradient for hydration that allows expansive growth of the radicle to occur. Although germination is a very complex phenomenon in which many environmental, biochemical, and physiological factors are involved, endosperm rupture is caused by enzymatic degradation. Endo-ß-D-mannanase is one of the more studied enzymes expressed at the time of endosperm rupture. The poor germination performance of immature seeds at 25-30 DAA has been correlated with low endo-ß-D-mannanase activity, one of the many enzymes responsible for endosperm weakening. However, the activity of endo-ß-D-mannanase in muskmelon cultivar Top Mark peaks at 40-50 DAA and is correlated with maximum germinability (Welbaum, 1999).

Light, predominantly associated with the seed dormancy in many species (Copeland and McDonald, 2001), affects the timing of seed germination in soil (Desai, 2004). Both light intensity and light quality influence germination. The greatest promotion of germination occurs with red light (660-700 nm), while far-red (above 700 nm) is generally inhibitory (Copeland and McDonald, 2001; Flint and McAlister, 1937). The lack of difference in the T_{50} for 40 DAA 'Top Mark' seeds harvested under dark and light suggests that immature 40 DAA seeds were less influenced by light environment (Table 1). In vigorous and fully germinable 55 DAA 'Top Mark' seeds, there was a significant difference in the T_{50} of seeds harvested and processed in the dark versus those harvested and dried in sunlight. The T_{50} of seeds exposed to sunlight was significantly higher compared to seeds harvested in the dark, which implies that light exposure during harvest caused mature 'Top Mark' seeds to germinate slower. We were unable to measure the phytochrome concentration in these seeds, since phytochrome deficient mutants were not available for muskmelon and a phytochrome antibody was unavailable. The difference in T_{50} of seeds harvested at different developmental stages, suggests that light environment is a factor that contributes to 'Top Mark' seed vigor in mature seeds.

Treatments with either red or far-red light at 55 DAA applied to 'Athena' seeds significantly affected T_{50} values (Fig. 6). The T_{50} of dark harvested seeds were much higher, because they took longer to germinate. Light treatment (sunlight, red light, and far-red light), decreased T_{50} compared to dark harvested seeds (Fig. 6) in Athena. This positive effect of light treatment at harvest on seed vigor could be due to the fact that 'Athena' has a very different genetic background compared to 'Top Mark', 'Top Mark' is categorized as a US shipping melon by Purseglove (1984). This cultivar is distinct

from ‘Athena’, which was developed for humid areas of the eastern US, in that it was developed for desert regions in the western US.

For 55 DAA ‘Athena’ seeds, exposure to light decreased T_{50}, and caused faster germination (Table 2). Exposing ‘Top Mark‘ seeds to light during seed harvest increased the mean time to germination, which is the opposite effect observed for ‘Athena‘. Therefore, within the same species, cultivars respond differently to light.

The correlation between green seed coats and chlorophyll content is well established in such seeds as canola and rapeseed. Ward et al., (1992, 1994) reported that immature canola seeds contained high amounts of chlorophyll in the seed coat, and the seed performance of these ‘green-seed’ was significantly lower than non-green seeds. Suhartanto (2003) used fluorescent microscopy to observe that most of the chlorophyll was located in the seed coat and only small amounts were detected in the embryo of immature tomato seeds. Suhartanto (2003) also reported that high CF in the seed coat disappears as the seeds mature. The majority of the CF in muskmelon was found to be within the de-coated seed (in the endosperm and embryo) (Table 3). The CF observed for the seed coat was low since the background signal was 120 pA. The lack of chlorophyll in the seed coat was perhaps due to the fact that 55 DAA mature ‘Top Mark’ seeds were used to measure tissue specific CF. If tissue specific CF was measured for muskmelon seeds at different developmental stages, we would have likely observed higher CF in the seed coat of immature seeds, similar to what was observed in tomato (Suhartanto, 2003).

A strong negative relationship between seed germination and chlorophyll content in the seed coat has been established with carrot (Steckel et al., 1989), oilseed, and turnip rape (Ward et al., 1992, 1995). However, the relationship between chlorophyll and seed

maturity has never been compared in muskmelon seeds. Open-pollinated muskmelon seeds are produced in large fields, and fruits from different stages of development are combined in a single seed lot (Kelly and Raymond, 1998). Mixing seeds from different maturity stages in a single lot makes it difficult to separate low and high vigor seeds. Separation of immature tomato seeds by CF has increased germination from 90 to 97% by sorting out 13% of the seeds with very high CF (Jalink, 1999). In muskmelon, the drop in CF occurred at approximately the same time as physiological maturity. This suggests that conventional density gradient technologies may be just as effective as CF at separating mature and immature seeds (Fig.3, Welbaum and Bradford, 1988).

A frequency histogram of CF signals of cabbage seed lots showed that the majority of the seeds had a low to medium CF value, but about 10% of the seeds showed a CF signal more then twice that of the median (Jalink et al., 1998). This demonstrated that the seed lot was not uniform for the amount of chlorophyll, due to seed-to-seed variation. The results in Fig. 5 for muskmelon agree with results attained by Suhartanto (2003), where the standard deviation of CF values in a tomato seed lot decreased when seeds attained maximum germinability and vigor. The CF distribution showed that the variation in CF values decreased as seeds matured. However, the decrease in distribution of CF signals with maturity demonstrates that the lot is composed of a population of individual seeds, each with unique characteristics. Mean population CF comparisons do not adequately represent the performance of the seed lots since the variance of the population can vary more than population means.

A number of crop management practices are known to influence the chlorophyll content in harvested seed (Ward et al., 1990). These include sowing rate, sowing date,

and swathing procedures (Cenkowski, 1989; Ward et al., 1992). Lower temperatures during seed maturation (Ward, 1992; Cenkowski et al., 1993) cause slower chlorophyll degradation, resulting in the 'green-seed' problem. The rate of chlorophyll degradation in oilseed rape was reported to be faster in a warm environment (Ward et al., 1994).

The concomitant increase in ethylene production and chlorophyllase activity has been documented in a variety of plant tissues undergoing chlorophyll loss, including climacteric fruits (Frankel, 1972), and senescing cucumber cotyledons (Abeles and Dunn, 1989). The increase in ethylene production in muskmelon of botanical variety *cantalupensis*, a climacteric fruit, has been established to be around 35 DAA (Lyons et al., 1962). The climacteric rise in ethylene production in muskmelon occurs just after maximum seed dry weight accumulation and the onset of fruit ripening (Lyons et al., 1962). Chlorophyll degradation is regulated by ethylene, which is known to accelerate senescence in many species and enhance chlorophyllase activity (Drazkiewicz, 1994; Takamiya et al., 2000). The rapid decrease in CF values after 35 DAA (Fig. 3) occurs at the same time as the climacteric burst of ethylene. The decrease in CF in muskmelon seeds parallels the chlorophyllase activity occurring in other plant species' tissues in response to ethylene. A study by Ward et al., (1994) reported a positive correlation between the chlorophyll content of oilseed rape (*Brassica napus*) seed and the rate of ethylene evolution during seed ripening. However, the study also showed that the ethylene production peaked early during seed ripening, which occurred after seed chlorophyll breakdown had begun. The study concluded that there was a distinct correlation between the increase in ethylene and increase in the chlorophyllase activity. However the ethylene did not act as a trigger to the breakdown of chlorophyll (Ward et

al., 1994). Oilseed rape is not a climacteric species and does not produce an ethylene burst during fruit development as does muskmelon. However, this relationship between ethylene and chlorophyll degradation would be an interesting association to be studied in future research. Studies by Ward et al., (1994) also looked at the relationship between moisture loss during seed maturation and chlorophyll breakdown. According to Welbaum and Bradford (1988), the water content of muskmelon seeds peaks at 20 DAA and gradually decreases, and at 35 DAA it plateaus until 65 DAA. This coincides with the shape of the CF curve in Fig. 3, in which CF peaks at about the same stage of development as seed water content (Welbaum and Bradford, 1988). This result is supported by data from Ward et al., (1994) where a highly significant positive correlation was found between the moisture content of ripening canola seed and chlorophyll content. Seed chlorophyll in canola was rapidly degraded at 65 to 34% seed moisture, and the rate slowed at lower seed moisture contents. However, seed chlorophyll breakdown in canola did continue below 35% seed moisture until all or most of the chlorophyll had disappeared at 10% moisture content (Ward et al., 1994; Johnson-Flanagan and Spencer, 1996). In the case of muskmelon, no chlorophyll breakdown was observed after 40 DAA (Fig. 3), which is when the moisture content of the seed is approximately 40% (Welbaum and Bradford, 1988). During seed ripening, both moisture and chlorophyll levels declined, the rate of chlorophyll degradation was high when the chlorophyll levels were high, and the rate slowed as more and more chlorophyll was lost (Fig. 3). This agrees with the results of Cenkowski et al., (1993), who reported that seed chlorophyll declines exponentially as canola seed ripens.

We can conclude that chlorophyll in muskmelon seeds was predominately located in the embryo and/or endosperm tissues in the mature 55 DAA seeds. Seed chlorophyll was high early in development and declined as the seeds matured. Therefore, seed chlorophyll in muskmelon is a marker for seed maturity. By the time of natural seed desiccation when seeds are released from fruit after 60 DAA, chlorophyll levels are low in most seeds and likely do not damage seeds by imposing oxidative stress. The decline in seed chlorophyll correlated to the drop in seed moisture content and climacteric ethylene production in the fruit as the seeds mature.

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Chapter 3: The effect of phytochrome on tomato seed chlorophyll content and seed performance

3.1 INTRODUCTION

The tomato is an important annual vegetable because of its wide popularity and economic importance. Many studies have investigated tomato seed quality. Both qualitative and quantitative differences in the light received by the mother plant during seed development cause a wide variation in germination responses to light (Casal and Sanchez, 1998). Tomato seeds characteristically germinate best in the dark, and light will inhibit germination (Heuvelink, 2005). This phenomenon is thought to be caused by the ratio of the two phytochrome forms (P_{fr}, physiologically active form: P_r, physiologically inhibitory form) accumulated in the maternal plant (Casal and Sanchez, 1998), suggesting that the presence of P_{fr} in tomato seeds is a prerequisite for germination (Heuvelink, 2005).

Biochemical changes during seed development influence seed performance. Tomato seeds of the third truss reached physiological maturity earlier than those of the first and second truss (Demir and Ellis, 1992). Jalink et al., (1999) showed that differences in the time it takes seeds to reach physiological maturity are correlated with differences in chlorophyll fluorescence (CF) among individual seeds. Seeds from the third truss showed lower CF than those from the first and second truss. In Jalink et al.,'s 1999 study, CF declined with tomato seed maturity.

Production of high quality tomato seeds depends upon the timing of fruit harvest. However, the timing of maximum seed germination performance development and its relationship with plant and fruit developmental markers shows variability among

cultivars and geographic locations (Dias et al., 2006). Kwon and Bradford (1987) reported that germination rate and percent of tomato seeds increased as the fruits developed from the mature green to mature-red stages and then declined as fruits became overripe. On the other hand, Valdes and Gray (1998) found that seeds from fruit harvested as early as the breaker stage of development were fully germinable, but that seeds from red fruits had higher vigor because they germinated more rapidly. Demir and Ellis (1992) reported that maximum seed quality occurred after maximum seed dry matter accumulation, with no mention of the stage of fruit ripeness.

Steckel et al., (1988) reported a negative linear correlation between the percent germination and seed moisture content, chlorophyll a and b content in the seed, and seed distortion of carrot ('Chantenay' and 'Amsterdam'). Jalink et al., (1996) created a non-invasive and non-destructive seed sorting technique that measures the relative chlorophyll content in the outer layers of seeds using CF. A Laser Induced Fluorescence (LIF) technique was introduced as a high-speed sorting method to detect differences in seed quality by evaluating the magnitude of the CF signal of individual seeds (Jalink et al., 1999). The study, conducted with *Brassica oleracea,* showed that the magnitude of the CF signal was inversely related to the quality of the seeds as measured by germination percentage, germination rate, and other indices of germination performance. For example, a tomato seed lot was improved from 90 to 97% normal seedlings, by sorting out 13% of the seeds with very high CF (Jalink et al., 1998).

Studies by Suhartanto (2003) showed that higher tomato seed quality, as measured by total percent germination, percentage normal seedling, and germination rate, was achieved when the CF in developing seeds reached a minimum and leveled off.

There are variations in maturation among individual seeds in a fruit during development, which resulted in large standard deviations among individual seed CF values within the same fruit. However, the standard deviation for CF became smaller when seeds attained maximum quality. Using fluorescence microscopy, Suhartanto (2003) showed that in tomato seeds most of the chlorophyll was located in the seed coat, and small amounts were detected in the embryo as well. The study also demonstrated that chlorophyll in tomato seeds is photosynthetically active. In vitro analysis showed photosynthetic oxygen evolution and Rubisco activity in young tomato seeds. In addition, carotenoid compounds were present in young tomato seeds as well, suggesting active photosynthesis at this stage of development.

Light intensity and day length might be expected to influence seed chlorophyll content. Suhartanto (2003) showed that tomato seeds, developed in darkness contained lower amounts of chlorophyll, which suggests that high light input is required for elevated chlorophyll synthesis. Chlorophyll synthesis occurs both in low and high light environments. However in low light, the reduction of protochlorophyllide, the key step in chlorophyll synthesis, is not driven by light but by chemical energy. This process is capable of synthesizing only small amounts of chlorophyll, while the reduction of protochlorophyllide in presence of light is required for the synthesis of larger quantities of chlorophyll. Chlorophyll biosynthesis is controlled by phytochrome, since the synthesis of chlorophyll precursor, δ-aminolevulinic acid (ALA) is regulated by P_{fr} (Masoner and Kasemir, 1975).

The control of seed germination by red and far-red light is one of the earliest documented phytochrome-mediated processes. Phytochrome is one of the most studied

photoreceptors. At present there are five known phytochrome genes in *Arabidopsis thaliana* and in tomato, phytochrome B is present in dry seeds and affects germination of dark-imbibed seeds (Casal and Sanchez, 1998). The absorption of the two photo-interconvertible forms of phytochrome, the red-light-absorbing form (P_r) and the far-red-light-absorbing form (P_{fr}), are dependent upon the presence of either wavelength as long as other pigments absorbing in a similar spectral region do not distort the light. Absorption maxima of P_r and P_{fr} are 660 nm and 730 nm, respectively. However, the considerable overlap between the absorption spectra for the two forms produces a mixture of P_r and P_{fr}. Dry seeds show little photoreversible absorption change attributable to phytochrome. During imbibition, there is a rapid increase in phytochrome signal due to the rehydration of inactive phytochrome (P_r), which is thought to be synthesized during the formation of seeds. The phytochrome form found in mature seeds is largely dependent on the light environment of the mother plant (Casal and Sanchez, 1998). Studies presented by Cresswell and Grime (1981) show a negative relationship between germination in darkness and the chlorophyll content of extra-embryonic tissues. Seeds that retained green tissues around the embryo had most of their phytochrome in the P_r form. The Pfr form of phytochrome B in seeds is important for germination of dark-imbibed seeds. Arabidopsis seeds over-expressing phytochrome B germinates well in darkness since phytochrome B enhances germination in seeds. When the mother plant is grown under low red/far-red ratios, the amount of phytochrome B P_{fr} is reduced (McCormac et al., 1993).

The objectives of this study were to investigate if chlorophyll in immature tomato seeds can be used as a marker for poor seed quality. Another object was to determine

whether chlorophyll in mature seeds could damage seeds by causing oxidative stress during desiccation. The third objective was to study the role of phytochrome in chlorophyll synthesis and breakdown, by using phytochrome deficient mutants.

Two hypotheses were tested;

Hypothesis 1

- Tomato mutants that lack phytochrome B contain less chlorophyll in their seeds.
- The rate of chlorophyll degradation is unaffected in phy B mutant seeds, compared to wild type.

Hypothesis 2.

- Post harvest, predrying, and prestorage light treatments improve tomato seed vigor.
- Post harvest light treatments alter the amount of chlorophyll present in seeds.

3.2 MATERIALS AND METHODS:

3.2.1 Plant Material

Tomato plants (*Solanum lycopersicum* L.) 'Moneymaker', phytochrome A mutant *fri*$^{-1}$, and phytochrome B mutant *tri*$^{-1}$ (Tomato Genetic Resource Center, Davis CA) were grown in a field at the Kentland research farm of Virginia Tech near Blacksburg, VA during summer of 2007. Open pollinated flowers were tagged, and the fruits were harvested at 40, 50, or 60 DAA. The fruits were transported to the laboratory and seeds were extracted from the fruits on the same day that the fruits were harvested. The seeds were removed from the fruit and acid washed with 15% HCl solution in complete darkness. The seeds were then subjected to four light treatments for 45 min:

sunlight (2000 μmol m^{-2} s^{-1}), red light (between 625nm and 675 nm, 11.0 μmol m^{-2} s^{-1}), far-red light (between 700nm and 750 nm, 18.00 μmol m^{-2} s^{-}), or dark (no light treatment). Light intensities were measured using a Li-250 light meter from LI-COR Biosciences (LI-COR Biosciences, Lincoln, NE). The seeds were then dried in the dark to a WC of 6.0% (dry weight basis) and stored at 20°C until germination experiments were performed the next day.

3.2.2 Germination and Vigor Testing

The mean time to germination (T_{50}) was used as an indicator of vigor. Germination and vigor analysis was conducted by placing three replicates of 25 seeds each on one layer of germination paper in 90 mm Petri dishes moistened with 6 ml of 0.2% KNO_3. The dishes were placed in self-sealing plastic bags and incubated in the dark at 25 °C. For 14 days, the germinated seeds were counted every 12 hr and removed from the Petri dish. The seeds were considered to have germinated when 2 mm of the radical was visible.

3.2.3 Statistical Analysis

The mean time to germination (T_{50}) was calculated according to $t=\Sigma n_i t_i / \Sigma n_i$, where n_i is the number of newly germinated seeds at time t_i. The time values were $\log_{10}$ transformed to produce log-normal distributions from the positively skewed cumulative germination time courses (Scott et al., 1984). The germination percentage was calculated according to germination % = (germinants/total seed)*100. The data were compared statistically by ANOVA to determine if the data were statistically significant at $\alpha = 0.10$.

3.2.4 Chlorophyll Fluorescence

Approximately 1000 seeds from each stage of development (40, 50, or 60 DAA) were sent to Satake USA Inc. (Houston, TX), and their CF was quantified using the SeedMaster Analyzer, where the fluorescence was reported in pA units. For measurement, the seeds were isolated on a rotary vibrator and fed individually to the optical CF measuring unit and ejector. The CF unit was comprised of a laser diode and a lens system, which contains an interference filter and a photodiode. The laser diode produced radiation with a wavelength of 670 nm, the maximum absorption of chlorophyll a; and the chlorophyll fluorescence signal was captured by the lens system. The CF signal was fed into a computer to obtain either a frequency histogram or to control the ejector for sorting seeds (Jalink et al., 1999).

3.3 RESULTS

3.3.1 Germination Performance

The germination percent of all genotypes ('MoneyMaker', *tri*$^{-1}$, and *fri*$^{-1}$) gradually increased with maturity. At 40 DAA, *tri*$^{-1}$ had 70% germination, where both 'MoneyMaker' and *fri*$^{-1}$ germination percent exceeded 85% (Fig. 1). The cultivar 'MoneyMaker' was used as a control genotype for this study, since mutants, *tri*$^{-1}$ and *fri*$^{-1}$, were of 'MoneyMaker' background. Seed vigor, measured as the mean time to germination (T_{50}), similar to germination percent, increased as seeds matured; however the increase was greater in 'MoneyMaker' compared to the two phytochrome mutants

(Fig. 2). The phytochrome B mutant *tri*$^{-1}$ had the highest vigor of the three genotypes in this experiment while *fri*$^{-1}$ was intermediate.

Chlorophyll fluorescence decreased as the seeds matured. The CF for 'MoneyMaker' and *fri*$^{-1}$ was around 2500 pA at 40 DAA, but decreased to 1500 pA at 60 DAA (Fig. 3). The mutant *tri*$^{-1}$ had the least change in CF compared to the other two genotypes. The immature seeds exhibited higher CF values at 40 DAA, however the CF signal decreased as seeds matured. The CF of the mutants *tri*$^{-1}$ *and fri*$^{-1}$ decreased by 30% from 50 to 60 DAA. The change in CF observed for 'MoneyMaker' was less dramatic, and remained relatively constant with only a slight decrease from 50 to 60 DAA (Fig. 3).

3.3.2 Seed vigor in relative to maturity and light treatment

There were no significant changes in seed vigor and germination when seeds were treated separately during harvesting with either: red light (between 625 and 675 nm, 11.0 μmol m^{-2} s^{-1}), far-red light (between 700 and 750 nm, 18.00 μmol m^{-2} s^{-}), and dark (no light treatment) for 45 min. There was no evidence to suggest that CF changed as the seeds were treated with different light sources during harvesting, since the mean CF was constant for all light treatments (Table 1). This indicates that seed chlorophyll in tomato was developmentally regulated and not dependent on the light environment during seed harvest.

There were significant differences in seed vigor among the different genotypes. The T_{50} of *tri*$^{-1}$ was lower then 'MoneyMaker' and *fri*$^{-1}$, which means *tri*$^{-1}$ consistently germinated faster than the others regardless of light treatment applied (Table 1). Differences in CF were observed among genotypes regardless of light treatment. The CF

of *fri*$^{-1}$, the phytochrome A mutant, was consistently low compared to the others. (Table 1).

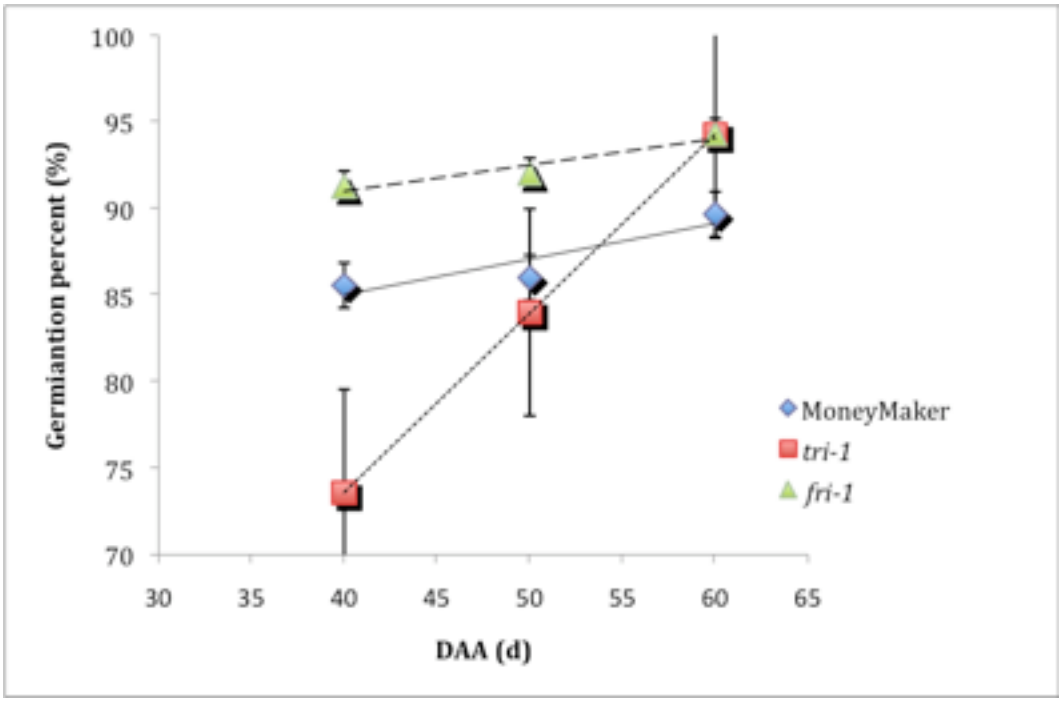

Figure 1: Changes in germination percentage of cultivar 'MoneyMaker', phytochrome B mutant *tri*$^{-1}$ and phytochrome A mutant *fri*$^{-1}$seeds during development. Germination percentage was measured via an AOSA standard test for *Solanum lycopersicum* L. Means represent three to four replicates of 25 seeds each. Error bars indicate standard error of the mean.

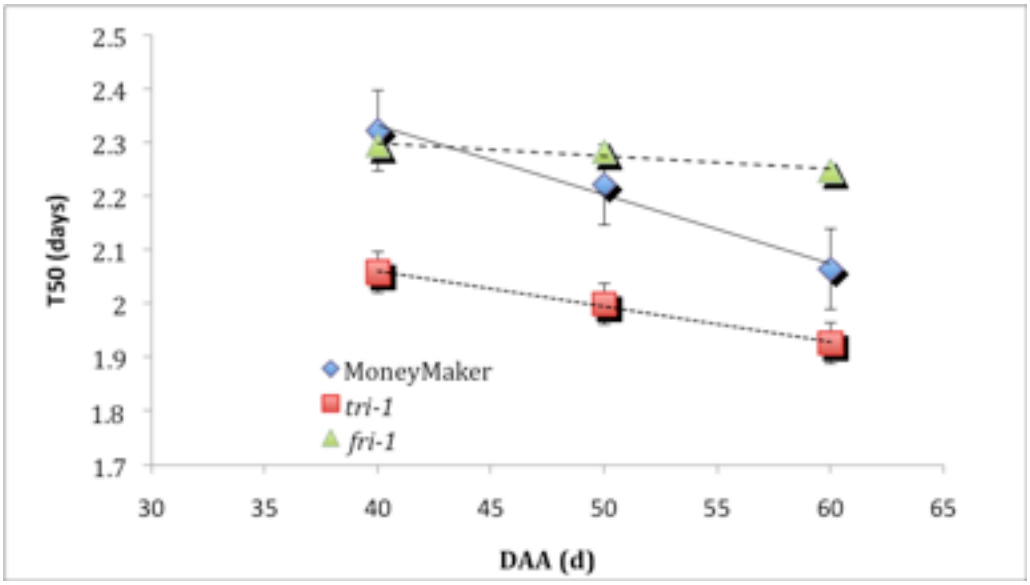

Figure 2: Changes in vigor of cultivar 'MoneyMaker', phytochrome B mutant *tri*$^{-1}$ and phytochrome A mutant *fri*$^{-1}$seeds during development. T_{50} was determined to assess vigor. Means represent three to four replicates of 25 seeds each. Error bars for 'MoneyMaker' ± 0.074; *tri*$^{-1}$± 0.038; and *fri*$^{-1}$± 0.014. Error bars indicate standard error of the mean.

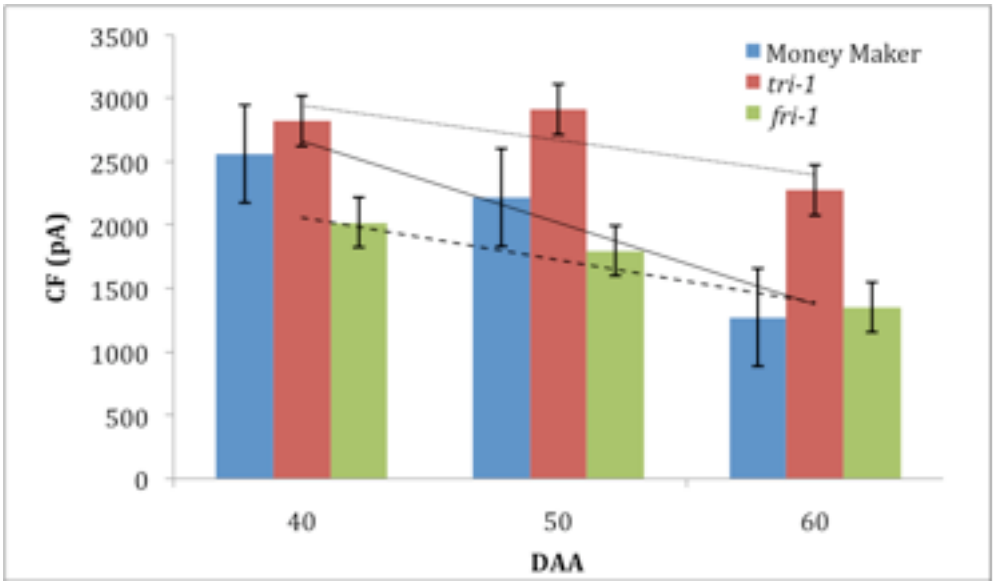

Figure 3: CF values for 'MoneyMaker', phytochrome B mutant *tri*$^{-1}$ and phytochrome A mutant *fri*$^{-1}$ seeds at different stages of development as detected with a SeedMaster Analyzer. CF mean values were measured in pA and the average of approximately 25 seed measurements. Error bars indicate standard error of the mean. The r^2 value for 'MoneyMaker' = 0.932, *tri*$^{-1}$ =0.627 and *fri*$^{-1}$ = 0.964.

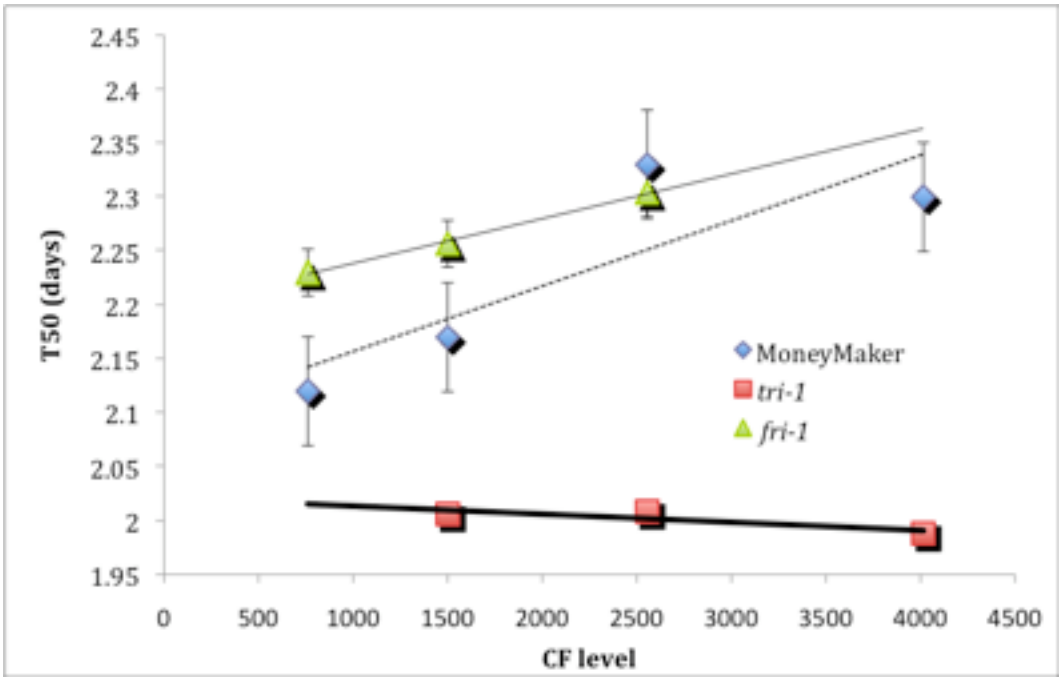

Figure 4: Changes in T_{50}, a measurement of vigor, of 'MoneyMaker', phytochrome B mutant *tri*$^{-1}$ and phytochrome A mutant *fri*$^{-1}$ seeds at 50 DAA according to CF measured with a SeedMaster Analyzer. The T_{50} was measured in accordance with the AOSA vigor test, and the CF mean values were measured in pA and the average of approximately 25 seed measurements. Error bars indicate standard error of the mean The r^2 value for 'MoneyMaker'=0.719, *tri*$^{-1}$ =0.739 and *fri*$^{-1}$ =0.994**.**

Table1: Seed germination percentage, T50 and mean CF of three tomato cultivars at 50 DAA in response to different light treatments. (L= light; D = dark; R = 45-min exposure to red light; FR = 45-min exposure to far-red light).

	MoneyMaker			***Tri*$^{-1}$**			**Fri^{-1}**		
Light Treatment	**D**	**R**	**FR**	**D**	**R**	**FR**	**D**	**R**	**FR**
Germination percent (%)	90^{az}	86^{a}	88^{a}	83^{a}	91^{a}	80^{a}	92^{a}	96^{a}	90^{a}
T_{50}	2.23^{a}	2.19^{a}	2.21^{a}	1.96^{b}	2.03^{b}	2.03^{b}	2.29^{a}	2.19^{a}	2.31^{a}
Mean CF (pA)	1914^{a}	2153^{a}	2704^{a}	2704^{a}	2549^{a}	2761^{a}	1678^{b}	1644^{b}	1744^{b}

^{z}Means represent the average of three replication of 25 seeds with separation by $LSD_{0.05}$.

3.4 DISCUSSION

The germination percent of cultivar 'MoneyMaker' displayed an exponential increase from 36 to 48 DAA and remained high after that time point (Suhartanto, 2003). A sharp increase in the germination percent early in seed development was not detected, but percentages were already high after 48 DAA (Fig. 1). However, the germination percent results of phytochrome B mutant show an exponential increase from 40 to 60 DAA, where the other two cultivars exhibit a linear increase. This dramatic increase in germination percent by the phytochrome B mutant could indicate that the maturation of phytochrome B mutant seeds was slower then the other two cultivars (Fig. 1). The belated increase in germination percent correlates with the exponential increase in germination

percent reported by Suhartanto (2003). The development of germination percentage in the current study showed the same trend as seed dry weight accumulation reported by Suhartanto (2003). Dry weight accumulation gradually increased from 24 to 48 DAA when it peaked and remained high thereafter. This correlation indicates that seed germinability for tomato is strongly related to seed weight and physiological maturity (Downie et al., 1999). The results were consistent with reports by Demir and Ellis (1992) who reported maximum seed quality occurred only after maximum seed dry matter accumulation. Phytochrome A mutant *fri*$^{-1}$ had been expected to perform in a similar manner to 'MoneyMaker' since phytochrome A according to Casal and Sanchez (1998) does not affect seed germination in tomato. Phytochrome B mutant *tri*$^{-1}$ was expected to be less viable then the control 'MoneyMaker' as it was unable to produce the phytochrome B, necessary for the induction of seed germination (Casal and Sanchez, 1998).

Another factor related to seed viability and vigor is the seed moisture content at seed harvest. According to Suhartanto (2003) fresh seed fluorescence of the tomato cultivar 'MoneyMaker' peaked at 24 DAA and decreased at an exponential rate, between 30 and 51 DAA and remaining constantly low afterwards. The results from my study indicates a similar trend with a sharp decrease in CF (Fig. 3). However since fruit harvest began at 40 DAA and ended at 60 DAA we observed neither the high CF values reported by Suhartanto (2003) at 24 DAA nor the plateau of the CF curve after 60 DAA. Both of the results are in accordance with the results presented by Ward et al., (1994) in which a high significant positive correlation was found between the moisture content of ripening canola seed and its chlorophyll level. Dias et al., (2006) reported that seed moisture

content -calculated on fresh weight basis- peaks at 25 DAA and reclines rapidly thereafter until 75 DAA, stabilizing around 50 and 53%. Seed chlorophyll of canola rapidly degraded when the crop was at 65-34% seed moisture, with the rate slowing at lower seed moisture contents. (Ward et al., 1994; Johnson-Flanagan and Spencer, 1996). During seed ripening, both moisture and chlorophyll levels declined and the rate of chlorophyll degradation was high when the chlorophyll levels were high, but the rate slowed as more and more chlorophyll was lost (Fig. 3). This agrees with the results of Cenkowski et al., (1993), who reported that seed chlorophyll declined exponentially as the canola seed matured.

The results obtained in this study indicate that the CF signal is high in seeds of round-fruited tomato cultivars (40 DAA) and decreases as the seeds develop and mature (Fig. 3). The study by Jalink et al., (1999) demonstrates an exponential decrease in CF signal from 30 to 40 DAA. Our study was only able to detect a decrease in CF signal after 40 DAA because of the quick degradation of CF between 30 DAA to 40 DAA had already occurred. The findings of the current study are consistent with the relationship found by Jalink et al., (1999) and the findings of Steckel et al., (1988) who showed a strong negative linear correlation between chlorophyll content and seed maturity in carrot seeds. This shows that chlorophyll can be used as a marker for maturity to separate out immature seeds from the seed lot. Jalink et al., (1999) showed that germination percent could be increased from 90 to 97% by sorting out 13% of the seeds with very high CF (Jalink, 1999).

Suhartanto (2003) reported that light levels during seed development could alter the chlorophyll content of the seed and subsequently tomato seed germination

performance. 45 DAA seeds from fruits covered and grown with limited light had higher mean CF, lower viability, and lower vigor, than seeds produced under normal light conditions (Suhartanto, 2003). This was slightly different from the results in this study (Table 1), since the light treatments (dark harvest, red light and far-red light) did not have any significant effect on the viability of the seeds. There was no significant effect on the T_{50} or the mean CF value following light treatments. However, there were some significant differences due to the genotype of the seeds (Table 1). The mean CF signal of *fri*$^{-1}$ was significantly lower than for 'MoneyMaker' or *tri*$^{-1}$, which suggests that phytochrome A plays a role in chlorophyll accumulation (Fig. 3). The fact that the CF signal for *fri*$^{-1}$ was lower than for 'MoneyMaker' or *tri*$^{-1}$ even at its peak (40 DAA) suggests that *fri*$^{-1}$ chlorophyll accumulation was impaired due to the absence of the phytochrome A gene. These results suggest that phytochrome A plays a role in chlorophyll biosynthesis. An investigation with mustard seedlings suggests that Pfr regulates the synthesis of δ-aminolevulinic acid (ALA), a very early precursor of chlorophyll synthesis (Masoner and Kasemir, 1975). Since there is no evidence that subsequent enzyme activity in the pathway is correlated with light, light-mediated chlorophyll accumulation is regulated at the level of phytochrome-controlled formation of ALA (Masoner and Kasemir, 1975; Ilag, 1994). The *aurea* (*au*) and *yellow-green-2* (*yg-2*) mutants of tomato are unable to synthesize the linear chromophore of phytochrome, resulting in plants with a pale, yellow-colored phenotype. Dark grown seedlings of both mutants have reduced levels of protochlorophyllide (chlorophyll precursor) due to an inhibition of ALA as a precursor (Terry and Kendrick, 1999). Chlorophyll synthesis is closely associated with phytochrome synthesis, since ALA, a

chlorophyll precursor, is regulated by the photoreception of phytochrome. Phytochrome-deficient mutants, such as *au* and *yg-2*, result in a phenotype that lacks green-pigment and chlorophyll deficient mutants (Terry and Kendrick, 1999).

The rate of CF signal decrease was similar in all three genotypes, which suggests that neither phytochrome A nor B affects the rate of chlorophyll degradation in seeds. The phytochrome B mutant, *tri*$^{-1}$, on the other hand, showed lower T_{50} values, and hence greater vigor, compared to the other two genotypes (Table 1). Moreover, the T_{50} of *tri*$^{-1}$ did not change inversely with changing CF levels, rather, seeds with high CF germinated with the same T_{50} as seeds with low CF in this mutant (Fig. 4). This result suggests firstly, that the presence of phytochrome B may exert an inhibitory influence on vigor in tomato seeds, and secondly, that the persistent presence of chlorophyll during seed development does not affect vigor.

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Chapter 4: GENERAL DISCUSSION

4.1 GENERAL DISCUSSION

The objective of this study was to investigate if chlorophyll levels vary with seed maturity in muskmelon and tomato seeds, as suggested by Jalink et al. (1998, 1999) for *Brassica oleracea*. The study was able to confirm that CF is high in immature muskmelon and tomato seeds and it decreases as the seeds mature (Chapter 2, Figure 3). The relationship between seed fluorescence at 665 nm and seed chlorophyll content has been established previously (Jalink et al., 1998, 1999). Although many compounds in seeds fluoresce at 665 nm, chlorophyll A is the predominant compound producing a signal at 665 nm and a vast majority of the signal is due to the fluorescence of chlorophyll A. The study by Suhartanto (2003) has investigated the pigments detected at 665 and 450 nm by HPLC chromatogram. His results have demonstrated that at 450 nm, numerous pigments such as chlorophyll B, Neoxanthine, Lutein, Zeaxnthine and β-carotene are detected at various concentrations. However, at 665nm chlorophyll A was the only detected pigment. This indicates that the fluorescence measured by LIF at 665 nm is primarily chlorophyll A (Suhartanto, 2003).

The second objective of this study was to assess the use of CF as a tool to improve the overall vigor and viability of seed lots. There was a strong negative correlation between viability and vigor of melon seeds and CF measured by the laser-induced fluorescence (LIF) (Chapter 2, Fig. 4). According to these results, we can speculate that removing those high CF seeds from the lot would remove the low vigor, low viability seeds, increasing the overall performance of the seed lot, and thus proving our first hypothesis to be true for muskmelon seeds. CF appears to primarily be an

indicator of seed maturity and not a factor responsible for low vigor in mature seeds. The data do not suggest that oxidative stress caused by free radicals generated by chlorophyll during seed drying is responsible for seed damage. Instead, seeds with high CF do not germinate well because they are not fully developed.

In the case of tomato seeds, the range of fruit maturity used in this study was not sufficiently broad to provide an overall picture of changes in CF during seed maturity. However, based on previous studies performed by Suhartanto (2003) and Jalink et al. (1999) tomato seeds show a strong negative correlation between CF signal and seed vigor. This means that CF can be used to sort out immature, low vigor tomato seeds to improve seed lot performance. Studies by Jalink et al. (1999) have demonstrated that the seed lot was improved from 90 to 97% normal seedlings by sorting out 13% of the seeds with very high CF signals. It is important to note that other seed sorting equipment, such as seed density sorters, can effectively remove immature seeds based on their physical properties. CF sorting may be most useful in removing seeds that have obtained maximum dry mass, but which are still physiologically immature. In muskmelon seeds, for example, 35 and 40 DAA seeds attained their maximum dry mass but still contained higher-than-baseline chlorophyll and exhibited low seed vigor. CF, and not density gradient, sorting could remove these poorly performing seeds from a seed lot.

The third objective of this study was to assess whether post-harvest light treatments during seed harvest and processing of muskmelon and tomato improves seed germination performance. This study showed that light environment during seed harvest affected seed vigor, but not the viability of muskmelon seeds (Chapter 2, Table 1 and 2). The vigor, measured as T_{50} (mean time to germination), increased in some cases

depending on the genotype and the maturity of the seed. Light had a positive effect on mature 'Athena' 55 DAA seeds, as the T_{50} was lower compared to dark harvested seeds (Chapter 2, Table 2). On the other hand, light had a negative effect on mature 55 DAA 'Top Mark' seeds, since the T_{50} increased by harvesting seeds in the light as opposed to harvesting them in the dark (Chapter 2 Table 2). Curiously enough, the vigor of immature 'Top Mark' seeds at 40 DAA was not affected by the light, as there was no significant change in vigor in response to light environment (Chapter 2, Table 1). We were unable to determine whether this change in vigor due to light environment during seed harvest was due to a change in chlorophyll concentration in the seeds. However, this would be an interesting phenomenon to be studied further in future studies.

Another goal of this study was to determine whether chlorophyll developed within the dark fruit or whether it formed during exposure to light during harvesting and processing. While we were unable to accurately determine the answer to this question, we found that both light-harvested and dark-harvested seeds had a similar mean CF signal, which suggests that chlorophyll development in seeds occurs in the absence of light in muskmelon seeds. In the light-independent pathway of chlorophyll synthesis, the protochlorophyllide reduction occurs by an "enzymatically generated electronically excited state" (Raskin and Schwartz, 2003). In the absence of light, protochlorophyllide reduction is achieved via protochlorophyllide oxidoreductase by using chemical energy generated in the enzymatic ascorbate peroxidase system (Raskin and Schwartz, 2003). This allows the plant to bypass the light requirement for chlorophyll biosynthesis and allows for the protochlorophyllide reduction to occur even in the absence of light.

We analyzed whether chlorophyll accumulation in developing seeds is mediated by phytochrome. The analysis was performed with tomato phytochrome mutants since there are no known muskmelon phytochrome mutants. The results in Fig 3 (Chapter 3) showed that the CF signal for *tri*$^{-1}$, phytochrome B mutant decreased faster then the other genotypes. This suggests that phytochrome B affects the rate of chlorophyll degradation in seeds. The result agrees with our hypothesis that phytochrome B influences chlorophyll degradation. The mechanism by which phytochrome mediates chlorophyll degradation was not within the scope of this study. The phytochrome B mutant also showed lower T_{50} values, and hence greater vigor, compared to the other two genotypes (Chapter 3, Table 1). Moreover, the T_{50} of *tri*$^{-1}$ did not change inversely with changing CF levels. Instead, seeds with high CF germinated with the same T_{50} as seeds with low CF in this mutant (Fig 4.). This result suggests that the presence of phytochrome B may exert an inhibitory influence on vigor in tomato seeds, and that the persistent presence of chlorophyll during seed development does not affect vigor.

The distribution of CF signals became narrower as muskmelon seeds matured, indicating that there is less variation in CF values within the seed lot (Chapter 2, Figure 5). Mature seeds were more vigorous, perhaps because the variation within the population was smaller, leading to an overall improvement in the performance of the seed lot. However, the change in distribution of the CF signals as the seeds matured demonstrates that the lot is composed of a population of individual seeds, each with separate characteristics. Mean population comparisons do not adequately represent the performance of the seed lot and the population variance must also be considered.

Findings by Suhartanto (2003) showed that chlorophyll was located in immature (40DAA) tomato seed coats. However at 60 DAA, chlorophyll in coats of the mature seeds in the current study was very low. The decrease in chlorophyll fluorescence of mature seed coats is associated with cell death in the testa (Werker, 1997; Berry and Bewley, 1992). Our results in Chapter 2 (Table 3) demonstrated that chlorophyll in muskmelon seeds is predominantly localized in the endosperm and embryo, and not in the seed coat. This could largely be due to the fact that we used fully mature muskmelon seeds (55 DAA) to localize chlorophyll. Curiously, our study did find that the CF of the seed embryo rapidly decreases when the testa is removed from a 55 DAA muskmelon seed. This decrease in CF may be largely due to the rapid disintegration of chlorophyll in the embryo upon exposure to light. Future experiments should be conducted to separate the endosperm from the embryo and measure the CF of each tissue individually to determine if the chlorophyll is mainly concentrated in the embryo or in the endosperm.

Suhartanto (2003) demonstrated in his study that chlorophyll is not only present in young seeds, but is also photosynthetically active. The potential to assimilate CO_2 was proven by the increase in Rubisco activity, coinciding with the transient peak in starch content (Suhartanto, 2003). Photosynthetic oxygen evolution occurred at a light intensity of greater than 40 $\mu mol.cm^{-2}.s^{-1}$, which lead to the conclusion that the photosystem II is functional in the chloroplast of young tomato seeds. Goffman et al. (2005) showed that chlorophyll in young seeds is photosyntheticaly active and the seeds accumulate photosynthate. Seed chlorophyll content and photosynthetically activity were lower in domesticated tomato species compared to the wild relatives of the species (Suhartanto, 2003).

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