AN ABSTRACT OF THE DISSERTATION OF

Duangporn Angsumalee for the degree of Doctor of Philosophy in Crop Science presented on July 28, 2016.

Title: Effect of Plant Growth Regulator and Irrigation on Physiological and Harvest Maturity of Red Clover in Relation to Seed Quality.

Abstract approved:

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Sabry G. Elias

Red clover (*Trifolium pratense* L.) seed yield can be affected by plant growth regulators (PGR) and irrigation; however, the effects of these factors on physiological maturity (PM), harvest maturity (HM), and seed quality are unknown. The objectives of this study were to: 1) determine how irrigation and trinexapac-ethyl (TE, a PGR) affect PM, HM, seed viability, and seed vigor of red clover at different stages of maturity, 2) evaluate the effect of irrigation, TE and their interaction on seed yield, its components, and the quality of red clover seeds at harvest, 3) investigate changes in gibberellic and abscisic acid contents in red clover during seed development and maturation, and 4) determine the potential of red clover seed storability under different storage conditions over two years. A field study was conducted over a two-year period at Hyslop Research Farm, Corvallis, Oregon. A single irrigation was applied at first flowering stage (BBCH 55). Five rates of TE, ranging from 0 to 700 g a.i. ha^{-1}, were applied at stem elongation and bud

emergence stages (BBCH 32 and BBCH 51, respectively). Seed viability and vigor tests were conducted at Oregon State University Seed Laboratory to measure the effects of treatments on seed quality.

Irrigation delayed PM by four days compared to the non-irrigated treatment. The TE applications did not alter seed maturation. At PM, the flower heads contained light brown petals with brownish-green sepals and seeds were pale green to pale yellow. Heads at HM contained dark brown petals and sepals, whereas seeds turned to yellow or yellow-dark grayish purple. Seed dry weight did not change significantly from PM to HM. Seed moisture content at maximum seed dry weight (PM) ranged from 340 to 540 g kg^{-1} and decreased to below 140 g kg^{-1} at HM. Seed quality as determined by tetrazolium (TZT), standard germination (SGT), and cold tests (CT) were gradually increased during seed development and maturation. The accelerated aging test (AAT) was not a reliable indicator for evaluating vigor of young seeds. At HM, seeds reached maximum quality for all treatments, with 92 - 98% viability by TZT and SGT, and 90 - 94% vigor by CT.

Seed yield was increased by irrigation and TE application, but the interaction between these two treatments was not significant. Irrigation increased seed yield in both years by 10% due to the greater seed weight. However, TE increased seed yield by up to 18% only when applied at stem elongation stage in the second year. The increase in seed yield by TE was attributed to greater number of heads per stem. Neither irrigation nor TE had significant effect on above-ground biomass or stems m^{-2}. Seed viability and vigor were slightly correlated with

thousand-seed weight and stems m^{-2}, respectively. However, none of them significantly affected seed quality. The study revealed that seed yield can be increased by: 1) a single irrigation application during first flowering stage (BBCH 55) in both years; and 2) TE application at a rate of 280 g a.i. ha^{-1} at the stem elongation stage (BBCH 32) in the second-year stand of red clover.

Gibberellic acid (GA_3) and abscisic acid (ABA) are two major phytohormones that affect seed germination. Changes in the contents of GA_3 and ABA from seed development to maturation was conducted using seeds from untreated, TE-treated, irrigated, and TE plus irrigated plots. The GA_3 and ABA were extracted from seeds using the solid phase method and were quantified by the liquid chromatography-tandem mass spectrometry (LC-MS/MS). The ABA content was high (1242 pg g^{-1} DW) at the early stage of seed development, and then gradually decreased to 388 pg g^{-1} DW at HM. The GA_3 content did not change significantly during seed development until HM, ranging from 173 to 187 pg g^{-1} DW. Irrigation and TE application did not significantly affect the endogenous production of GA_3 and ABA in the seeds. The ABA:GA_3 ratio was high (6.7) at the early stage of seed development, but seed germination was low (24%). When seeds reached HM, the ABA:GA_3 ratio dropped to 2.2 and seed germination increased to 93%. These results suggest that physiological dormancy is not a substantial concern in red clover seeds. However, before scarification, seed with hard seed coat at HM was approximately 34%. Hard seeds were scarified before conducting the germination tests.

Maintaining seed quality during storage is essential to ensure value until the time of planting. Two red clover seed lots, untreated and field treated with TE, were stored for 24 months in three conditions: 1) uncontrolled environment of open warehouse (WH), 2) controlled room temperature (RT) at 20°C, and 3) controlled cold storage (CS) at 10°C. Seed quality, i.e., viability and vigor, was determined at 6-month intervals to measure the rate of deterioration after each storage period. Relative humidity (RH) was observed as 55% in RT and 90% in CS. Average seed viability of both seed lots stored in WH and RT and were 96% and 95%, respectively, throughout the 24-month storage period. Seeds stored at RT for 24 months maintained high vigor of 87% as determined by the AAT, whereas seeds stored at WH maintained vigor of 81% for 18 months and then dropped to 67% at the end of the 24-month storage period. In CS, seed viability and vigor gradually dropped, reaching 0% at the end of the 24-month storage period due to the adverse effect of the high RH (90%) in the CS. Seed maintained acceptable viability and vigor standards of above 80% when seed moisture content was less than 10%. This study suggests that red clover seeds from untreated and TE-treated plots can be stored safely under similar WH conditions used in this study for 18 months and in RT for 24 months when the initial seed moisture content is under 10%. The results of this study improved our understanding of the potential storability of the red clover seed in response to TE application.

Effect of Plant Growth Regulator and Irrigation on Physiological and Harvest Maturity of Red Clover in Relation to Seed Quality

by
Duangporn Angsumalee

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Duangporn Angsumalee, Author

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TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF TABLES (Continued)

Effect of Plant Growth Regulator and Irrigation on Physiological and Harvest Maturity of Red Clover in Relation to Seed Quality

CHAPTER 1: Introduction and Literature Review

1.1 Red Clover

Red clover (*Trifolium pratense* L.) is the most important forage legume seed crops and is widely used as rotation crop in Oregon. According to the Extension Economic Information Office at Oregon State University, the estimated sale value of red clover seed produced in 2015 was 12.1 million US dollars from approximately 6,000 hectares of harvested area. Red clover seed production in Oregon was first in the national ranking. It supplied approximately 75% of the U.S. market needs (Oregon Department of Agriculture, 2015).

1.2 Plant Growth Regulators

Foliar applied plant growth regulators (PGRs) have been widely used on temperate grass seed crops in Oregon and other parts of the world. This practice has been adopted as a result of documented seed yield increases and reduction in lodging (Chastain et al., 2014; Chynoweth et al., 2008; Rolston et al., 2010; Zapiola et al., 2006). However, little research has been conducted on the use of PGRs on legume seed crops, such as red clover in Oregon. During the late 1990s, Silberstein et al. (1996) reported a reduction in stem length, a decrease in lodging, and an increase in seed yield with soil applications of paclobutrazol and uniconazole PGRs. These soil applied products are no longer available for commercial use due to longevity in the soil and residual activity on subsequent crops (Hampton, 1996). More recently, the acylcyclohexanedione PGRs

have been introduced as growth retardant for turfgrasses and as a lodging control agent in cereals. One of these compounds is registered for use as a lodging control in grass seed crops in Oregon – Trinexapac-ethyl (TE) [4-(cyclopropyl-α-hydroxymethylene)-3,5-dioxocyclohexane-carboxylic acid ethyl ester].

Trinexapac-ethyl benefits in reducing mowing, suppressing seed head formation, and enhancing turfgrass quality (Fagerness and Yelverton, 2001). The TE is used in seed production to reduce lodging and consequently increase seed yield of grass seed crops, such as perennial ryegrass (*Lolium perenne* L.) (Borm and van den Berg, 2008; Chastain et al., 2014; Chynoweth et al., 2008; Rolston et al., 2010), and tall fescue [*Schedonorus arundinaceus* (Schreb.) Dumort.] (Chastain et al., 2015). The TE blocks 3β-hydroxylation, preventing the formation of active gibberellic acids (GAs) from inactive forms (Rademacher, 2000). The TE suppresses GA biosynthesis. Since GA promotes cell elongation, reducing GA production results in limiting plant height (Rademacher, 2015).

A study conducted in Norway reported that seed yield was increased by 21% in red clover when TE was applied at the stem elongation stage (Øverland and Aamlid, 2007). Based on these results, this product was registered for use on red clover seed production in Norway. Anderson et al. (2015) reported that red clover seed treated with TE increased seed yield up to 16% when TE was applied at 500 g a.i. ha^{-1} at the stem elongation stage under New Zealand and Oregon conditions.

1.3 Irrigation

A single irrigation applied at flowering stage doubled seed yield over the non-irrigated control in red clover (Oliva et al., 1994). Yield components most associated with the irrigation-induced increase in seed yield were the number of seeds per floret and seed weight (Oliva et al., 1994).

Irrigation reduces drought stress and increases seed yield in several crops, such as field bean (*Phaseolus vulgaris* L.) (Efetha et al., 2011), castor (*Ricinus communis* L.) (Severino and Auld, 2013), and red clover (Oliva et al., 1994). Under drought stress, abscisic acid (ABA) endogenous production accumulates at high levels in plant cells. For example, Masoumi et al. (2011) reported that water deficit significantly increased ABA content in soybeans (*Glycine max* L.). Weldearegay et al. (2012) found that ABA content in wheat (*Triticum aestivum* L.) spikelet was significantly less when irrigation was applied. In addition, they found that the increase in ABA content in wheat spikelet was highly correlated with seed set reduction.

1.4 Endogenous Phytohormones

Gibberellin (GA) is a group of plant hormones, which promotes plant growth and development. This group is a tetracyclic diterpenoid acid compound (Gupta and Chakrabarty, 2013). It includes a large number of compounds, abbreviation as GA followed by number in the chronological order of its discovery. However, only a few of the GAs have biological activity. The GA_3, also known as gibberellic acid, is one of the bioactive forms of gibberellin, which is widely studied (Gupta and Chakrabarty, 2013;

Rademacher, 2015; Taiz et al., 2015). One of the functions of GA in plants is to promote cell elongation, resulting in an increase in plant height. Besides promoting longitudinal growth, GA stimulates seed germination, induces flowering, determines sex expression, and enhances pollen, fruit and seed development (Gupta and Chakrabarty, 2013; Rademacher, 2015; Taiz et al., 2015).

Abscisic acid (ABA) is a growth inhibitor phytohormone which contains a 15-carbon terpenoid. It accumulates at high levels in plants exposed to some abiotic stresses during seed development, such as cold, drought, and high salinity (Nambara and Marion-Poll, 2005). The physiological functions of ABA include: a) stomata closure to limit transpiration, b) metabolism modification to tolerate dryness and low temperatures, and c) inhibition of seedling growth. The ABA also suppresses seed germination (Bentsink and Soppe, 2008; Finch-Savage and Leubner-Metzger, 2006; Finkelstein et al., 2008; Kermode, 2005) and enhances seed maturation and dormancy (Taiz et al., 2015).

1.5 Seed Quality, Dormancy, and Storability

Seed quality, as defined by viability and vigor, is maximum when seed reaches either physiological maturity in several crops, such as soybean (Bishnoi et al., 2007) and cuphea (Berti et al., 2007), or harvest maturity, such as in canola (*Brassica napus* L.) (Elias and Copeland, 2001) and red clover (Chapter 2). If seeds were left in the field after full maturation, they gradually deteriorated, especially under adverse weather conditions.

Seed dormancy can be classified as morphological, physiological, physical, and combinational types of dormancy (Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006). Morphological dormancy involves an immature embryo and requires additional time to develop and germinate. Physiological dormancy relates to ABA and GA balance (hormone-mediated seed dormancy). Physical dormancy involves the water-impermeable seed coat (Baskin and Baskin, 2004). Dormancy in red clover and other legume species is well known as hardseededness. This is a type of physical dormancy, which induced by hard seed coat that restricts water imbibition. The water-impermeable seed coat is a genetic trait, which is modified by environment conditions. Hard seed coat is expressed during seed dehydration, the last step of seed development and maturation. It involves the development of tightly bound palisade cell with phenolic and suberin layers (Smýkal et al., 2014). This type of dormancy can be released naturally by temperature changes or artificially by mechanical or chemical scarification (Smýkal et al., 2014; Taiz et al., 2015). However, there is still limited knowledge of hormone-mediated seed dormancy, i.e., physiological dormancy, in red clover.

In most cases during seed development, the GA content is low at the beginning of seed development and increases over time until seed reaches maturation. Unlike GA, the ABA content starts low and increases quickly to the peak level during the early stage of seed development, and then decreases later as seeds mature (Liu et al., 2010; Yang et al., 2006). The ratio of ABA to GA is the major determining factor of physiological dormancy and the ability of seeds to germinate (Taiz et al., 2015). The balance between

the ABA and GA activities in seeds is affected by developmental and environmental factors. During the early stage of seed development, the ABA:GA ratio is high, which favors seed dormancy. After that, ABA decreases while GA increases, which favors seed germination (Taiz et al., 2015). In addition, environmental factors, such as temperature, light, and chemical treatments, can affect the balance between ABA and GA in seed, which either promote or inhibit seed germination (Jha et al., 2010; Kim et al., 2009; Taiz et al., 2015).

Seed deterioration is an irreversible process. Storage conditions should be well managed to ensure seed quality and delay deterioration as much as possible. Seeds are typically stored after harvest until the planting time, which ranges from several months to several years. Ideal storage condition to maintain seed quality is dry and cold storage. Factors that affect seed storability are initial seed quality, seed moisture content, storage temperature and relative humidity, length of storage, and protection from storage fungi and insects (Elias et al., 2007; Vertucci and Roos, 1993). Red clover seed can be safely stored in ambient conditions for up to three years (Taylor and Quesenberry, 1996). Evans (1957) found that moisture content of red clover seed was the most important factor influencing its life span. Seed quality was maintained in a high temperature storage when the relative humidity was low (Evan, 1957).

CHAPTER 2: Effect of Plant Growth Regulator and Irrigation on Physiological and Harvest Maturity of Red Clover (*Trifolium pratense* L.) in Relation to Seed Quality

ABSTRACT

Red clover seed yield is affected by plant growth regulators (PGRs) and irrigation; however, the effects of these factors on physiological maturity (PM), harvest maturity (HM), and seed quality are unknown. This field study determined how trinexapac-ethyl (TE, a PGR) and irrigation affect PM, HM, seed viability, and seed vigor of red clover at different stages of maturity in two harvest years. Five TE rates, ranging from 0 to 700 g a.i. ha^{-1}, were applied at stem elongation (BBCH 32) and bud emergence stages (BBCH 51) and irrigation was applied at first flowering stage (BBCH 55). Physiological and visual indicators of seed maturity were recorded to determine PM and HM. Standard germination (SGT), tetrazolium (TZT), accelerated aging (AAT), and cold (CT) tests were conducted to evaluate seed viability and vigor at weekly intervals beginning after anthesis (BBCH 65) until harvest. Irrigation resulted in a four-day delay in PM compared to the non-irrigated treatment. The TE applications did not affect seed maturation. At PM, the flower heads contained light brown petals with brownish-green sepals and seeds were pale green to pale yellow. Heads at HM contained dark brown petals and sepals, whereas seeds were yellow or yellow-dark grayish purple. Seed dry weight did not change significantly from PM to HM. Seed moisture content at maximum seed dry weight (PM) ranged from 340 to 540 g kg^{-1} and decreased to below 140 g kg^{-1} at HM. Seed quality as determined by SGT, TZT, and CT were gradually increased during

seed development and maturation. At HM, seeds reached maximum quality for all treatments, with 92 - 98% viability by TZT and SGT and 90 - 94% vigor by CT. The AAT was not a reliable indicator for evaluating vigor of young seeds.

Abbreviations: AAT, accelerated aging test; BBCH, Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie (a phenological development stage of a plant); CT, cold test; DAA, days after anthesis; HM, harvest maturity; PGR, plant growth regulator; PM, physiological maturity; SDW, seed dry weight; SGT, standard germination test; SMC, seed moisture content; TE, trinexapac-ethyl; TZT, tetrazolium test; WAA, weeks after anthesis.

2.1 INTRODUCTION

Red clover is the most important forage legume seed crop and is widely used as a rotation crop for grass seed crops and wheat in Oregon. According to the Extension Economic Information Office at Oregon State University, the estimated sale value of red clover seed produced in 2015 was 12.1 million US dollars from approximately 6,000 hectares of harvested area. Oregon was first in the national ranking in red clover seed production and supplied approximately 75% of the U.S. market needs (Oregon Department of Agriculture, 2015).

Foliar applied plant growth regulators (PGRs) have been widely used on temperate grass seed crops in Oregon and other parts of the world. This practice has been adopted due to well documented seed yield increases and reduction in lodging (Chastain et al., 2014; Chynoweth et al., 2008; Rolston et al., 2010; Zapiola et al., 2006). Little research has been conducted on the use of PGRs on legume seed crops worldwide. Silberstein et al. (1996) reported stem length and lodging reduction, and seed yields improvement in Oregon red clover with soil applications of the triazole PGRs, uniconazole and paclobutrazol. These soil applied products are no longer available for commercial use due to persistence in the soil and residual activity on subsequent crops (Hampton, 1996).

More recently, acylcyclohexanedione PGRs have been introduced as growth retardant for turfgrasses grown for seed, and as a lodging control agent in cereals. One of these compounds is registered for use as a lodging control in grass seed crops in

Oregon – Trinexapac-ethyl (TE, Palisade) [4-(cyclopropyl-α-hydroxymethylene)-3,5-dioxocyclohexane-carboxylic acid ethyl ester]. This PGR inhibits gibberellin (GA) biosynthesis by acting as a structural mimic of 2-oxoglutaric acid, a cofactor in the 3β-hydroxylation of GA during the final step of GA biosynthesis (Rademacher, 2000).

A study conducted in Norway reported that seed yield was increased by 21% in red clover when TE was applied at stem elongation stage (Øverland and Aamlid, 2007). Based on these results, TE was registered for use on red clover seed crop in Norway. Anderson et al. (2015) reported that red clover seed production under New Zealand and Oregon environments increased seed yield up to 16% when TE was applied at 500 g a.i. ha^{-1} at the stem elongation stage.

Field observation over the two years of on-farm trials indicated that TE treatment increased seed yield and promoted earlier maturation of the crop, allowing more timely harvest operations (Anderson et al., 2012). However, the effect of early maturation on seed quality of red clover treated with TE is unknown. Moreover, we hypothesized that using PGRs may alter seed development and maturation time with potential effect on seed quality. No work has been done regarding the effect of PGRs on PM and HM in red clover seed crop. However, techniques employed in canola (*Brassica napus* L.) (Elias and Copeland, 2001) were adapted to explain the relationship of PM, HM, and seed quality in red clover seed crops.

Oliva et al. (1994) reported that a single irrigation applied at flowering stage doubled seed yield over the non-irrigated control in red clover. Yield components most

associated with the irrigation-induced increase in seed yield were seed number and seed weight (Oliva et al., 1994). It has been unknown, however, whether irrigation has an effect on maturation and quality of red clover seed.

The objectives of this study were to: 1) determine the effects of PGR and irrigation on the time to reach PM and HM of red clover seed; 2) develop visual and physiological indicators of PM and HM for the benefits of the red clover seed growers; and 3) evaluate the influences of PGR and irrigation on seed quality during PM and HM, as well as the relationship between seed maturity and quality of red clover.

2.2 MATERIALS AND METHODS

2.2.1 Overview and Plant Materials

Field trials were conducted at Oregon State University's Hyslop Crop Science Research Farm (44°38'21"N, 123°11'40"W) Corvallis, Oregon. The soil is classified as Woodburn silt-loam (fine-silty, mixed, mesic Aquultic Argixerolls). Non-certified "common" diploid red clover variety (commonly used by red clover seed growers in Oregon) was planted on 26 Sept. 2011 at a rate of 9 kg ha^{-1} using a 15-row plot-sized drill with 15-cm spaced rows. The field was monitored for a two-year period. The field was mowed and residue was removed from the plots in 15 May 2012 and 3 May 2013, prior to bud emergence. Four honey bee (*Apis mellifera* L.) hives were placed in the trial area to assist pollination during the flowering period. Natural bumble bees (*Bombus* spp.) were also observed in the study field. Plots were harvested for seed yield on 22 Aug. 2012 and 9 Aug. 2013 with a small-plot swather (modified JD 2280) and threshed by a Hege 180 small-plot combine.

2.2.2 Experimental Design

The experimental design was a randomized complete block (RCBD) with a split-plot arrangement of treatments with four replications. The TE treatments were subplots within irrigated and non-irrigated main plots. Individual subplot size was 3.4 m x 15.2 m. The TE was applied to subplots at two application timings and four rates in the first year stand and five rates of TE in the second year stand (Table 2.1). Irrigation was applied to

main plots at first flowering stage (BBCH 55) with 100 mm of water by using a custom-designed Pierce AcreMaster linear system with minimum-drift Nelson sprinklers.

2.2.3 Data Collection

Red clover reached peak flowering stage when 80% of the plants in the trial had open flowers (BBCH 65) in 22 July and 12 July, which was 68 and 70 d after mowing in the 2012 and 2013, respectively. At BBCH 65, five flower heads were randomly selected and tagged with ribbons as representatives for sampling. During July and August of 2012 and 2013, five heads were randomly sampled from each subplot twice each week starting four days after anthesis (BBCH 65) until harvest, following the protocol established by Elias and Copeland (2001). The accumulation of growing degree days (GDDs), the heat accumulation units needed for crop development and maturation, were recorded from the start of flowering through seed development until harvest. The GDD was calculated using the following formula:

$$GDD = \sum \left\{ \left(\frac{T_{max} + T_{min}}{2} \right) - T_{base} \right\}$$

Where T_{max} and T_{min} are the maximum and minimum daily temperatures, respectively, and T_{base} is the base temperature at which the crop grows. The base temperature of 10°C was used. If daily GDD resulted in a negative value, the GDD was recorded as zero (McMaster and Wilhelm, 1997). The accumulated GDDs were calculated by summation of GDD for each day during a period of plant growth and development.

2.2.3.1 Physiological assessment of seed maturity

Determination of seed dry weight: Seed dry weight was evaluated twice each week. At each sample date, four-100 seed samples of each subplot were separated from flower heads by hand and oven dried at 130°C for 2.5 h until constant dry weight was obtained. Seed dry weight was measured by four-digit electronic balance and recorded as mg seed^{-1}. The PM was determined when seed attained maximum dry weight.

Seed moisture determination: Seed moisture content was calculated from each sample date, twice each week, and was determined on a wet-weight basis from four replications of 100 seeds each. The equation below was used to compute seed moisture content (AOSA, 2007):

$$\%\ Moisture\ content = \frac{Seeds\ fresh\ weight - Seeds\ dry\ weight}{Seeds\ fresh\ weight}\ x\ 100$$

The HM was determined when seed moisture content dropped to less than or equal to 140 g kg^{-1}.

2.2.3.2 Visual indicator of PM and HM

Head and seed colors were observed at weekly intervals from anthesis to harvest. Munsell color charts (Munsell, 1977) were used to indicate the color at each maturity stage. Munsell color notation is a numerical scale based on three color attributes; hue (color itself), value (lightness to darkness), and chroma (color saturation) and is written as Hue Value/Chroma or symbolically H V/C (Munsell, 1977).

2.2.3.3 Chlorophyll content determination

Chlorophyll content was estimated by the Minolta Soil Plant Analysis Development (SPAD) 502 chlorophyll meter (Spectrum Technologies, Inc., Aurora, IL). The SPAD value was proportional to the amount of chlorophyll present in the leaves. It determined the green color intensity in the leaves by non-destructive reading based on the quantification of light intensity absorbed by tissue samples. Four top leaves were randomly selected to measure the SPAD value from each plot at the beginning of seed development until PM at weekly intervals. The SPAD value was recorded in the field from 0800 to 1000 h under sunlight condition.

2.2.3.4 Seed quality evaluation

Seed quality was determined at weekly intervals from anthesis to harvest. Seed quality determination included seed viability, including standard germination and tetrazolium tests, and seed vigor, including cold and accelerated aging tests. All tests were replicated four times and adapted from the protocol of the Association of Official Seed Analysts (AOSA, 2009; AOSA, 2012) as follows:

Standard germination test (SGT): Four replications of 25 seeds each were planted on the top of two layers of moistened germination paper, which were placed into transparent boxes inside a 20°C growth chamber. The number of normal seedlings was recorded 7 d after planting.

Tetrazolium test (TZT): Four replications of 25 seeds each were evaluated by a TZT test. Seeds were placed on the top of moistened blue blotter paper at 20°C for 12 h.

Seeds were then placed in a 1% TZ solution at 35°C for 5 h. The evaluation was conducted by observing the pattern and intensity of the stained tissues under a stereo microscope. Seeds were classified into viable and non-viable.

***Cold test (CT)*:** Four replications of 25 seeds each were evaluated by a CT test. Seeds were placed on the top of moistened germination paper and incubated at 10°C for 7 d, and then moved into 20°C for 7 d. Afterward, the number of normal seedlings was recorded.

***Accelerated aging test (AAT)*:** Seeds were placed in a single layer on mesh tray inside a plastic box containing 50 ml of water. The plastic boxes were incubated in a chamber at 41°C for 72 h. Four replications of 25 seeds each were planted and evaluated after 7 d as described in the standard germination test.

Hard seeds are generally found in mature red clover and many other crops in Fabaceae (Copeland and McDonald, 2001). Hard seeds are impermeable to water and gases. At the end of each seed quality test, seeds with hard seed coats were scarified to allow water imbibition. The process was conducted with a mechanical PSS1000 pneumatic seed scarifier (Mater International, Inc., Corvallis, OR) using air pressure of 40 psi for 60 seconds. The scarified seeds were retested to determine their quality as explained above. Relationship between the highest seed quality (when seed reach maximum viability and vigor) and optimum harvest time was determined.

2.2.4 Data Analysis

Analysis of variance (ANOVA) was conducted to determine the effects of treatments (irrigation and TE) and seed maturity stages on seed moisture content, dry weight, SPAD value, and seed qualities using the statistical package MSTAT (Michigan State Univ., East Lansing, MI). Means of those response variables were separated by Fisher's protected LSD test at the 0.05 probability level, whenever the effects of factors were significant. A regression analysis was conducted to determine the relationship between the accumulated GDDs and DAA.

2.3 RESULTS AND DISCUSSION

2.3.1 Growing Season Environment

Monthly precipitation in the red clover field during the growing season (May, June, July, and August) was 71, 58, 12, and 1 mm in 2012; and 58, 33, 0, and 8 mm in 2013, respectively (Fig.2.1 and Table 2.2). The total precipitation in the four-month growing season was 142 and 99 mm in 2012 and 2013, respectively, which was lower than the 119-year average by 27 mm in 2012 and 70 mm in 2013. The total precipitation for the growing season in 2013 was 30% lower than that in 2012. In addition, the precipitation in winter and early spring months preceding regrowth in May was less in 2013 than in 2012 (Fig.2.1). However, precipitation in both years was sufficient to promote vegetative growth. Nearly zero precipitation was measured during seed development and maturation in either year, which was ideal for seed maturation. The dry summer allowed native bumble bee and honey bee to effectively assist in the pollination process, resulting in high seed yield.

The average monthly temperature during the growing season was 17°C in 2012 and 18 °C in 2013, which was higher than the 119-year average (16°C) by 1°C and 2°C in 2012 and 2013, respectively (Fig. 2.2, Table 2.2). The accumulated GDD during the growing season was higher in 2013 than in 2012, which was 419 and 372, respectively (Fig. 2.3).

2.3.2 Physiological Characteristics and Visual Indicators of PM and HM

2.3.2.1 Seed dry weight

Seed dry weight (SDW) was significantly affected by seed maturity stage, TE application, and irrigation. The interactions among these factors were also significant (Table 2.3). The SDW gradually increased during seed development for all treatments. The SDW reached maximum, 1.75 mg seed^{-1}, at 3 weeks after anthesis (WAA), which was defined as PM, and stayed without significant change until HM. The average SDW was 0.43, 1.18, 1.75, 1.77, and 1.76 mg seed^{-1} for 1, 2, 3, 4, and 5 WAA, respectively (Table 2.4).

Higher TE rates resulted in lower SDW. The average SDW was 1.45, 1.40, 1.38, 1.34, and 1.32 mg seed^{-1} for 0, 140, 280, 420, and 560 g TE ha^{-1}, respectively. The average SDW from irrigated plots was 1.40 mg seed^{-1}, which was slightly heavier than those from non-irrigated plots, 1.36 mg seed^{-1} (Table 2.4).

2.3.2.2 Seed moisture content

Seed moisture content (SMC) significantly changed during seed maturity stage, between years and between irrigated and non-irrigated plots. The interactions among these factors was also significant. However, moisture content was not altered by the application of TE (Table 2.3). In general, SMC decreased as the seeds developed and matured regardless of the treatments included in the study. The SMC at maximum SDW (3 WAA) ranged from 340 to 540 g kg^{-1} and dropped to nearly 140 g kg^{-1} at 4 and 5 WAA, which was defined as HM (Fig. 2.4 and Fig. 2.5). The average SMC from irrigated plots

was 422 g kg^{-1}, which was higher than those from non-irrigated plots, 382 g kg^{-1} (Table 2.4).

2.3.2.3 Time to PM and HM

The periods from anthesis (BBCH 65) to reach PM and HM were different between irrigation and non-irrigation treatments as well as between years. However, they were similar with all TE treatments. As a result, PM and HM are presented separately by irrigation treatments and years in Fig. 2.4 and 2.5. In non-irrigated plots, PM was attained at 3 WAA in 2012 and 3 d earlier in 2013 (Fig. 2.4). The PM of irrigated plots in 2012 and 2013 also followed same pattern (Fig. 2.5). Irrigation resulted in an approximate four-day delay in PM and HM compared to the non-irrigated treatment in both years (Fig. 2.4 and 2.5). The early seed maturation in 2013 might have been caused by higher temperature and less precipitation compared to 2012 (data shown in section 2.3.1). The time at which red clover seeds reached HM was 7 d after PM in all treatments in both years.

Although the time from anthesis to PM was different between 2012 and 2013, accumulated GDD was similar. The relationship between GDD and days after anthesis (DAA) was explained by the following regression equations: 1) For 2012: $Y = 10.23X - 12.60$ ($R^2 = 0.996$, $P < 0.001$); and 2) For 2013: $Y = 10.57X + 8.38$ ($R^2 = 0.998$, $P < 0.001$); where Y = GDD and X = DAA. The GDD from anthesis to PM for non-irrigated red clover was similar at 202 and 199 in 2012 and 2013, respectively. The GDD from anthesis to PM for irrigated plots was 233 and 230 for 2012 and 2013, respectively. The GDD from

anthesis to HM for the non-irrigated treatment was slightly higher in 2012 than in 2013 at 274 and 262, respectively, whereas those for irrigated plots were consistent and similar in both years at 305 and 304, respectively. The variability in environments between years is not unusual. Such variation was reported in several studies, such as in canola (Elias and Copeland, 2001), soybean (*Glycine max* L.) (Crookston and Hill, 1978; Tekrony et al., 1979) and cuphea (*Cuphea viscosissima* Jacq.) (Berti et al., 2007).

2.3.2.4 Visual indicator of PM and HM

Gradual change in seed color was observed with progressive development and maturity (Table 2.5). At the very early stages of seed formation, seeds were light green [5GY (7/6 – 8/6)]. With further development, the seeds turned to pale yellowish green [5GY (6/4) – (7/4)]. At PM, the seeds ranged from pale green to pale yellow [2.5Y (5/4 – 8/6)] and heads contained light brown petals with brownish green sepals. The seeds were firm but not hard and could be smashed with fingernails. At HM, seeds contained yellow [5Y (8.5/6)] or yellow – dark grayish purple [5Y (8.5/6) – 5RP (3/2)], whereas heads turned to dark brown. Seeds were hard and easy to separate from the heads. Therefore, the change in seed color and accompanying changes in head color can be dependable indicators of PM and HM in red clover. Visual indicators of PM have also been recommended for canola (Elias and Copeland, 2001), soybean (Crookston and Hill, 1978; Tekrony et al., 1979; Gibkpi and Crookston, 1981), bird vetch (*Vicia cracca* L.) (Wang et al., 2008), Zinnia (*Zinnia violacea* Cav.) (Miyajima, 1997) and physic nut (*Jatrapha curcas* L.) (Silva et al., 2011).

2.3.2.5 Chlorophyll content by SPAD value

The degree of green canopy that is reflected is a magnitude of chlorophyll present, therefore the color of the canopy was used as visual indicator of PM and HM. The SPAD value, represented as chlorophyll content, significantly changed during seed development and between irrigated and non-irrigated treatments (Table 2.3). The TE application and year had no significant effect on the magnitude of chlorophyll in the red clover plants. The interaction between irrigation and seed maturity stage and between TE rate and seed maturity were significant.

The average SPAD value across all treatments decreased over time of seed development (Table 2.4). The SPAD value of non-irrigated plots was lower than irrigated ones. The values were 50, 46, and 42 in non-irrigated plots compared to 53, 51, and 50 in irrigated plots for 1, 2, and 3 WAA, respectively (Fig. 2.6). The faster drop in SPAD values is explained by the earlier seed maturation found in the non-irrigated treatment.

The average SPAD value for TE-treated plots did not differ from untreated plots for each maturity stage. However, the value differed among seed maturity stages. The SPAD values decreased towards seed maturation for all TE rates. At early stage of seed development, SPAD values were not altered by TE application, i.e., 52 and 49 for all TE treatments in 1 and 2 WAA, respectively. At 3 WAA, the SPAD values of TE-treated plots were slightly lower at 46, compared with the untreated control (48) (Fig. 2.6). This result was in contrast to the study of Espindula et al. (2009) in flag leaves of wheat (*Triticum aestivum* L.) where SPAD values were increased by TE application. Espindula et al. (2009)

reported that TE may have reduced cell elongation and increased tissue density in wheat. However, TE did not increase SPAD values in this red clover study. A possible explanation is that TE reduced stem length in red clover (data shown in chapter 3) but did not affect tissue density in red clover leaves.

2.3.3 Seed Quality Characteristics

Red clover seed from all treatments attained maximum quality for viability and vigor at HM. Changes in quality during seed development and maturation was affected by irrigation and TE application as follows:

2.3.3.1 Tetrazolium test

Seed viability determination by the TZT was significantly affected by TE rate, irrigation, seed maturity stage, and year. The interactions among these factors were also significant (Table 2.3). Timing of TE application did not significantly affect TZT results (Table 2.4). The TE rate slightly altered TZT results. The viability percentages were 87.2%, 89.1%, 88.5%, 90.9%, and 88.8% for 0, 140, 280, 420, and 560 g TE ha^{-1}, respectively (Table 2.4). The average percentage of viability for irrigated plots over all other treatments was 84.7%, which was significantly lower than the non-irrigated (93.1%). This may be due to the fact that seed viability in irrigated plots increased at a slower rate than in non-irrigated plots during seed maturation in both years. For example, in 2013, the result of TZT was 74% - 88% at 3 WAA for irrigated plots and was 89% - 97% for non-irrigated plots (Table 2.6). However, seed viability by TZT at HM (4 WAA) was not significantly different among TE rate, TE timing, and irrigation treatments,

ranging from 94% to 98% (Table 2.6 and Fig. 2.7). This suggests that red clover seeds reach maximum viability at HM. This agrees with an earlier report for canola (Elias and Copeland, 2001) which indicated maximum seed quality at HM. This results disagree, however, with TeKrony et al. (1984) who reported maximum seed quality for soybean being achieved at PM.

2.3.3.2 Standard germination test

Seed viability by SGT was significantly affected by TE rate, irrigation, seed maturity, and year. The interactions among these factors were also significant (Table 2.3). Timing of TE application did not affect SGT results significantly (Table 2.4). The TE rates slightly altered SGT results. The germination percentages were 84.5%, 85.8%, 85.6%, 87.4%, and 87.3% for 0, 140, 280, 420, and 560 g TE ha^{-1} (Table 2.4). The percentage of germination for irrigated plots averaged 81.9%, which was significantly lower than the non-irrigated plots (90.3%). This is because seed viability in irrigated plots increased as seeds matured at a slower rate than those in non-irrigated plots in both years (Table 2.6). For example, in 2013, the SGT result at 3 WAA was increased to 74% - 80% for irrigated plots and was increased to 77% - 86% for non-irrigated plots (Table 2.6). However, seed viability by SGT at HM was not significantly different among TE rate, TE timing, and irrigation treatments, ranging from 92% to 97% (Table 2.6 and Fig. 2.7). The SGT results were similar to those obtained by the TZT. Both SGT and TZT can be used to estimate seed viability in red clover.

2.3.3.3 Cold test

Seed vigor by CT was significantly affected by TE rate, irrigation, seed maturity, and the interactions among them (Table 2.3). Timing of TE applications did not significantly affect CT results. The vigor percentages were 68.2% and 67.5% for application at stem elongation and bud emergence stages, respectively. The TE rates slightly altered CT results, which were 67.4%, 67.5%, 69.4%, 66.3%, and 68.7% for 0, 140, 280, 420, and 560 g TE ha^{-1} (Table 2.4). The average seed vigor for irrigated plots was 60.9%, which was significantly lower than non-irrigated plots (74.8%). This may be a result of the slower increase in vigor rate of irrigated plots, compared to non-irrigated plots (Table 2.6). For example, in 2013, CT results ranged between 10% and 26% 3 WAA for irrigated plots compared to 14% - 33% for non-irrigated plots. However, seed vigor by CT at HM was not significantly different among TE and irrigation treatments. Seed reached maximum vigor of 90% - 94% at HM (Table 2.6 and Fig. 2.7). This indicates that the cold stress (10°C) in CT did not affect the vigor of red clover seed at HM. Similar results were found in canola (Elias and Copeland, 2001).

2.3.3.4 Accelerated aging test

Unlike the other seed quality tests, seed vigor percentage by AAT fluctuated greatly among all treatments (data not shown). The result of the first year study showed that the AAT was not a reliable method to measure vigor in developing seed of red clover. Therefore, this test was excluded from the second year of the study. However, this test is valuable with fully matured seeds (AOSA, 2009).

Seed quality improved after PM and reached maximum quality at HM for all treatments. For example, in 2013, the average seed viability at PM was 82% by TZT and 77% by SGT, while seed vigor by CT was 19% (Fig. 2.7). At HM, seed viability increased to 96% and 94% by TZT and SGT, respectively. The vigor by CT result greatly improved to 92%. Several studies observed that maximum seed quality was attained at PM in various crops, such as soybean (Bishnoi et al., 2007; Tekrony et al., 1984), cuphea (Berti et al., 2007), triticale (*Triticale hexaploid* L.) (Bishnoi, 1974), wheat (Rasyad et al., 1990), maize (*Zea mays* L.) (Tekrony and Hunter, 1995), physic nut (*Jatropha curcas* L.) (Silva et al., 2011), perennial legume plant (*Vicia cracca* L.) (Wang et al., 2008), and sweet pepper (*Capsicum annuum* L.) (Vidigal et al., 2011). However, maximum seed quality was attained at HM in other crops, such as canola (Elias and Copeland, 2001), tomato (*Solanum lycopersicum* L.) (Demir and Ellis, 1992; Demir and Samit, 2001), rice (*Oryza sativa* L.) (Ellis et al., 1993), common bean (*Phaseolus vulgaris* L.) (van de Venter et al., 1996; Ghassemi-Golezani and Mazloomi-Oskooyi, 2008), lignosus bean (*Dipogon lignosus* L. Verdc.) (Fakir et al., 2013) and cowpea (*Vigna sinensis* L.) (Eskandari, 2012). The difference in attaining maximum seed quality among crops may be due to a difference in physiological changes during seed development and maturation, such as changes in seed hormone ratio, especially gibberellic and abscisic acids (data shown in chapter 4).

2.4 CONCLUSIONS

Neither rate nor timing of TE application had significant effect on attaining PM or HM of red clover compared to untreated control. Irrigation treatment, however, delayed PM and HM by approximately four days. Seed quality, viability and vigor, reached maximum levels at HM and was not significantly different among TE and irrigation treatments. To achieve maximum seed yield, harvesting after PM and not beyond HM can help to avoid the negative effects of unfavorable weather conditions, e.g., rainfall and wind, to reduce seed loss by shattering. The crop can be swathed approximately three to four weeks after anthesis and threshed one week later.

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Table 2.1. Trinexapac-ethyl (TE) application timing and rate used in the first- and second-year stands of red clover grown for seed.

Application timing (BBCH scale)	TE application rate In the first-year stand (g a.i. ha^{-1})	TE application rate In the second-year stand (g a.i. ha^{-1})
Untreated control	0	0
Stem elongation (BBCH 32)	140	140
	280	280
	420	420
	560	560
	-	700
Bud emergence (BBCH 51)	140	140
	280	280
	420	420
	560	560
	-	700

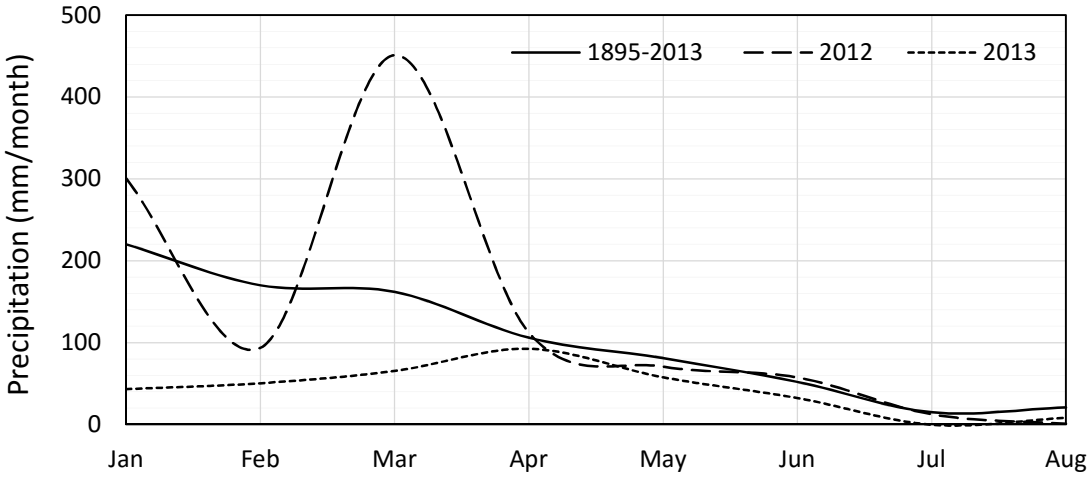

Figure 2.1. Monthly precipitation (mm) during the study period and long-term (1895-2013) monthly mean precipitation for Corvallis, Oregon.

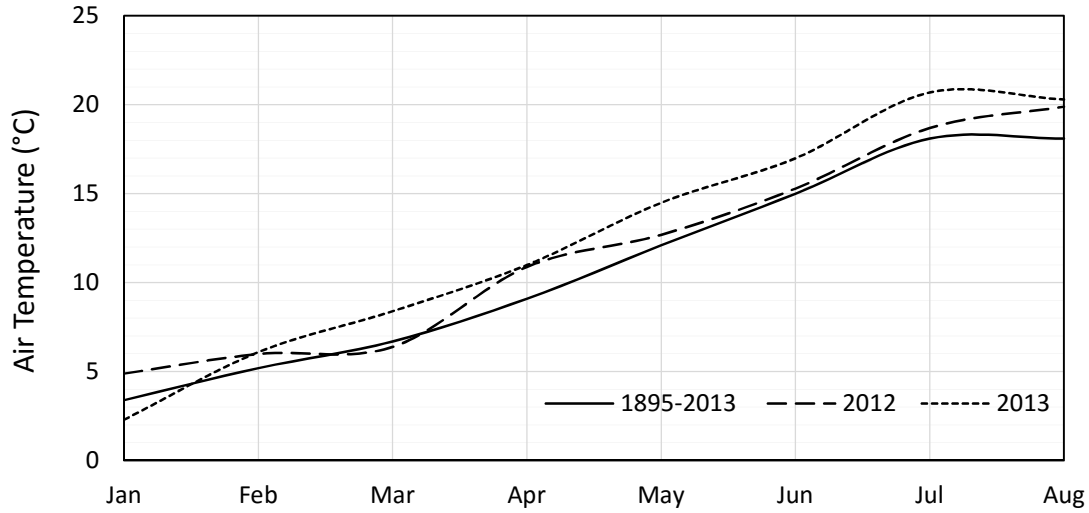

Figure 2.2. Monthly mean temperature (°C) during the study period and long-term (1895-2013) monthly mean temperature for Corvallis, Oregon.

Table 2.2. Total precipitation (mm) and monthly mean temperature (°C) during growing season in 2012 and 2013.

	May	Jun	July	Aug	Sum/Average †	Anomaly ‡
Total Precipitation	----------	----------	----------	mm	----------	----------
1895-2013	81	52	15	21	169	0
2012	71	58	12	1	142	-27
2013	58	33	0	8	99	-70
Mean temperature	----------	----------	----------	°C	----------	----------
1895-2013	12	15	18	18	16	0
2012	13	15	19	20	17	+1
2013	15	17	21	20	18	+2

† Sum for precipitation/average for monthly temperature.

‡ Anomaly, a departure from the 119-year average of rainfall and temperature for Corvallis, Oregon.

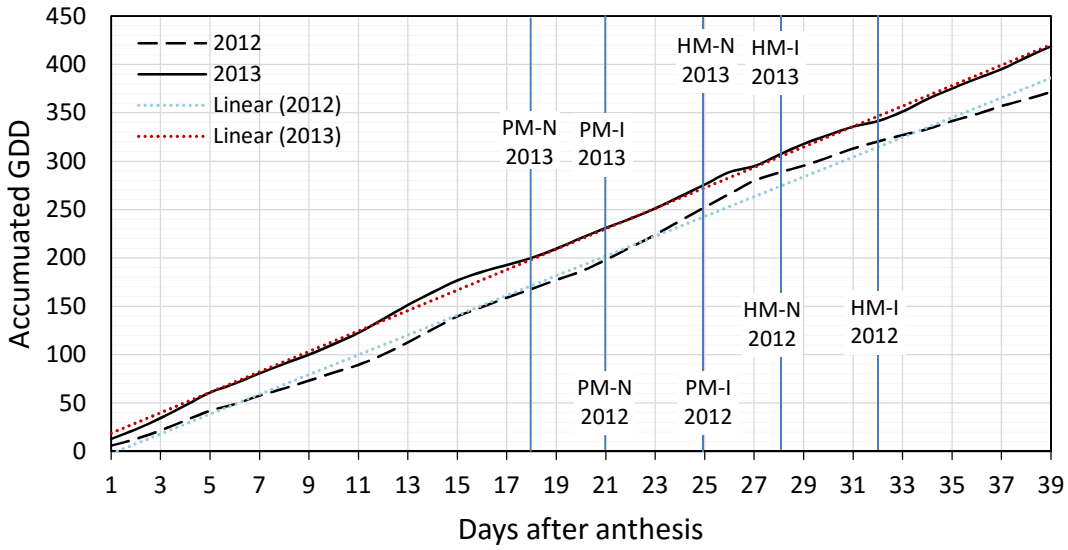

Figure 2.3. Relationship between accumulated growing degree days (GDD) and days after anthesis (DAA) in 2012 and 2013.
For 2012, $Y = 10.23X - 12.60$, where X = DAA and Y = GDD, $P < 0.001$, $R^2 = 0.996$.
For 2013, $Y=10.57X + 8.38$, where X = DAA and Y = GDD, $P < 0.001$, $R^2 = 0.998$.
PM = Physiological maturity, HM = Harvest maturity, N = non-irrigation, and I = irrigation.

Table 2.3. Analysis of variance for the effects of year, irrigation and trinexapac-ethyl (TE) application on red clover seed dry weight (SDW), moisture content (SMC), chlorophyll content (CC), and seed quality by tetrazolium (TZT), standard germination (SGT), and cold (CT) tests.

Source of variation	df	DW	SMC	df	CC	TZT	SGT	CT
Year (Y) †	1	ns††	**	1	ns	***	***	ns
Irrigation (I) ‡	1	*	***	1	***	***	***	***
YI	1	ns	**	1	ns	***	***	***
Timing of TE application (T) §	1	***	ns	1	ns	ns	ns	ns
YT	1	***	ns	1	ns	ns	ns	ns
IT	1	ns	ns	1	ns	ns	ns	ns
YIT	1	ns	ns	1	ns	ns	ns	ns
Rate of TE application (R) ¶	4	***	ns	4	ns	**	*	*
YR	4	***	ns	4	ns	ns	ns	ns
IR	4	ns	ns	4	ns	***	**	*
YIR	4	**	ns	4	ns	**	*	**
TR	4	***	ns	4	ns	*	ns	ns
YTR	4	***	ns	4	ns	ns	ns	*
ITR	4	ns	ns	4	ns	*	ns	**
YITR	4	ns	ns	4	ns	*	ns	ns
Seed maturity (M) #	4	***	***	2	***	***	***	***
YM	4	***	***	2	***	***	***	***
IM	4	***	***	2	***	***	***	***
YIM	4	***	***	2	*	***	***	***
TM	4	ns	ns	2	ns	ns	ns	ns
YTM	4	ns	ns	2	ns	ns	ns	ns
ITM	4	**	ns	2	ns	ns	ns	ns
YITM	4	**	ns	2	ns	ns	ns	ns
RM	16	***	ns	8	*	***	ns	ns
YRM	16	***	ns	8	**	*	ns	ns
IRM	16	***	ns	8	ns	**	ns	***
YIRM	16	*	ns	8	ns	**	**	***
TRM	16	ns	ns	8	ns	*	ns	ns
YTRM	16	ns	ns	8	ns	***	ns	***
ITRM	16	ns	ns	8	ns	ns	ns	ns
YITRM	16	ns	ns	8	ns	ns	ns	**

*, **, *** Significant at the 0.05, 0.01, and 0.001 probability level, respectively.

† Year: 2012 and 2013.

‡ Irrigation: none and single irrigation.

§ Timing of TE application: at stem elongation (BBCH32) and bud emergence (BBCH51) stages.

¶ Rate of TE application: 0, 140, 280, 420, 560 g TE ha^{-1}.

Seed maturity: five stages (1 – 5 WAA) for DW and MC and three stages (1 – 3 WAA and 3 – 5 WAA) for CC and quality tests, respectively.

†† ns: nonsignificant.

Table 2.4. Means of seed dry weight (SDW), moisture content (SMC), chlorophyll content (CC), and seed quality by tetrazolium (TZT), standard germination (SGT), and cold (CT) tests of red clover as affected by year, irrigation, trinexapac-ethyl (TE) timing and rate, and seed maturity.

Factor	SDW	SMC	CC	TZT	SGT	CT
	mg seed^{-1}	g kg^{-1}	SPAD	----- Viability, % ----		Vigor, %
Year						
2012	1.39 a †	426 a	50.1 a	84.5 b	82.2 b	68.2 a
2013	1.36 a	377 b	47.5 a	93.3 a	90.0 a	67.5 a
Irrigation						
Single irrigation	1.40 a	422 a	51.3 a	84.7 b	81.9 b	60.9 b
None irrigation	1.36 b	382 b	46.3 b	93.1 a	90.3 a	74.8 a
TE timing						
Stem elongation	1.39 a	400 a	48.7 a	89.2 a	86.0 a	68.2 a
Bud emergence	1.36 b	403 a	48.9 a	88.6 a	86.2 a	67.5 a
TE Rate (g a.i. ha^{-1})						
0	1.45 a	402 a	48.8 a	87.2 b	84.5 b	67.4 ab
140	1.40 b	404 a	48.6 a	89.1 ab	85.8 ab	67.5 ab
280	1.38 c	397 a	48.7 a	88.5 b	85.6 ab	69.4 a
420	1.34 d	401 a	49.2 a	90.9 a	87.4 a	66.3 b
560	1.32 d	405 a	48.7 a	88.8 b	87.3 a	68.7 a
Seed maturity (WAA)						
1	0.43 c	733 a	51.5 a	-	-	-
2	1.18 b	616 b	48.7 b	-	-	-
3	1.75 a	435 c	46.1 c	79.4 c	72.8 c	31.1 c
4	1.77 a	137 d	-	90.2 b	89.1 b	80.6 b
5	1.76 a	88 e	-	97.2 a	96.5 a	91.9 a

† Within each column and by factor, means followed by the same letter are not significantly different by Fisher's protected LSD values ($P \leq 0.05$).

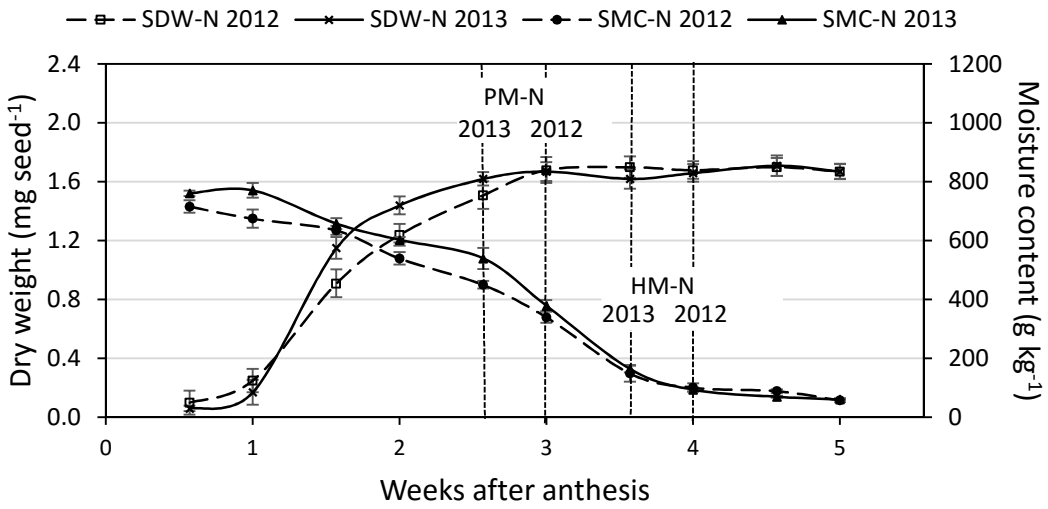

Figure 2.4. Seed dry weight (SDW) and moisture content (SMC) of non-irrigated (N) red clover during seed development in 2012 and 2013.
If error bars regions do not overlap, treatments are significantly different. Seed dry weight and moisture content curves represent the mean of all PGR treatments in the non-irrigated plots. (PM=physiological maturity and HM=harvest maturity).

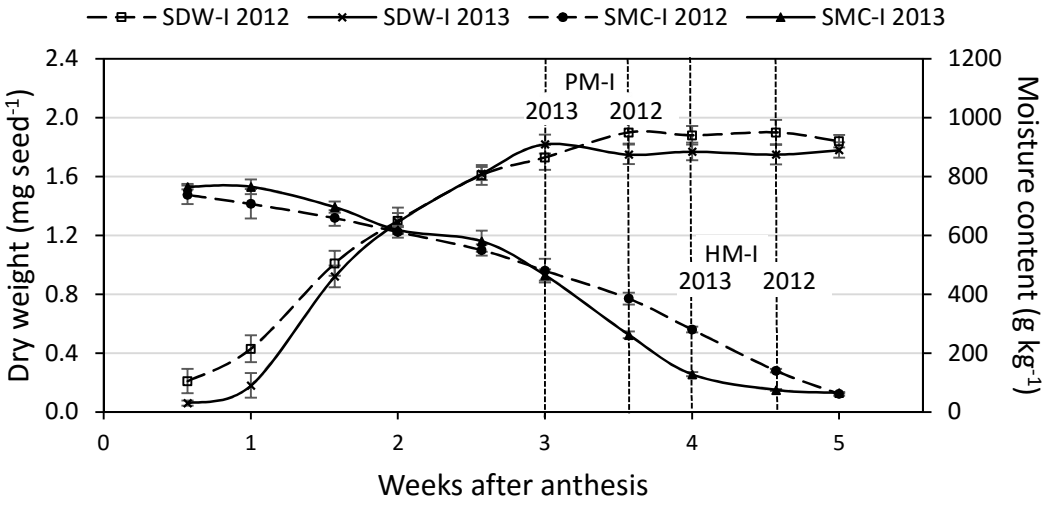

Figure 2.5. Seed dry weight (SDW) and moisture content (SMC) of irrigated (I) red clover during seed development in 2012 and 2013.
If error bars regions do not overlap, treatments are significantly different. Seed dry weight and moisture content curves represent the mean of all PGR treatments in the irrigated plots. (PM=physiological maturity and HM=harvest maturity).

Table 2.5. Seed color at different stages of seed development of red clover averaged over two years (2012 and 2013).

Weeks after anthesis (WAA)	Seed color [Munsell code]	Head color
1	Light green [5GY (7/6 – 8/6)] §	Pale pink petals with green sepals
2	Pale yellowish green [5GY (6/4 – 7/4)]	Pale to light brown petals with green sepals
3 (PM)†	Pale green to pale yellow [2.5Y (5/4 – 8/6)]	Light brown petals with brownish-green sepals
4-5 (HM)‡	Yellow or yellow-dark grayish purple [5Y (8.5/6), 5Y (8.5/6) - 5RP (3/2)]	Dark brown petals with light to dark brown sepals

† PM, physiological maturity.

‡ HM, harvest maturity.

§ Color code as indicated in Munsell color charts.

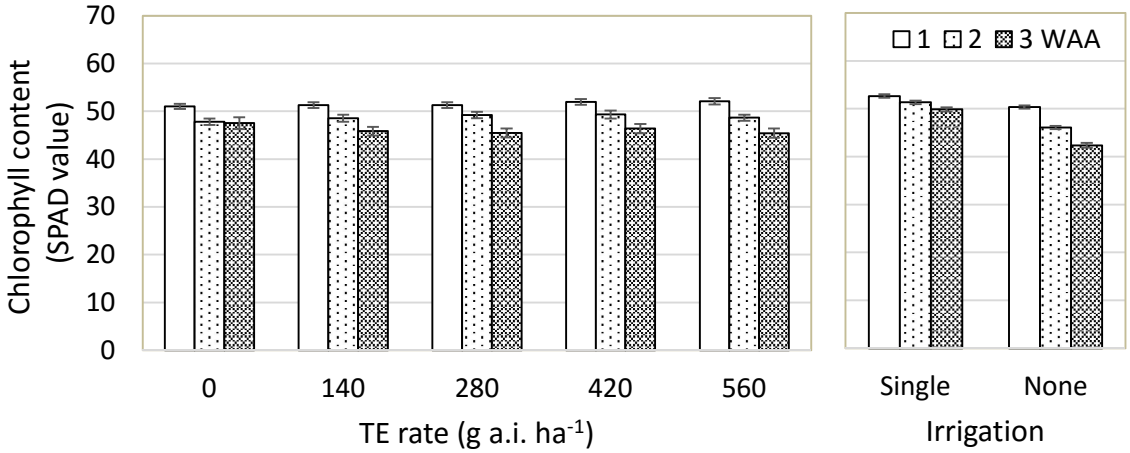

Figure 2.6. Change in chlorophyll content (SPAD value) during seed development (1, 2, and 3 weeks after anthesis, WAA) of red clover affected by trinexapac-ethyl (TE) and irrigation application.
Data were the average of two years (2012 and 2013). If errors bars regions do not overlap, treatments are significantly different.

Table 2.6. Effects of irrigation and trinexapac-ethyl (TE) application on seed quality at different stages of seed development and maturation of red clover as measured by tetrazolium (TZT), standard germination (SGT), and cold (CT) tests in 2012 and 2013.

Year	Irrigation	TE Rate	TZT			SGT			CT		
			wk			wk			wk		
			3	4	5	3	4	5	3	4	5
		g a.i. ha^{-1}	viability (%)			viability (%)			vigor (%)		
2012	Single	0	50	66	98	47	66	95	18	48	93
		140	58	76	98	46	73	96	18	56	92
		280	57	73	97	54	71	97	33	54	91
		420	82	80	97	61	81	97	19	50	92
		560	75	68	98	69	72	97	28	48	94
	None	0	79	98	98	77	94	96	71	89	95
		140	82	96	98	80	97	98	64	85	91
		280	79	97	98	77	95	96	53	89	95
		420	80	92	95	79	93	97	58	85	92
		560	79	95	97	78	95	96	62	88	94
			LSD = 8.25 †			LSD = 7.43			LSD = 7.66		
2013	Single	0	78	95	98	76	93	96	16	92	89
		140	84	96	97	80	94	96	14	93	93
		280	81	96	97	74	92	96	26	91	91
		420	88	97	97	80	94	98	10	91	90
		560	74	94	97	76	94	98	17	91	89
		700	84	96	96	77	94	96	19	92	92
	None	0	90	98	98	82	95	97	14	92	92
		140	91	97	96	77	96	98	23	90	91
		280	94	96	98	86	95	96	24	93	92
		420	89	97	97	78	97	96	26	93	90
		560	97	95	95	80	96	98	28	94	92
		700	91	98	98	79	96	96	33	90	95
			LSD = 5.51			LSD = 5.90			LSD = 5.94		

† Within each test and by years, any two means exceed the LSD value are significantly different at $P \leq 0.05$.

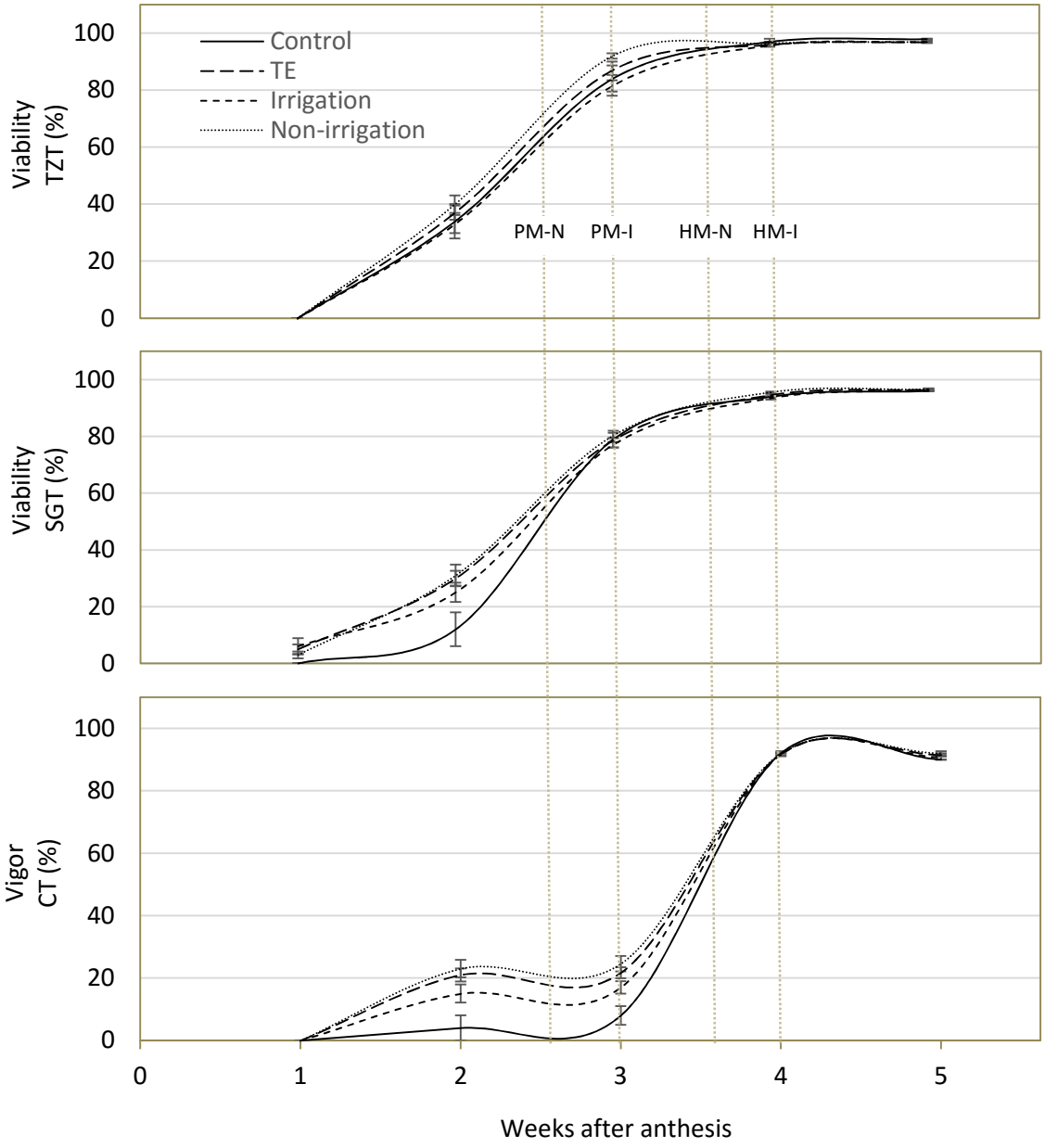

Figure 2.7. Tetrazolium (TZT), standard germination (SGT), and cold (CT) tests on red clover seed in 2013.
Control data were averaged from untreated subplots. TE data were averaged over rate and timing of TE applications, irrigation data were averaged over irrigated treatment, and non-irrigation data were averaged over non-irrigated treatment. If error bars regions do not overlap, treatments are significantly different.

CHAPTER 3: Plant Growth Regulator and Irrigation Effects on Seed Yield and Yield Components of Red Clover (*Trifolium pratense* L.) in Relation to Seed Quality

ABSTRACT

Red clover seed yield and quality can be affected by plant growth regulators (PGRs) and irrigation. This study determined the effect of irrigation, trinexapac-ethyl (TE, a PGR) [4-(cyclopropyl-α-hydroxymethylene)-3,5-dioxocyclohexane-carboxylic acid ethyl ester], and their interaction on seed yield, yield components, and the quality of red clover seed. Five TE rates, ranging from 0 to 700 g a.i. ha^{-1}, were sprayed at stem elongation (BBCH 32) and bud emergence stages (BBCH 51). The TE treatment subplots were randomly arranged in a randomized complete block design within irrigated and non-irrigated main plots. Single irrigation was applied at first flowering stage (BBCH 55). Observations were recorded for seed yield, canopy characteristics, yield components, including above-ground biomass, harvest index, seed weight, number of flower heads per stem, number of stems per m^2 and seed size. Seed quality, i.e., viability and vigor, was evaluated by standard germination test and cold test. The study was conducted over two-year period. Seed yield was increased by irrigation and TE application, but the interaction between these two treatments was not significant. The increase in seed yield of irrigated plots in both years was a result of increased seed weight. However, TE increased seed yield only when applied at stem elongation in the second year. The increase in seed yield by TE was attributed to greater number of flower heads per stem. Neither irrigation nor TE had a significant effect on above-ground biomass or number of

stems per m^2. Seed quality from all treatments was similar with viability and vigor between 92 and 97%. Viability and vigor were slightly correlated with seed weight and stem m^{-2}, respectively. However, none of the measured yield components significantly affected seed quality. This study revealed that seed yield can be increased by: 1) single irrigation application during first flowering stage; and 2) TE application at a minimum rate of 280 g a.i. ha^{-1} at stem elongation stage in the second year stand of red clover.

Abbreviations: BBCH, Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie (a phenological development stage of a plant); GA, gibberellin; HI, harvest index; PGR, plant growth regulator; TE, trinexapac-ethyl.

3.1 INTRODUCTION

Red clover is one of the important forage legume crops and widely used as a rotation crop in Oregon. According to The Extension Economic Information Office at Oregon State University, the estimated sale value of red clover seed produced in 2015 is 12.1 million US dollars from approximately 6,000 hectares of harvested area. Oregon led the US in red clover seed production and supplied approximately 75% of the US market needs (Oregon Department of Agriculture, 2015).

Foliar applied plant growth regulators (PGRs) have been widely used on temperate grass seed crops in Oregon and other parts of the world. This practice has been adopted due to documented seed yield increases in grasses and reduction in lodging (Chastain et al., 2014; Chynoweth et al., 2008; Rolston et al., 2010; Zapiola et al., 2006). However, little research has been conducted on the use of PGRs on legume seed crops, such as red clover in Oregon. During the late 1990s, Silberstein et al. (1996) reported a reduction in stem length, a decrease in lodging, and an increase in seed yield with soil applications of paclobutrazol and uniconazole PGRs. These soil applied products are no longer available for commercial use due to persistence in the soil and residual activity on subsequent crops (Hampton, 1996).

More recently, the acylcyclohexanedione PGRs have been introduced as growth retardant for turfgrasses, and as a lodging control agent in cereals. Trinexapac-ethyl is one of these compounds which registered for use as a lodging control in grass seed crops in Oregon. This PGR inhibits gibberellin (GA) biosynthesis. Their structure

mimic 2-oxoglutaric acid, a cofactor in 3β-hydroxylation of GA. This is the final step of GA biosynthesis (Rademacher, 2000).

A study conducted in Norway reported that seed yield was increased by 21% in red clover when TE was applied at stem elongation stage (Øverland and Aamlid, 2007). Based on these results, this product was registered for use on red clover seed production in Norway. Anderson et al. (2015) reported that red clover seed production under New Zealand and Oregon environments produced an increase of 16% seed yield when 500 g TE ha^{-1} was applied at stem elongation stage.

Oliva et al. (1994) reported that a single irrigation applied at flowering stage doubled seed yield over the non-irrigated control in red clover. Yield components most associated with the irrigation-induced increase in seed yield were the number of seeds per floret and seed weight component (Oliva et al., 1994).

The previous studies with irrigation and TE applied to red clover implied that there might be possibilities for improving seed yield by combining these management practices. However, the mechanism by which the seed yield increase is not fully understood. Also, the combination effects of TE, timing and rate of application, and irrigation on seed yield and seed quality are still unknown. Therefore, the objective of this study was to determine the influences of TE and irrigation on red clover vegetative growth, seed yield, yield components, and seed quality.

3.2 MATERIALS AND METHODS

3.2.1 Overview and Plant Materials

Field trials were conducted at Oregon State University's Hyslop Crop Science Research Farm (44°38'21"N, 123°11'40"W), Corvallis, Oregon. The soil is classified as Woodburn silt-loam (fine-silty, mixed, mesic Aquultic Argixerolls).

Non-certified "common" diploid red clover, commonly used by red clover seed growers in Oregon, was planted on 26 Sept. 2011 at a rate of 9 kg ha^{-1} using a 15-row plot-sized drill with 15-cm spaced rows. The field was monitored over a two-year period. The field was mowed and residue was removed from the plots in mid-May of 2012 and 2013, prior to bud emergence. Four honey bee (*Apis mellifera* L.) hives were placed near the red clover field to assist in the pollination process during the flowering period. Natural bumble bees (*Bombus* spp.) were also observed in the study field. The red clover was harvested for seed yield on 22 Aug. 2012 and 9 Aug. 2013 by a small-plot swather (modified JD 2280) and threshed by a Hege 180 small-plot combine.

3.2.2 Experimental Design

The experimental design was a randomized complete block with a split-plot arrangement of treatments with four replications. The TE treatments were subplots (3.4 m x 15.2 m) within irrigated and non-irrigated main plots. The TE applications were applied to the subplots at two application timings and four rates in the first-year stand and at five rates of TE in the second-year stand (Table 3.1). Irrigation, at approximately

100 mm of water, was applied to the main plots at first flowering stage (BBCH 55) by a custom-designed Pierce AcreMaster linear system with minimum-drift Nelson sprinklers.

3.2.3 Data Collection

3.2.3.1 Crop canopy characteristics

Red clover stems originate from the crowns that are located at or slightly above the soil surface. The crowns are developed from the complex of axillary buds, which enlarged as the plants grow. Stem length was measured in all plots at peak flowering stage (BBCH 65).

3.2.3.2 Seed yield and yield components

Seeds were harvested from the center 180 cm of each subplot and cleaned using a laboratory size cleaner machine (Clipper M-2B, A.T. Ferrell, Saginaw, MI) to determine clean seed yield.

Above-ground biomass was measured by sampling of two 30-cm^2 quadrats at peak flowering stage (BBCH 65). Samples were oven dried at 65°C for 48 h and then weighed to determine above-ground biomass. Number of stems per square meter and number of heads per stem were also determined at this growth stage by counting all stems and heads within each sample.

Harvest index (HI) is defined as the ratio of seed yield to the total above-ground biomass that has been produced. The HI was determined for each plot dividing clean seed yield by total above-ground biomass and presented the ratio as a percentage.

Seed weight was determined by counting two 1000-seed replicates and measuring the weight. Seed size was determined using a Vernier Caliper by randomly collecting ten-seed samples from each subplot and recording length, width, and thickness.

3.2.3.3 Seed quality evaluation

Seed quality determination included seed viability by the standard germination test and seed vigor by the cold test. All tests were replicated four times and adapted from the protocol of the Association of Official Seed Analysts (AOSA, 2009; AOSA, 2012) as follows:

Standard germination test: Four replications of 25 seeds each of all treatments were planted on the top of two layers of moistened germination paper, which were placed into transparent boxes at 20°C. The number of normal seedlings was recorded 7 d after the planting.

***Cold test*:** Four replications of 25 seeds each of all treatments were evaluated by a cold test. Seeds were placed on the top of moistened paper, which were placed into transparent boxes and incubated at 10°C for 7 d, and then moved into 20°C for 7 d. Afterward, the number of normal seedlings was recorded.

Hard seeds are generally found in mature red clover and many other crops in Fabaceae (Copeland and McDonald, 2001). Hard seeds are impermeable to water and gases. At the end of each seed quality test, seeds with hard seed coats were scarified to allow for water imbibition. The process was conducted with a mechanical PSS1000

pneumatic seed scarifier (Mater International, Inc., Corvallis, OR) with air pressure of 40 psi for 60 seconds. The scarified seeds were retested according to the above protocols.

3.2.4 Data Analysis

Analysis of variance (ANOVA) was used to determine the effect of treatments (irrigation and TE application) over two years, and the interaction among these factors using the statistical package MSTAT (Michigan State Univ., East Lansing, MI). Treatment, i.e., irrigation and PGR, means were separated by Fisher's protected LSD test at the 5% level of significance, whenever the effects of the treatment were significant. Regression analysis was calculated to determine the relationship between yield components and seed quality.

3.3 RESULTS AND DISCUSSION

3.3.1 Growing Season Environment

The growing season environments were observed during the field trial with rainfall during the May-August period ranging from 99 to 142 mm, which decreased 27 to 70 mm from the 119-year average (Table 3.2). The observed average monthly temperatures were 16.6°C and 18.1°C, which were warmer than the 119-year average by 0.8°C and 2.3°C in 2012 and 2013, respectively.

3.3.2 Seed Yield, Above-Ground Biomass, and Harvest Index

Seed yield was affected by year, irrigation, TE application timing, TE rate, and the interactions among TE timing, TE rate, and year (Table 3.3). Above-ground biomass was not affected by any treatments, whereas harvest index was affected by irrigation, TE timing, interaction between TE timing and TE rate, and interaction between TE timing and year. However, interactions between irrigation and TE for seed yield and any yield components were not significant.

Seed yield was higher in 2012 than in 2013 at 929 and 802 kg ha^{-1}, respectively (Table 3.4). This might be due to the infestation by the clover crown borer (*Hylastinus obscurus*), which frequently found in the second-year stand of red clover (Rao et al., 2012 and Rao et al., 2013).

Irrigation resulted in seed yield increase by an average of 9.9% in both years. Harvest index was also increased by irrigation by an average of 10.2% in both years. The amount of rainfall during the growing season was noticeably different between the two

years; however, red clover seed yield response to irrigation was positive and similar in both years. The higher rainfall in 2012 was not sufficient to cause significant increase in seed yield. Rainfall in both growing seasons was below the average for the region. Because of the relatively dry conditions, irrigation was needed to maximize seed yield in both years. These results confirmed the previous report by Oliva et al. (1994) that irrigation significantly increased red clover seed yield by 72 to 160% compared to the non-irrigated control.

The TE applied at stem elongation stage increased seed yield and harvest index in the second-year stand, but it had no significant effect in the first-year stand (Fig. 3.1). The application of low TE rate (140 g a.i. ha^{-1}) at stem elongation stage increased seed yield by 10.5% over the untreated control, whereas higher rates (280 to 700 g a.i. ha^{-1}) increased seed yield by an average of 17.9%. On the other hand, The TE application at bud emergence stage in the second-year stand had no effect on seed yield at the 140 g a.i. ha^{-1} TE rate, whereas higher rates of TE (280, 420, 560, and 700 g a.i. ha^{-1}) resulted in seed yield reduction by 8.4, 12.1, 13.4, and 21.2%, respectively (Fig. 3.1). The reduction in seed yield by TE at bud emergence is likely related to lower seed weight and number of heads per stem (data shown in section 3.3.4). The second year results confirm the previous report by Øverland and Aamlid (2007) and Anderson et al. (2015). Øverland and Aamlid (2007) found that red clover seed production under Norway condition was increased by 21% when TE was applied at a rate of 250 g a.i. ha^{-1} at stem elongation stage. However, Anderson et al. (2015) reported that red clover seed production under

New Zealand and Oregon environments produced highest seed yield by up to 16% when TE was applied at a rate of 500 g a.i. ha^{-1} at stem elongation stage.

Above-ground biomass was not significantly affected by irrigation or TE application. However, irrigation increased stem length and TE application decreased it (data shown in section 3.3.3). These results were in contrast with the findings of Oliva et al. (1994), who reported that irrigation improved above-ground biomass in red clover seed production. Oliva et al. (1994) added that the response of red clover was different from other forage legume seed crops in that seed yield of red clover was maximized even when the amount of above-ground biomass was high.

3.3.3 Crop Canopy Characteristics and Stem Length

Normal growth of red clover plants was presented in Fig. 3.2A. The stem remained upright until late flowering stage (BBCH 69). At this growth stage, stems lodged because of heavy heads during seed filling. However, lodging in this study occurred only in the lower part of the main stems, while the upper parts, including branches and flower heads, bended upward and stayed vertical as shown in Fig. 3.2B. The average height of erect plants from all plots was 67 cm, whereas the height of canopy after lodging was approximately 50 cm (data not shown). Lodging did not affect seed yield.

The ANOVA revealed that stem length was significantly affected by irrigation, TE application timing, TE rate, year, and the interactions among them (Table 3.3). The

effects of each factor are presented in Table 3.4, whereas the interaction effects are presented in Fig. 3.3 and Fig. 3.4.

Stem length decreased when TE was applied at stem elongation stage in both years of the study. The TE application at bud emergence also reduced stem length in the second-year stand (Fig. 3.3). The higher rates of TE resulted in shorter stem lengths. This is the direct effect of TE inhibiting GA biosynthesis and consequently reducing cell elongation (Rademacher, 2000). Similar effects of TE on stem length reduction were reported in red clover study in New Zealand and Oregon (Anderson et al., 2015). The reduction in stem length by TE was also reported in other crops, such as perennial ryegrass (*Lolium perenne* L.) (Borm and van den Berg, 2008; Chastain et al., 2014; Chynoweth et al., 2008; Rolston et al., 2010), tall fescue [*Schedonorus arundinaceus* (Schreb.) Dumort.] (Chastain et al., 2015), and wheat (*Triticum aestivum* L.) (Espindula et al., 2009).

Stem length increased with irrigation in the second-year stand, but it was not affected in the first-year stand (Fig. 3.4). This might be because of less rainfall and higher temperatures recorded in the second year compared to the first year (Table 3.2). Therefore, irrigation in the second year promoted more vegetative growth resulting in longer stem length. These results were similar to the findings of Oliva et al. (1994) where irrigation increased stem length of red clover seed production. The greatest reduction in stem length was observed when TE was applied at stem elongation stage

without irrigation (Fig. 3.4). This is, in part, occurred because irrigation promoted stem length, whereas TE application reduced it.

3.3.4 Number of Stems per m^2, Number of Flower Heads per Stem, and Seed Weight

Neither irrigation nor TE had significant effect on number of stems per m^2. However, irrigation affected heads $stem^{-1}$ and seed weight. The TE application only influenced seed weight. The interaction effect of irrigation and TE application on any seed yield components was not significant at $P \leq 0.05$ (Table 3.3). This indicated that TE behaved similarly under both irrigated and non-irrigated conditions.

Irrigation resulted in an increase of 5.3% in seed weight over the two-year period. On the other hand, irrigation resulted in an average decrease of 9.8% in number of heads per stem. The decrease in the number of heads per stem was compensated by an increase in seed weight, which was a contributing factor for the seed yield increase of irrigated plots (Table 3.4).

The TE application reduced seed weight. The higher rates of TE application resulted in the greater reduction in seed weight. In addition, seed weight was more negatively affected by TE applied at bud emergence than stem elongation stage. These results agreed with Øverland and Aamlid (2007), who also reported that seed weight was reduced by TE application at a rate of 250 g a.i. ha^{-1} at bud emergence stage. The number of heads per stem was inconsistent with TE timing over the two years (Fig. 3.5). However, number of heads per stem increased at the high rate of TE application at stem

elongation stage to the second-year stand. In summary, the application of TE at stem elongation stage in the second-year stand resulted in the decrease in seed weight; however, it was compensated by the increase in number of heads per stem, and it was a contributing factor for increasing seed yield.

3.3.5 Seed size

Seed size was significantly affected by irrigation, TE timing, TE rate, and the interactions among them (Table 3.5). The effects of each factor are presented in Table 3.6, whereas the interaction effects are presented in Fig. 3.6.

Irrigation increased seed size by length (L) and thickness (T), but not by width (W). Seeds from irrigated plots were longer and thicker than those from non-irrigation, i.e., 2.09L x 1.39W x 1.05T and 1.97L x 1.37W x 1.02T in mm for irrigated and for non-irrigated plots, respectively (Table 3.6). The TE application reduced seed size in all dimensions (i.e., L, W, and T). Higher rates of TE resulted in smaller seed size (Fig. 3.6). Seed size in the untreated control was 2.13L x 1.48W x 1.08T in mm. The smallest seed size was from the highest rate of TE application (700 g a.i. ha^{-1}), which was 1.94L x 1.28W x 1.00T in mm. Larger seeds (wider and thicker) were found when TE was applied at stem elongation compared to bud emergence, 2.03L x 1.40W x 1.05T and 2.03L x 1.36W x 1.03T in mm, respectively. In addition, seed size was significantly related to seed weight with correlation coefficient of 0.247, 0.666, and 0.416 at $P = 0.015$, <0.001, and < 0.001 for L, W, and T, respectively.

3.3.6 Yield Components and Seed Quality

The relationship between yield components and seed quality is presented in Table 3.7. Seed quality from all treatments was over 92% (Table 3.4). Most yield components did not significantly correlate with viability and vigor of seeds. However, the correlation between seed weight and seed viability was significant. The correlation between number of stems per m^2 and seed vigor was also significant.

The linear regression equation for the relationship between seed weight and seed viability was $Y = 110.07 – 10.10X$ (where, Y is the viability percentage and X is the seed weight (g) at $P = 0.018$, $R^2 = 0.0579$) (Fig. 3.7A). Such equation showed that seed viability remained as high as 91-93% regardless of whether seed weight was high (1.87 g) or low (1.66 g) for the untreated control and TE treatment, respectively. Larger seeds resulted in higher seed weight and smaller seeds resulted in lower seed weight. The change in seed weight and seed size did not affect seed viability in this study. This result contrasted with other studies, where larger seeds produced higher germination and vigor rates compared to smaller seeds in various crops, such as oat (*Avena sativa* L.) (Willenborg et al., 2005), triticale (xTriticosecale Witm. cv. Presto) (Kaydan and Yagmur, 2008), soybean (*Glycine max* L.) (Hampton et al., 2005; Hanley et al., 2007), pinto bean (*Phaseolus vulgaris* L.) (Gholami et al., 2009), and lentil (*Lens culinaris* Medik.) (Hojjat, 2011). This might be because water uptake of large seeds occurs at a higher rate than smaller seeds, as it is the case in safflower (*Carthamus tinctorius* L.) (Farhoudi and Motamedi, 2010). Also, the amount of carbohydrates and other nutrients in large seeds

is higher than those in smaller seeds (Gunaga et al., 2011). Vaughan and Delouche (1968) reported that viability was affected by seed size. Intermediate to large seeds generally produced higher germination than smaller seeds. However, no consistent relationship between seed size and viability was reported. In contrast, Wang and Hampton (1989) found that smaller seeds of red clover had greater vigor than larger seeds.

The linear regression equation for the correlation between the number of stem per m^2 and seed vigor was $Y = 87.72 + 0.02X$ (where, Y is the vigor percentage and X is the number of stem per m^2; $P = 0.044$, $R^2 = 0.0423$) (Fig. 3.7B). Such equation showed that seed vigor remained high at 87% regardless of the number of stem per m^2. However, plant density was found to have adverse effect on seed vigor in soybean (Rahman et al., 2005). They found that seed vigor reduced when plant density increased. However, plant density had no effect on seed germination.

In summary, the relationships between seed quality and yield components were weak. Seed size as a component of seed weight did not affect seed viability or vigor. Plant density, number of stem per m^2, also did not affect any seed quality in this study.

3.4 Conclusions

Irrigation and TE independently increased seed yield in red clover; however, the interaction between these two factors was not significant. Irrigation increased seed yield in the first and second years by an average of 10% due to the greater seed weight. However, TE increased seed yield by up to 18% when applied at stem elongation stage in the second-year stand under Oregon conditions due to the greater number of heads per stem. Seed quality from all treatments were similar with high percentage of viability and vigor, which was slightly correlated with seed weight and number of stem per m^2, respectively. However, none of them significantly affected seed quality. This study revealed that seed yield can be increased by: 1) single irrigation application during first flowering stage; and 2) TE application at a minimum rate of 280 g a.i. ha^{-1} at stem elongation stage in the second year stand of red clover.

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Table 3.1. Timing and rate of trinexapac-ethyl (TE) applications used in the first- and second-year stands of red clover grown for seed.

Application timing (BBCH scale)	TE application rate In the first-year stand (g a.i. ha^{-1})	TE application rate In the second-year stand (g a.i. ha^{-1})
Untreated control	0	0
Stem elongation (BBCH 32)	140	140
	280	280
	420	420
	560	560
	-	700
Bud emergence (BBCH 51)	140	140
	280	280
	420	420
	560	560
	-	700

Table 3.2. Total rainfall and monthly temperature during the growing season (May to August) in the first- and second-year stands of red clover.

Harvest year	Stand age (year)	Rainfall (mm)		Temperature (°C)	
		Total	Anomaly †	Average	Anomaly
2012	1st	142	-27	16.6	0.8
2013	2nd	99	-70	18.1	2.3

† Anomaly, a departure from the 119-year average of rainfall and temperature for Corvallis, Oregon.

Table 3.3. Analysis of variance for the effect of irrigation and trinexapac-ethyl (TE) application on red clover seed yield, above-ground biomass, harvest index (HI), stem length, number of stems per m^2, number of heads per stem, seed weight, seed viability, and seed vigor.

Source of variation	df	Seed yield	Above-ground biomass	HI	Seed length	Stems m^{-2}	Heads $stem^{-1}$	Seed weight	Seed viability	Seed vigor
Year (Y) †	1	*	ns	ns	**	ns	ns	**	ns	ns
Irrigation (I) ‡	1	**	ns	*	*	ns	**	**	ns	ns
TE timing (T) §	1	***	ns	***	***	ns	ns	***	ns	ns
TE rate (R) ¶	4	*	ns	ns	***	ns	ns	***	ns	ns
Interactions #										
YI	1	ns††	ns	ns	*	ns	**	ns	ns	ns
YT	1	***	ns	**	***	ns	***	ns	ns	ns
IT	1	ns	ns	ns	**	ns	ns	ns	ns	ns
YIT	1	ns	ns	ns	*	ns	ns	ns	ns	ns
TR	4	***	ns	*	***	ns	ns	***	ns	ns
YTR	4	***	ns	ns	***	ns	ns	ns	ns	ns

*, **, *** Significant difference at the 0.05, 0.01, and 0.001 probability level, respectively.
† Year, 2012 and 2013.
‡ Irrigation, none and single irrigation.
§ Timing of TE application, at stem elongation (BBCH 32) and bud emergence (BBCH 51) stages.
¶ Rate of TE application, 0, 140, 280, 420, and 560 g TE ha^{-1}.
# Interactions not shown in this table were not significantly different.
†† ns, nonsignificant.

Table 3.4. Means of red clover seed yield, above-ground biomass, harvest index (HI), stem length, number of stems per m^2, number of heads per stem, seed weight, seed viability, and seed vigor as affected by year, irrigation, and trinexapac-ethyl (TE).

Factor	Seed yield	Above-ground biomass	HI	Stem length	Stems m^{-2}	Heads $stem^{-1}$	Seed weight	Seed viability	Seed vigor
	------ kg ha^{-1} -------		-- % --	cm	no. m^{-2}	no. $stem^{-1}$	mg $seed^{-1}$	--------- % ---------	
Year									
2012	929 a†	10064 a	9.56 a	63.6 b	290 a	2.72 a	1.73 b	96.3 a	92.9 a
2013	802 b	9694 a	8.91 a	71.1 a	337 a	2.32 a	1.78 a	96.7 a	91.9 a
Irrigation									
Single	907 a	9865 a	9.67 a	68.4 a	307 a	2.39 b	1.79 a	96.5 a	92.4 a
None	825 b	9894 a	8.79 b	66.3 b	320 a	2.65 a	1.70 b	96.6 a	92.2 a
TE timing‡									
BBCH 32	913 a	9722 a	9.97 a	65.1 b	311 a	2.51 a	1.79 a	96.4 a	92.9 a
BBCH 51	818 b	10036 a	8.50 b	69.6 a	316 a	2.52 a	1.73 b	96.7 a	91.7 a
TE rate (g a.i. ha^{-1})									
0	849 c	9787 a	9.27 a	71.8 a	303 a	2.28 a	1.87 a	96.0 a	92.3 a
140	857 bc	10182 a	8.69 a	68.9 b	324 a	2.52 a	1.80 b	96.8 a	92.8 a
280	881 a	9509 a	9.79 a	66.7 c	301 a	2.55 a	1.76 c	96.0 a	92.0 a
420	870 abc	9851 a	9.14 a	65.9 d	309 a	2.62 a	1.70 d	96.8 a	91.9 a
560	873 ab	10068 a	9.27 a	63.5 e	329 a	2.63 a	1.66 e	97.1 a	92.8 a

† Within columns, means in each factor followed by the same letter are not significantly different by Fisher's protected LSD values ($P \leq 0.05$).

‡ Timing of TE application, at stem elongation (BBCH 32) and bud emergence (BBCH 51) stages.

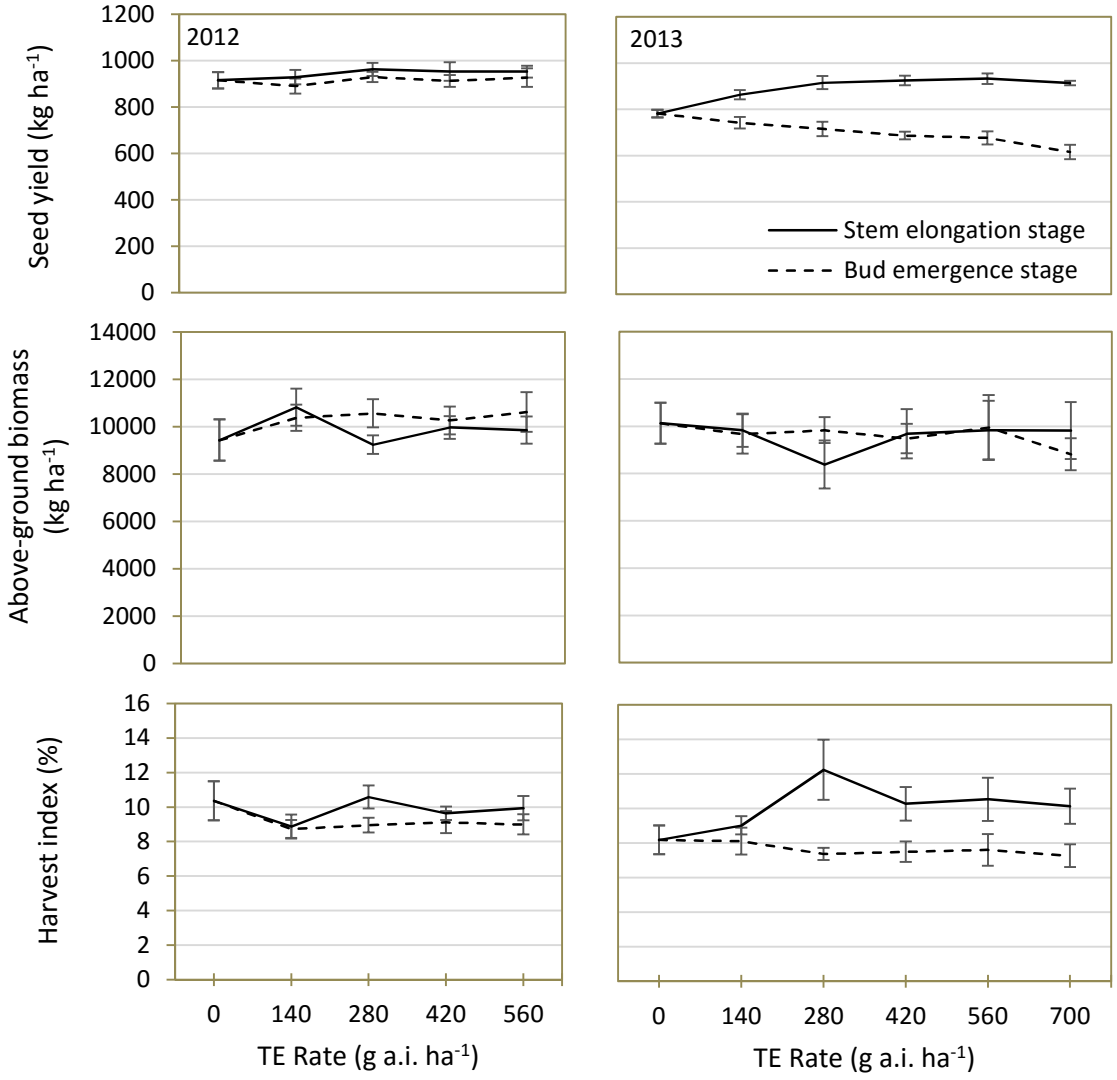

Figure 3.1. Effect of trinexapac-ethyl (TE) application timing and rate on seed yield, above-ground biomass, and harvest index in the first- and second-year stands of red clover in 2012 and 2013.

Figure 3.2. (A) Normal growth of red clover stems from a crown and (B) red clover stem bended upward after lodging.

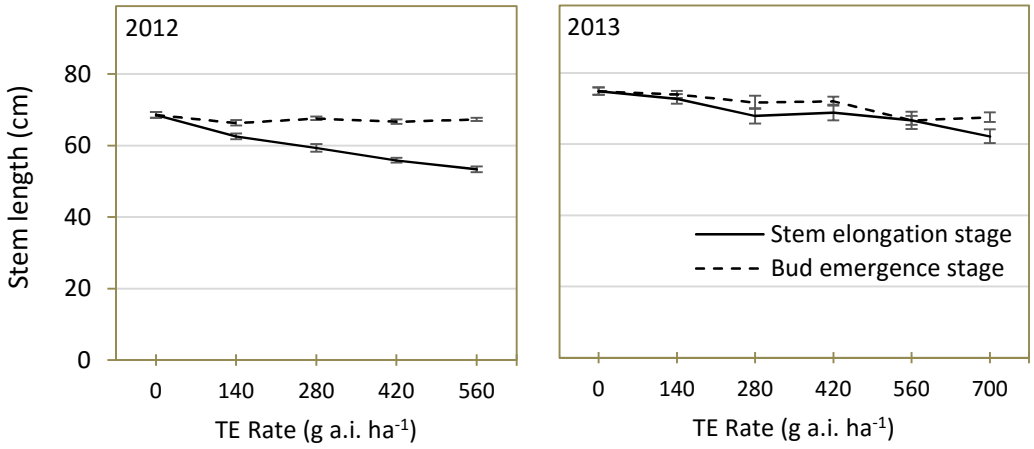

Figure 3.3. Effect of trinexapac-ethyl (TE) application timing and rate on stem length in the first- and second-year stands of red clover in 2012 and 2013.

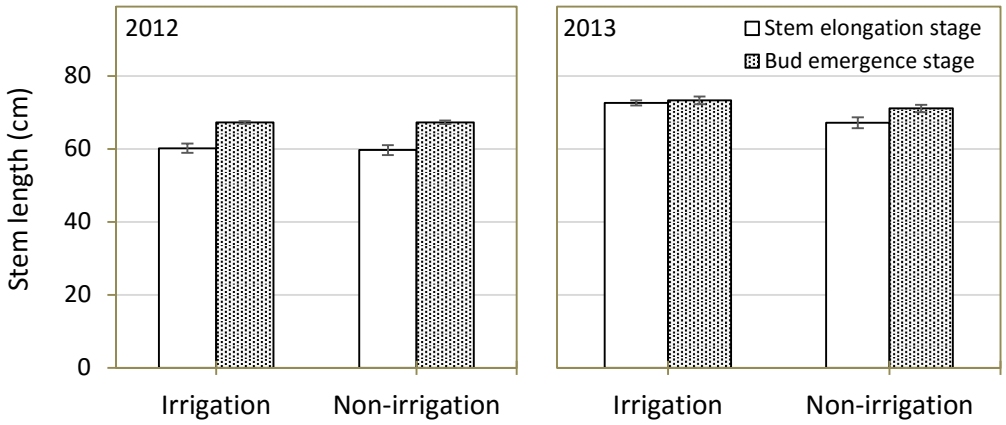

Figure 3.4. Interaction between irrigation and trinexapac-ethyl (TE) application timing on stem length in the first- and second-year stands of red clover in 2012 and 2013.

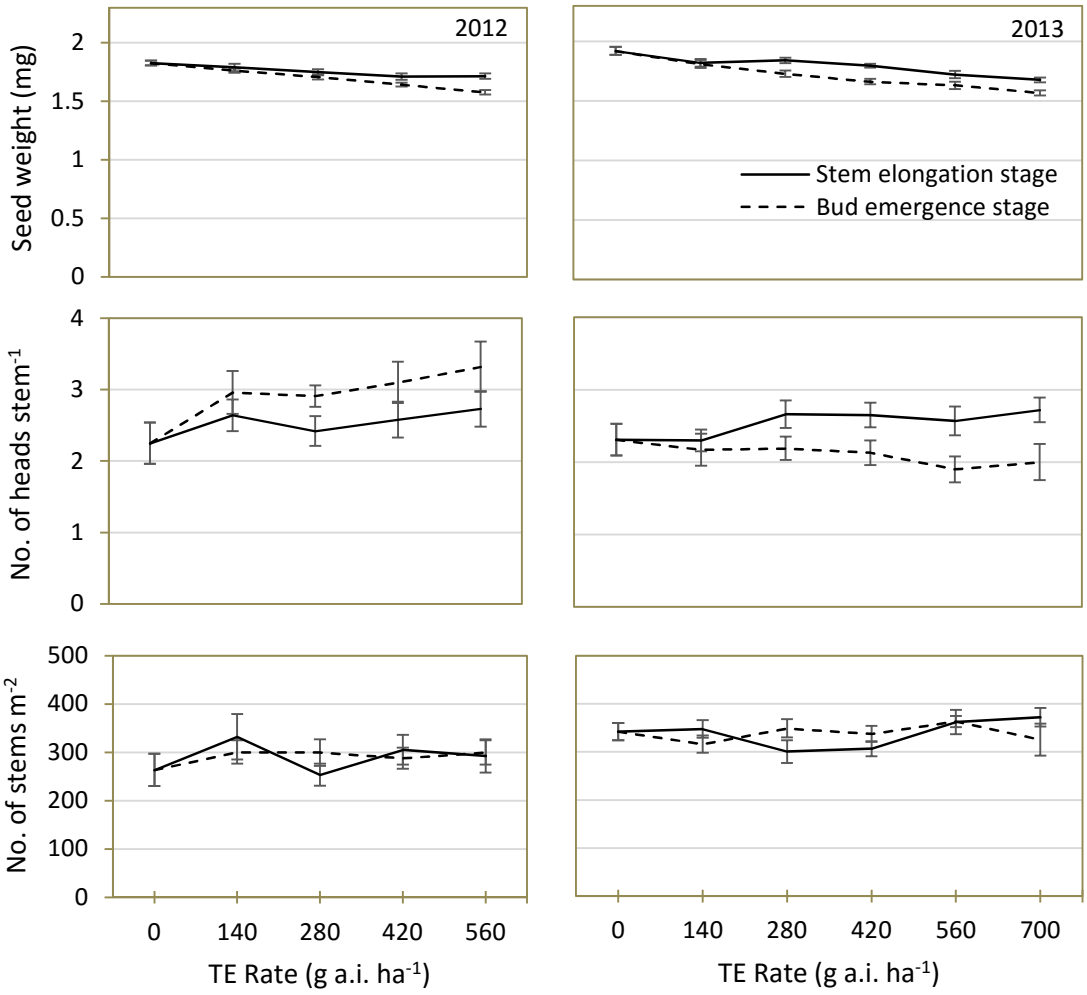

Figure 3.5. Effect of trinexapac-ethyl (TE) application timing and rate on seed weight, number of heads per stem, and number of stems per m^2 in the first- and second-year stands of red clover in 2012 and 2013.

Table 3.5. Analysis of variance for the effect of irrigation and trinexapac-ethyl (TE) application on seed size, including length (L), width (W), and thickness (T) in the second-year stand of red clover in 2013.

Source of variation	df	Seed length (L)	Seed width (W)	Seed thickness (T)
Irrigation (I) †	1	***	ns	**
TE timing (T) ‡	1	ns ¶	***	***
TE Rate (R) §	5	***	***	***
IT	1	ns	ns	***
IR	5	**	*	*
TR	5	ns	***	***
ITR	5	**	***	ns

*, **, *** Significant difference at the 0.05, 0.01, and 0.001 probability level, respectively.

† Irrigation, none and single irrigation.

‡ Timing of TE application, at stem elongation (BBCH 32) and bud emergence (BBCH 51).

§ Rate of TE application, 0, 140, 280, 420, 560, and 700 g TE ha^{-1}.

¶ ns, nonsignificant.

Table 3.6. Means of seed size, including length (L), width (W), and thickness (T) as affected by irrigation and trinexapac-ethyl (TE) in the second-year stand of red clover in 2013.

Factor	Seed length (L)	Seed width (W)	Seed thickness (T)
	---------- mm ----------		
Irrigation			
Single	2.09 a†	1.39 a	1.05 a
None	1.97 b	1.37 a	1.02 b
TE timing‡			
BBCH 32	2.03 a	1.40 a	1.05 a
BBCH 51	2.03 a	1.36 b	1.03 b
TE Rate (g a.i. ha^{-1})			
0	2.13 a	1.48 a	1.08 a
140	2.10 a	1.42 b	1.07 a
280	2.05 b	1.39 c	1.04 b
420	2.00 c	1.37 d	1.01 cd
560	1.96 d	1.34 e	1.02 c
700	1.94 d	1.28 f	1.00 d

† Within columns, means in each factor followed by the same letter are not significantly different by Fisher's LSD values ($P \leq 0.05$).

‡ Timing of TE application, at stem elongation (BBCH 32) and bud emergence (BBCH 51) stages.

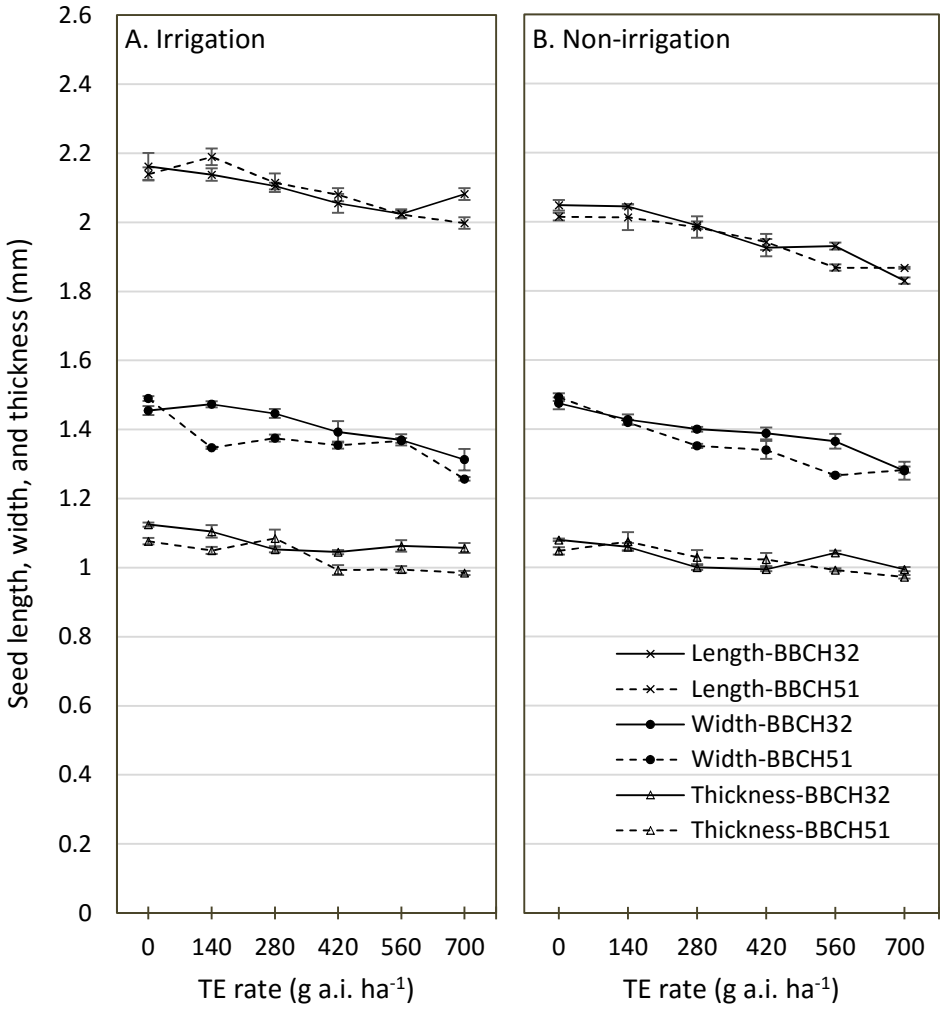

Figure 3.6. Seed length, width, and thickness in millimeter (mm) as affected by trinexapac-ethyl (TE) application at two timings and six rates with (A) Irrigation and (B) non-irrigation in 2013.
Two timings of TE application included stem elongation stage (BBCH 32) and bud emergence stage (BBCH 51). Six rates of TE application included 0, 140, 280, 420, 560, and 700 g a.i. ha^{-1}.

Table 3.7. Correlation coefficients (r) between yield components and seed quality of red clover.

Yield components	Seed viability	Seed vigor
Above-ground biomass	-0.064 ^{ns} †	-0.000 ^{ns}
Harvest index	0.029 ^{ns}	-0.064 ^{ns}
Seed weight	-0.241 *	0.087 ^{ns}
Heads stem^{-1}	0.025 ^{ns}	-0.103 ^{ns}
Stems m^{-2}	0.041 ^{ns}	0.206 *
Seed length	-0.069 ^{ns}	-0.062 ^{ns}
Seed width	-0.149 ^{ns}	-0.009 ^{ns}
Seed thickness	-0.120 ^{ns}	0.051 ^{ns}

* significant at $P \leq 0.05$.
† ns, nonsignificant.

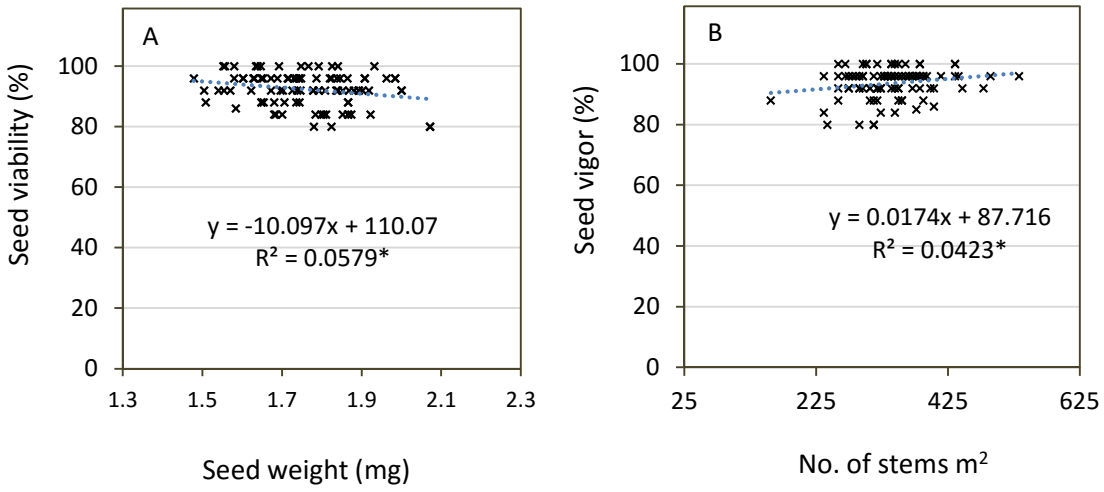

Figure 3.7. Relationship between seed quality and yield components; (A) seed viability and seed weight, (B) seed vigor and number of stems per m^2. Seed viability was evaluated by standard germination test and seed vigor was evaluated by cold test. * significant at $P \leq 0.05$.

CHAPTER 4: Changes in Gibberellic and Abscisic Acid Contents of Red Clover (*Trifolium pratense* L.) During Seed Development and Maturation

ABSTRACT

Gibberellic acid (GA_3) and abscisic acid (ABA) are two major phytohormones that affect seed germination. While a hard seed coat is the main physical dormancy form in red clover, it is unknown whether physiological dormancy plays any role. This study was conducted to: 1) determine the contents of GA_3 and ABA during seed development until maturation; 2) evaluate the effects of irrigation and trinexapac-ethyl (TE) plant growth regulator (PGR) on GA_3 and ABA contents in seeds; and 3) investigate the relationship between ABA:GA_3 ratio and seed germination. Seeds were collected from untreated, TE-treated, irrigated, and TE plus irrigated plots at weekly intervals after anthesis and germination tests were conducted. The GA_3 and ABA were extracted from seeds using the solid phase method and were quantified by the liquid chromatography-tandem mass spectrometry (LC-MS/MS). The ABA content was high (1242 pg g^{-1} DW) during early seed development and decreased to 388 pg g^{-1} DW at harvest maturity (HM). The GA_3 content did not change significantly during seed development until reaching HM, ranging from 173 to 187 pg g^{-1} DW. Irrigation and TE application did not have a significant effect on the endogenous production of GA_3 and ABA in seeds. The ABA:GA_3 ratio was high (6.7) at early seed development, but seed germination was low (24%). When seeds reached HM, the ABA:GA_3 ratio dropped to 2.2 and seed germination increased to 93%. These results suggest that physiological

dormancy is not a substantial concern in red clover seeds. However, before scarification, 34% hard seed coat at HM was found.

Abbreviations: ABA, abscisic acid; GA, gibberellin; GA_3, gibberellic acid; HM, harvest maturity; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PGR, plant growth regulator; PM, physiological maturity; TE, trinexapac-ethyl; WAA, weeks after anthesis.

4.1 INTRODUCTION

Red clover is a forage legume crop commonly used as a rotation crop in Oregon. The estimated sale value of red clover seed produced in 2015 was 12.1 million US dollars from approximately 6,000 hectares of harvested area. Red clover seed production in Oregon was the number one in national ranking. It supplied approximately 75% of the US market (Oregon Department of Agriculture, 2015).

Gibberellin (GA) is a group of plant hormones, which promote plant growth and development. This group is tetracyclic diterpenoid acid compound (Fig. 4.1) (Gupta and Chakrabarty, 2013). It includes a large number of compounds, abbreviation as GA followed by number in the chronological order of its discovery. However, only a few GAs have biological activity. Gibberellic acid (GA_3) is one of the bioactive forms of gibberellin, which is widely studied (Gupta and Chakrabarty, 2013; Rademacher, 2015; Taiz et al., 2015). One of the functions of GA_3 in plants is to promote cell elongation, resulting in an increase in plant height. Besides promoting longitudinal plant growth, GA_3 stimulates seed germination, induces flowering, determines sex expression, and enhances pollen, fruit and seed development (Gupta and Chakrabarty, 2013; Rademacher, 2015; Taiz et al., 2015).

Abscisic acid (ABA) is a growth inhibitor phytohormone, which contains a 15-carbon terpenoid (Fig. 4.1). It accumulates at high levels in plants exposed to some abiotic stresses during seed development, such as cold, drought, and high salinity (Nambara and Marion-Poll, 2005). The physiological functions of ABA include: a)

stomata closure to limit transpiration, b) metabolism modification to tolerate dryness and low temperatures, and c) inhibition of seedling growth. Also, ABA suppresses seed germination (Bentsink and Soppe, 2008; Finch-Savage and Leubner-Metzger, 2006; Finkelstein et al., 2008; Kermode, 2005) and enhances seed maturation and dormancy (Taiz et al., 2015).

Seed dormancy can be classified as morphological, physiological, physical, and combinational types of dormancy (Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006). Morphological dormancy involves an immature embryo and requires additional time to develop and germinate. Physiological dormancy relates to ABA and GA balance (hormone-mediated seed dormancy). Physical dormancy involves the water-impermeable seed coat (Baskin and Baskin, 2004). Dormancy in red clover and other legume species is well known as hardseededness. This is a type of physical dormancy, which is induced by hard seed coat that restricts water imbibition. The water-impermeable seed coat is a genetic trait, which is modified by environment conditions. Hard seed coat is expressed during seed dehydration, the last step of seed development and maturation. It involves the development of tightly bound palisade cells with phenolic and suberin layers (Smýkal et al., 2014). This type of dormancy can be released naturally by temperature changes or artificially by mechanical or chemical scarification (Smýkal et al., 2014; Taiz et al., 2015). However, there is limited knowledge of the hormone-mediated seed dormancy, i.e., physiological dormancy, in red clover.

During seed development, GA content in seed is usually low at the beginning of seed development and increases over time until seed reaches maturation. Unlike GA, the content of ABA starts low, increases quickly during early seed development, and then decreases later as seeds mature (Liu et al., 2010; Yang et al., 2006). The ratio of ABA to GA is the major determining factor of physiological dormancy and the ability of seeds to germinate (Taiz et al., 2015). The balance between the ABA and GA activities in seeds is affected by developmental and environmental factors. During early seed development, the ABA:GA_3 ratio is high, which favors seed dormancy. Later, ABA decreases while GA increases, which favors seed germination (Taiz et al., 2015). In addition, environmental factors such as temperature, light, and chemical treatments can affect the balance between ABA and GA in seed, which either promote or inhibit germination (Jha et al., 2010; Kim et al., 2009; Taiz et al., 2015).

Trinexapac-ethyl [4-(cyclopropyl-α-hydroxymethylene)-3,5-dioxocyclohexane-carboxylic acid ethyl ester] (TE, Palisade) is a plant growth regulator, commonly used in turfgrass management. The benefits of TE application are to reduce mowing, suppress seed head formation, and enhance turfgrass quality (Fagerness and Yelverton, 2001). Besides these benefits, it is used in seed production to reduce lodging and consequently increase seed yield of grass seed crops, such as perennial ryegrass (*Lolium perenne* L.) (Borm and van den Berg, 2008; Chastain et al., 2014; Chynoweth et al., 2008; Rolston et al., 2010) and tall fescue [*Schedonorus arundinaceus* (Schreb.) Dumort.] (Chastain et al., 2015). Recent research by Anderson et al. (2015) and previous research by Øverland and

Aamlid (2007) revealed that the application of TE can also increase seed yield in red clover. The TE blocks 3β-hydroxylation, preventing the formation of active GAs from inactive forms (Rademacher, 2000). The TE suppresses GA biosynthesis. Since GA promotes cell elongation, reducing GA production results in limiting plant height (Rademacher, 2015). However, very little is known about how TE affects the dynamics of the endogenous production of GA and ABA in seeds and its effect on seed germination and dormancy.

Irrigation reduces drought stress and increases seed yield in several crops, such as field bean (*Phaseolus vulgaris* L.) (Efetha et al., 2011), castor (*Ricinus communis* L.) (Severino and Auld, 2013), and red clover (Oliva et al., 1994). Under drought stress, ABA endogenous production accumulates at high levels in plant cells. For instance, Masoumi et al. (2011) reported that water deficit significantly increased ABA content in soybean (*Glycine max* L.). Weldearegay et al. (2012) found that ABA content in wheat (*Triticum aestivum* L.) spikelet was significantly increased in the non-irrigated treatment compared to the irrigated treatment. In addition, they found that the increase in ABA content in wheat spikelet was highly correlated with seed set reduction. These studies showed that drought stress increased ABA content in plant; however, limited studies have reported on the effect of irrigation on production of ABA and GA during seed development and maturation in red clover.

Knowledge of the dynamics of ABA and GA production during seed development in response to TE and irrigation will provide a better understanding on

whether the application of TE and irrigation affect seed dormancy and germination in red clover. Therefore, the objectives of this study were to: 1) determine the contents of GA_3 and ABA in red clover during seed development until harvest maturity; 2) evaluate the effect of irrigation and TE foliar application on GA_3 and ABA production in red clover seeds; and 3) investigate the correlation between ABA:GA_3 ratio and seed germination.

4.2 MATERIALS AND METHODS

4.2.1 Seed Materials

Non-certified "common" diploid red clover, commonly used by red clover seed growers in Oregon, was planted in late September 2011 at Hyslop Research Farm, Corvallis, Oregon, USA (44°38'21"N, 123°11'40"W). The soil at the research site is classified as Woodburn silt-loam (fine-silty, mixed, mesic Aquultic Argixerolls). Red clover heads were randomly collected during July and August 2013 at weekly intervals, starting during in the second week after anthesis and continuing up until harvest (2 – 5 WAA). Samples were collected from four different field treatments; TE, irrigation, TE plus irrigation, and untreated control. For the TE treatment, 280 g a.i. ha^{-1} of TE was sprayed at stem elongation stage (BBCH 32). For the irrigation treatment, approximately 100 mm of water was applied at first flowering stage (BBCH 55). Seeds were separated from flower heads by hand and stored in plastic Ziploc bags at -80°C until hormonal analyses were conducted.

4.2.2 GA_3 and ABA Extraction and Quantification

The GA_3 and ABA were extracted from red clover seeds by solid phase extraction method adapted from Gouthu et al. (2013). Fifty mg of homogenized and lyophilized seeds were extracted from each weekly seed collection in 3 ml of extraction solvent (methanol: formic acid: water, 15:1:4) with 20 ng each of the deuterated internal standards (d_2-GA_3 and d_6 ABA). The homogenate was extracted at 4°C on an orbital shaker for 24 h in darkness and centrifuged at 4415 x g for 15 min. After

centrifugation, supernatant was transferred to a fresh tube. The pellet was re-extracted with 0.5 ml additional extraction solvent and then combined with the first supernatant. To remove interfering compounds, the extract was passed through Oasis HLB 60 mg cartridge, which pre-equilibrated with acidified methanol (methanol: formic acid, 99:1) and acidified water (water: formic acid, 99:1) using a vacuum manifold. The elute was then evaporated overnight using a vacuum concentrator. The dried pellet was reconstituted with 2 ml of 1 M formic acid and passed through Oasis MCX 60 mg cartridge which pre-equilibrated with 1M formic acid. After a wash with 1M formic acid, GA_3 and ABA analytes were eluted with 100% methanol, evaporated overnight, and reconstituted with 200 µl of reconstitution solution (acetonitrile: water: formic acid, 15:85:0.1) for hormone analysis.

Seed extracts were quantified using a hybrid triple quadrupole/linear ion trap 4000 QTRAP liquid chromatography and tandem mass spectrometer (LC-MS/MS) instrument equipped with a Turbo V source. Chromatography separation was detected by an Agilent Zorbax Extend-C18 column. Gradient conditions were based on those optimized by Gouthu et al. (2013). Mass spectra for GA_3 and ABA were acquired in the negative mode. Calibration curves were generated from each standard sample using Analyst software version 1.5.1 (Applied Biosystems, Waltham, MA). The content of phytohormones were calculated by comparison to calibration curves.

4.2.3 Germination Determination

Standard germination tests were performed with four replicates of 100 seeds of each seed source (i.e., the weekly collected seeds and field treatments). Seeds were germinated on top of two layers of filter paper moistened with water. Seeds were incubated at 20°C with an alteration of 8 h of light and 16 h of dark for 7 d. After the 7-d incubation period, seeds with hard seed coats were scarified with a mechanical pneumatic seed scarifier model PSS1000 (Mater International, Inc., Corvallis, OR) using air pressure of 40 psi for 60 seconds to allow water imbibition of seeds. The number of normal seedlings was recorded for each replication in each treatment (AOSA, 2012).

4.2.4 Experimental Design and Data Analysis

For germination and hormonal analyses, a completely randomized design with three factors: two irrigation treatments (none and single), two TE applications (0 and 280 g a.i. ha^{-1}) and four seed maturity stages (2, 3, 4, and 5 WAA) was used. The GA_3 and ABA quantifications and the standard germination tests were replicated four times. Analysis of variance (ANOVA) was conducted to determine the effect of field treatment and seed maturity stage on the production of GA_3 and ABA, and germination, using the statistical package MSTAT (Michigan State Univ., East Lansing, MI). Means of GA_3 and ABA contents and germination percentage were separated by Fisher's protected LSD test at the 0.05 probability level, whenever the effects of factors were significant. Regression analysis was conducted to determine the relationship between the ABA:GA_3 ratio and seed germination.

4.3 RESULTS AND DISCUSSION

4.3.1 GA_3 and ABA Contents

Changes in endogenous GA_3 and ABA contents during seed development and maturation of red clover are presented in Fig. 4.2. Regardless of TE and irrigation treatments, GA_3 content did not differ significantly from the second week after anthesis until HM. The GA_3 content remained similar from early seed development (2 WAA) at 187 pg g^{-1} DW through full maturation (5 WAA) at 173 pg g^{-1} DW. These results differed from a study of Liu et al. (2010) who found fluctuation of GA_3 levels during soybean seed development.

In contrast to the GA_3 production pattern, ABA content was high, 1242 pg g^{-1} DW, during early seed development (2 WAA). However, it decreased rapidly to 547 pg g^{-1} DW in 3 WAA, which is 56% reduction from 2 WAA. Afterward, it gradually decreased to 388 pg g^{-1} DW in 4 WAA, which is 29% reduction from 3 WAA and to 331 pg g^{-1} DW in 5 WAA, which is 15% reduction from 4 WAA as seed reached maturation (Fig. 4.1). The high amount of ABA at early seed development prevents vivipary, i.e., preharvest sprouting, which is the germination of immature seed on the mother plant (Taiz et al., 2015). Vivipary is most known to occur in cereal crops (McCarty, 1995). The minimum level of ABA was found at harvest maturity (HM) (Fig. 4.2). The low level of ABA at HM enhanced seed germination (data shown in section 4.3.3). Similar trend of ABA reduction during seed maturation was found in soybean (Liu et al., 2010). However, our results were different from those reported by Liu et al. (2010) who observed that ABA

production started at a low level, then increased to its maximum level as seed developed, and declined to the minimum level at physiological maturity (PM). The timing of minimum ABA contents at PM and HM explains the maximum seed quality, i.e., germination, at full seed maturity. In soybean, Bishnoi et al. (2007) reported maximum seed germination at PM, whereas the maximum seed germination in red clover occurred at HM (data shown in section 4.3.3).

4.3.2 Effect of Field Treatment and Seed Maturity on GA_3 and ABA Production in Seeds

Neither field treatment (i.e., TE and irrigation) nor seed maturity stage had significant effect on GA_3 production in red clover seed (Table 4.1). During seed development and maturation (2 – 5 WAA), red clover seeds contained GA_3 ranging from 173 to 187 pg g^{-1} DW (Fig. 4.2). Also, TE and irrigation did not alter GA_3 content at any development stage of red clover seeds (Fig. 4.3A). For example, the amounts of GA_3 were 182 and 175 pg g^{-1} DW in the average of non-irrigated and irrigated plots, respectively. It is known that TE suppresses GA levels resulting in shorter plant stems (Anderson et al.,2015; Borm and van den Berg, 2008; Chastain et al., 2014; Chastain et al., 2015; Chynoweth et al., 2008; Rolston et al., 2010). However, TE and irrigation did not affect GA_3 contents in red clover during seed development and maturation.

Seed maturity stage, however, had significant effect on ABA content while TE and irrigation did not (Table 4.1). The ABA content was high at early seed development (1242 pg g^{-1} DW) and gradually decreased over time during seed development. It

dropped to the lowest level at 4 and 5 WAA to 388 and 331 pg g^{-1} DW, respectively, which coincided with HM (Fig. 4.2). The TE and irrigation did not alter ABA content in red clover seeds (Fig. 4.3B). Different findings were reported in other crops. For example, drought was found to increase the ABA accumulation in wheat seeds. The amount of ABA in seed from non-irrigated plots was greater than those from well-watered treatment (Yang et al., 2006). In this study, a single irrigation of 100 mm at early flowering, in addition to the 99 mm of total rainfall during growing season (May – August 2013), may not be sufficient to make any change in the level of ABA during seed development.

4.3.3 Seed Germination and ABA:GA_3 Ratio

Red clover seed germination was significantly affected by seed maturity stage, but not by TE and irrigation applications (Table 4.1). The germination was as low as 24% at early seed development (2 WAA). Gradually, it increased to 70% at 3 WAA and reached the maximum (93%) in 4 WAA and 91% in 5WAA (Table 4.3 and Fig. 4.3d). Similar results were reported by Elias and Copeland (2001) in canola (*Brassica napus* L.), but different from other crops such as soybean and cuphea (*Cuphea viscosissima* Jacq.), where seeds reached maximum quality at PM (Berti et al., 2007; Bishnoi et al., 2007). This is because of a different physiological change, i.e., seed hormone ratio. In general, as the ratio of ABA:GA_3 decreases, seed germination increases.

The ABA:GA_3 ratio negatively correlated with seed germination with a linear equation of $Y = 115.09 - 13.13X$ (where Y is the germination percentage and X is the

ABA:GA_3 ratio; r = -0.92, $P < 0.001$) (Fig. 4.4). Such equation suggests that maximum germination (93%) is achieved when ABA:GA_3 ratio is 1.7. The lower ABA:GA_3 ratio is necessary to break physiological dormancy and consequently increase seed germination.

During seed development, the ABA:GA_3 ratio and seed germination changed in opposite directions (Fig. 4.5). At early seed development, the ABA:GA_3 ratio was high (6.7) when seed germination was low (24%). At PM, the ABA:GA_3 ratio dropped to approximately 3.1, while seed germination increased to 70%, which was not the maximum quality. The maximum seed germination of 93% was recorded at HM which was one week after PM. At HM, the ABA:GA_3 dropped to 2.2 (Fig. 4.5). This means that when seed approached HM, seed reached maximum germination and no physiological dormancy to be concerned. However, before scarification, hard seed coat was commonly found at 34% in this study.

4.4 CONCLUSIONS

The ABA content was rapidly reduced by 56% 3 WAA and gradually dropped by an additional 29% 4 WAA. A further drop by 15% occurred 5 WAA. However, GA_3 content remained unchanged from early seed development until HM. Neither irrigation nor TE application had significant effect on the endogenous production of GA_3 and ABA during seed development and maturation. The ABA:GA_3 ratio was high at early seed development (6.7), but seed germination was low (24%). When seeds reached HM, the ABA:GA_3 ratio dropped to 2.2 and seed germination increased to 93%. These results suggest that physiological dormancy is not a substantial concern in red clover seeds.

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GA_3 ABA

Figure 4.1. Chemical structure of Gibberellic acid (GA_3) and Abscisic acid (ABA) (Rademacher, 2015).

Table 4.1. Analysis of variance for the effects of irrigation, trinexapac-ethyl (TE) and seed maturity stage on the production of gibberellic acid (GA_3), abscisic acid (ABA), ABA:GA_3 ratio and germination of red clover seeds.

Source of variation	df	GA_3	ABA	ABA:GA_3	Germination
Irrigation (I) †	1	ns ¶	ns	ns	ns
TE application (T) ‡	1	ns	ns	ns	ns
I x T	1	ns	ns	ns	ns
Seed maturity stage (M) §	3	ns	***	***	***
I x M	3	ns	ns	ns	ns
T x M	3	ns	ns	ns	ns
I x T x M	3	ns	ns	ns	ns

*** Significant at the 0.001 probability level.

† Irrigation; none and single irrigation.

‡ TE application; untreated control and 280 g TE ha^{-1} foliar application.

§ Seed maturity; harvested 2, 3, 4, and 5 weeks after anthesis.

¶ ns, nonsignificant.

Table 4.2. Means of quantities of gibberellic acid (GA_3), abscisic acid (ABA), ABA:GA_3 ratio, and germination percentage of red clover seeds during different stages of seed maturity (weeks after anthesis, WAA).

Seed maturity stage	GA_3	ABA	ABA:GA_3	Germination
---------- WAA ---------	-------- pg g^{-1} DW ----------			---- % -----
2	187 a †	1242 a	6.7 a	24 c
3	177 a	547 b	3.1 b	70 b
4	177 a	388 c	2.2 c	93 a
5	173 a	331 c	1.9 c	91 a

† Means within each column followed by the same letter are not significantly different by Fisher's protected LSD values ($P < 0.05$).

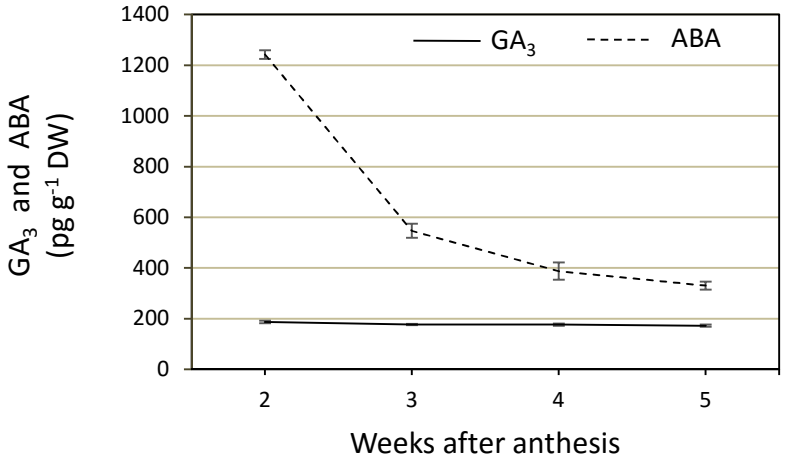

Figure 4.2. Gibberellic acid (GA_3) and abscisic acid (ABA) contents during red clover seed development and maturation. Data were the average of all field treatments. If error bars regions do not overlap, treatments are significantly different.

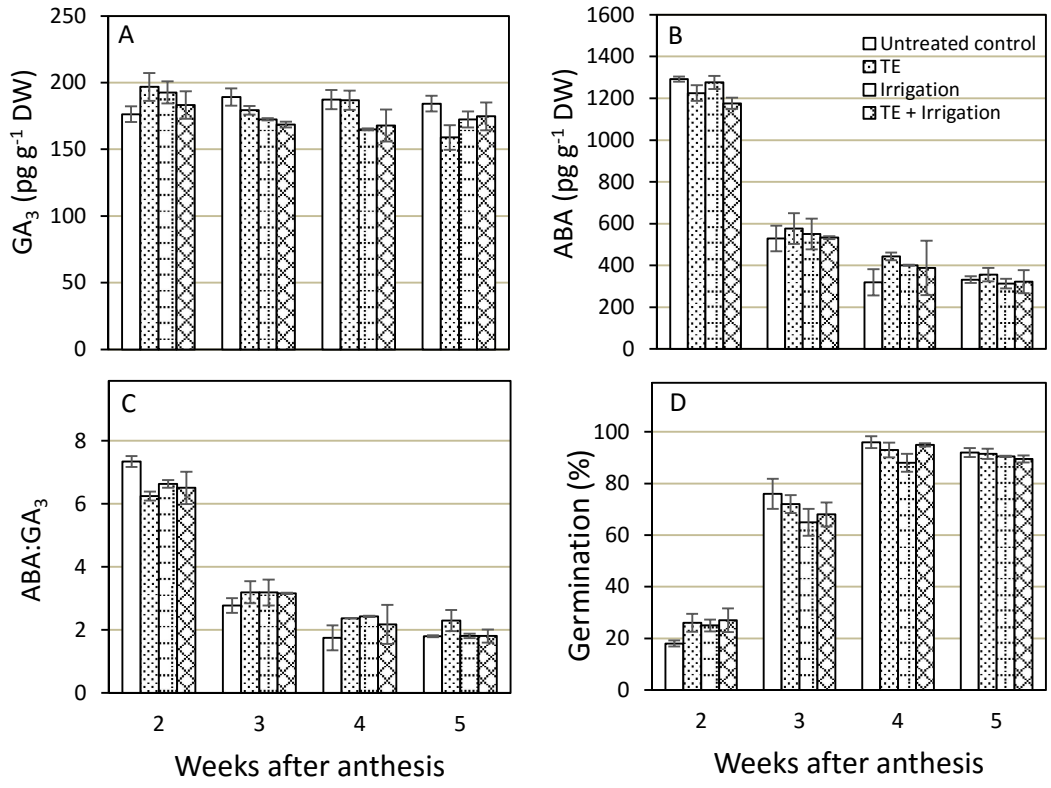

Figure 4.3. Change in (A) gibberellic acid (GA_3), (B) abscisic acid (ABA), (C) ABA:GA_3 ratio, and (D) germination in red clover seed harvested from four different field treatments during seed development and maturation. If error bars regions do not overlap, treatments are significantly different.

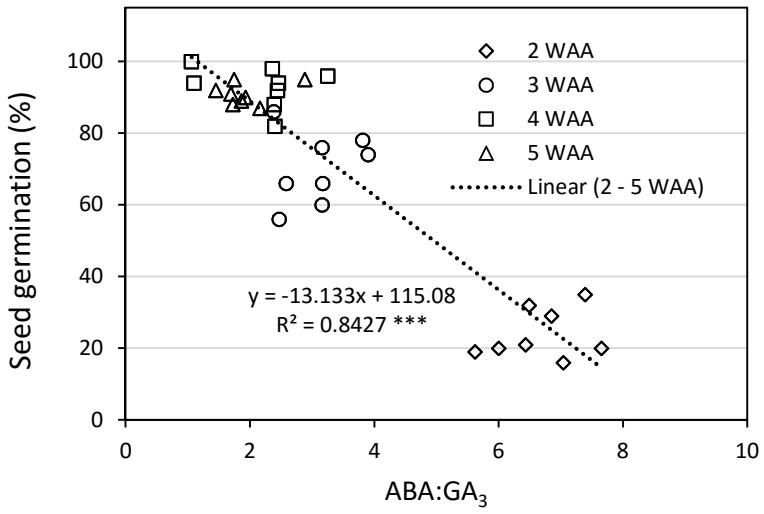

Figure 4.4. Relationship between seed germination and the ratio of abscisic acid to gibberellic acid (ABA:GA_3) in red clover seed.
Data from early seed development to harvest (2 – 5 weeks after anthesis, WAA).
*** significant at $P < 0.001$.

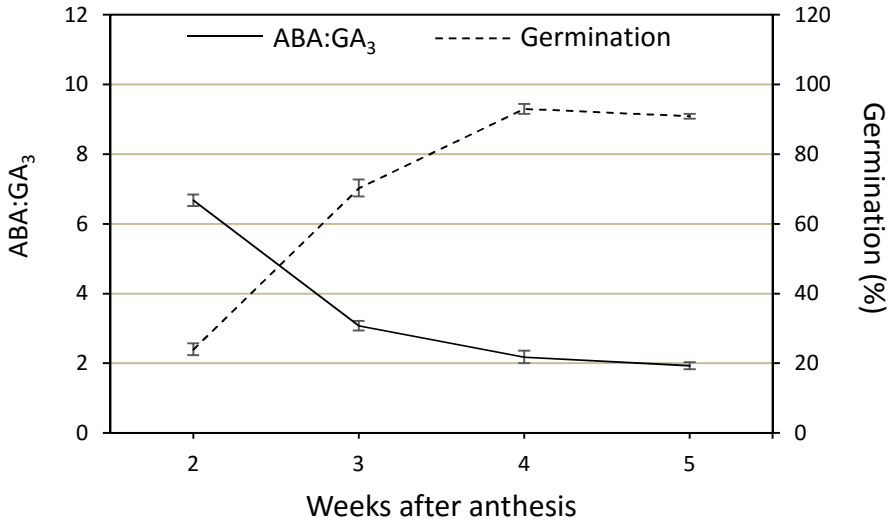

Figure 4.5. The ratio of abscisic acid to gibberellic acid (ABA:GA_3) and germination percentage of red clover during seed development and maturation.
If error bars regions do not overlap, treatments are significantly different.

CHAPTER 5: Effects of Storage Conditions on Viability and Vigor of Red Clover (*Trifolium pratense* L.) Seed

ABSTRACT

Maintaining seed quality during storage is essential to ensure its value until the time of planting. This study was conducted to determine seed viability and vigor of trinexapac-ethyl (TE)-field treated red clover seed stored at different conditions for 24 m and to measure the relationship among seed quality tests and identify the optimum tests to evaluate seed deterioration over time. Two red clover seeds lots, harvested from untreated and TE-field treated plots grown in the Willamette Valley, OR, were stored for 24 m under three storage conditions; 1) uncontrolled environment of open warehouse (WH), 2) controlled room temperature (RT) at 20°C, and 3) controlled cold storage (CS) at 10°C. Seed viability was determined by standard germination (SGT) and tetrazolium (TZT) tests. Seed vigor was determined by cold (CT), accelerated aging (AAT) tests, and electrical conductivity (EC). Seed moisture content (SMC), seed viability, and vigor were determined at 6-m intervals to measure the rate of deterioration after each storage period. In RT and CS, relative humidity (RH) was 55% and 90%, respectively. The average seed viability of both seed lots, untreated and TE-field treated seed, stored in RT and WH were 95% and 96%, respectively throughout 24 m of storage period. Seeds stored in RT conditions for 24 m maintained high vigor of 87% as determined by AAT, whereas seeds stored in the WH maintained vigor of 81% for only 18 m and then dropped to 67% at 24 m of storage period. In CS, seed viability and vigor gradually

dropped, reaching 0% at 24 m of storage period due to the adverse effect of the high RH in this storage environment. The SGT and TZT were valid tests for seed viability determination. The AAT was an appropriate test for seed vigor determination. Although CT was able to determine seed vigor, it had a tendency to overestimate seed vigor. Seed quality was closely correlated with SMC. Seed maintained acceptable viability and vigor standard of above 80% when SMC was less than 10%. The study suggests that red clover seeds from untreated and TE-treated plots can be stored safely under similar WH conditions used in this study for 18 m and in RT for 24 m when the initial SMC was under 10%.

Abbreviations: AAT, accelerated aging test; CT, cold test; CS, cold storage; EC, electrical conductivity; SMC, seed moisture content; PGR, plant growth regulator; RH, relative humidity; RT, room temperature; SGT, standard germination test; TE, trinexapac-ethyl; TZT, tetrazolium test; UC, untreated control; WH, open warehouse storage.

5.1 INTRODUCTION

Red clover is a forage legume crop that commonly used as a rotation crop in Oregon. Red clover seed production in Oregon was the number one in national ranking. It supplied approximately 75% of the US market. The estimated sale value of red clover seed produced in 2015 was 12.1 million US dollars from approximately 6,000 hectares of harvested area (Oregon Department of Agriculture, 2015).

Seed quality, as defined by viability and vigor, is maximum when seed reached either physiological maturity in several species, such as soybean (*Glycine max* L.) (Bishnoi et al., 2007) and cuphea (*Cuphea viscosissima* Jacq.) (Berti et al., 2007), or harvest maturity, such as in canola (*Brassica napus* L.) (Elias and Copeland, 2001) and red clover (Chapter 2). If seeds were left in the field after full maturation, they gradually deteriorated, especially under adverse weather conditions. Seed deterioration is an irreversible process. Therefore, storage condition needs to be well-managed to maintain seed quality and delay deterioration as much as possible.

Seeds are usually stored after harvest until the planting time, which may be a few months to a few years. The ideal storage condition to maintain seed quality is dry and cold storage. Factors that affect seed storability are initial seed quality, SMC, storage temperature and RH, length of storage, and protection from storage fungi and insects (Elias et al., 2007; Vertucci and Roos, 1993). Red clover seed can be safely stored in ambient condition for up to three years (Taylor and Quesenberry, 1996). Evans (1957) found that moisture content of red clover seed was the most important factor

influencing its life span. Seed quality was maintained in a high temperature storage when the RH was low (Evan, 1957).

Trinexapac-ethyl (TE) [4-(cyclopropyl-α-hydroxymethylene)-3,5-dioxocyclohexane-carboxylic acid ethyl ester] is a foliar applied plant growth regulator. The TE is an acylcyclohexanediones which interfere with the gibberellin biosynthesis by the structural mimics of 2-oxoglutaric acid, the co-substrate of dioxygenases. The TE blocks 3β-hydroxylation, thus preventing the formation of active gibberellins from inactive ones, resulting in gibberellin reduction, and consequently minimizing vegetative growth (Rademacher, 2000). The TE application reduces mowing and enhance turfgrass quality (Fagerness and Yelverton, 2001). Besides, it is commonly used in seed production, mainly for lodging reduction and consequently seed yield increase in grasses, such as perennial ryegrass (*Lolium perenne* L.) (Borm and van den Berg, 2008; Chastain et al., 2014; Chynoweth et al, 2008; Rolston et al., 2010) and tall fescue [*Schedonorus arundinaceus* (Schreb.) Dumort.] (Chastain et al., 2015). Recent research by Anderson et al. (2015) and previous research by Øverland and Aamlid (2007) revealed the TE application can efficiently increase seed yield in red clover. However, little is known about the effect of TE application on seed storability, especially in various storage conditions. The objectives of the study were 1) to determine seed viability and vigor of TE-field treated red clover seed stored at different conditions for 24 m and 2) to measure the relationship among seed quality tests and identify the optimum tests to evaluate seed deterioration over time.

5.2 MATERIALS AND METHODS

5.2.1 Seed Materials

Red clover seeds used in this study were harvested from untreated control (UC) and TE treated (TE) plots. The field study of the harvested seeds was conducted in the Hyslop Research Farm, Corvallis, Oregon, USA (44°38'21"N, 123°11'40"W) with the Woodburn silt-loam (fine-silty, mixed, mesic Aquultic Argixerolls) soil.

Both seed lots were from non-certified "common" diploid red clover, which is commonly used by red clover seed growers in Oregon. For TE treatment, 280 g a.i. ha^{-1} of TE was sprayed at stem elongation stage. After harvest, 300 g of cleaned seeds were placed in each woven polypropylene bags sealed with thread. Each seed lot was placed in three different storage conditions as described below.

5.2.2 Storage Conditions

Three storage conditions consisted of: 1) open warehouse (WH) with ambient condition, i.e., uncontrolled atmospheric and weather conditions in a warehouse, 2) room temperature (RT) with controlled temperature of 20°C and 55% RH, and 3) cold storage (CS) with controlled temperature of 10°C and 90% RH. For the WH, seeds were stored in an uncontrolled environment room at Hyslop research station in Corvallis, OR. For the RT and CS, seeds were stored at the Oregon State University Seed Laboratory.

5.2.3 Data Collection

Seed quality determination included seed viability, i.e., standard germination (SGT) and tetrazolium (TZT) tests, and seed vigor, i.e., cold (CT), accelerated aging (AAT) tests, and electrical conductivity (EC). Seed moisture content (SMC) and all quality tests were evaluated at each storage period, i.e., 0, 6, 12, 18, and 24 m. All tests were adapted from the protocols of the Association of Official Seed Analysts (AOSA, 2007; AOSA, 2009; AOSA, 2012) as follows:

5.2.3.1 Standard germination test

Four replications of 100 seeds each of all treatments were planted on the top of two layers of moistened germination paper, which were placed into transparent boxes at 20°C growth chamber with an alteration of 8 h of light and 16 h of dark for 7 d. The number of normal seedlings was then recorded.

5.2.3.2 Tetrazolium test

Four replications of 100 seeds each of all treatments were used. Seeds were placed on the top of moistened blue paper at 20°C for 12 h. Afterward, seeds were placed in 1% TZ solution at 35°C for 5 h. Evaluation was conducted by observing the pattern and intensity of the stained tissues under a stereo microscope. Seeds were classified into viable and non-viable. The number of viable seeds in each treatment was recorded.

5.2.3.3 Cold test

Four replications of 100 seeds each of all treatment were used. Seeds were placed on the top of moistened paper and incubated at 10°C for 7 d in darkness, and then moved into 20°C with an alteration of 8 h of light and 16 h of dark for 7 d. Afterward, the number of normal seedlings were recorded.

5.2.3.4 Accelerated aging test

Seeds were placed in a single layer on mesh tray inside a transparent plastic box containing 50 ml of water. This plastic boxes were incubated in a chamber at 41°C for 72 h. Then, four replications of 100 seeds each of all treatments were planted and evaluated after 7 d as described in the SGT.

When hard seeds were found in SGT, TZT, CT, or AAT, they were scarified to allow seeds to imbibe water. Scarification was conducted by a mechanical pneumatic seed scarifier model PSS1000 (Mater International, Inc., Corvallis, OR) using air pressure of 40 psi for 60 seconds. Scarified seeds were retested to determine their quality as explained above. Data were presented as total viability and vigor percentage, including the scarified seeds.

5.2.3.5 Electrical conductivity test

Four replications of 0.5 g of seeds were soaked in 100 ml of distilled water and stored at 20°C for 24 h. After adding water and before measuring conductivity, seeds were stirred for 10 sec to ensure uniformity of ion distribution in the solution. The EC

was measured using the Thermo Scientific Orion Versa Star Electrical Conductivity Meter (Waltham, MA). Results were reported in μS cm^{-1} g^{-1}.

5.2.3.6 Seed moisture content determination

Four replications of 2 g seeds for each treatment were placed in moisture vials and weighted using a 4-digit electronic balance. Samples were placed in an oven set at 130°C for two and a half hours (AOSA, 2007). The percentage of moisture was calculated on a wet weight basis by the following equation:

$$\%\ Moisture\ content = \frac{Seeds\ fresh\ weight - Seeds\ dry\ weight}{Seeds\ fresh\ weight}\ x\ 100$$

5.2.4 Experimental Design and Data Analysis

The experimental design was a completely randomized design with three factors: two seed lots (UC and TE), three storage conditions (WH, RT, and CS), and five storage durations (0, 6, 12, 18, and 24 m). All tests, i.e., SGT, TZT, CT, AAT, EC, and SMC were replicated four times.

Analysis of variance (ANOVA) was conducted to determine the effect of factors, i.e., seed lot, storage condition, and storage duration using the statistical package MSTAT (Michigan State Univ., East Lansing, MI). Factor (seed lots, storage condition and storage duration) means were separated by Fisher's protected LSD test at the 5% level of significance, whenever the effect of factor was significant. Regression analysis was conducted to determine the relationship among the seed quality tests.

5.3 RESULTS AND DISCUSSION

5.3.1 Temperature and RH During Storage Period

The temperatures in WH fluctuated among the four seasons of each year, they ranged between 1 and 29°C. The daily mean temperature was 16°C. The RH ranged from 36 to 79% and the daily mean RH was 54%. For RT, temperature was controlled at 20°C and the average RH was 55%, whereas the temperature in CS was controlled at 10°C and the average RH was 90% (Table 5.1).

The dynamics of temperature and relative humidity during WH storage were in opposite direction. During the first six months of WH storage (1 – 6 m, which was fall and winter), the RH was high whereas the temperature was low (61.7% and 10.8°C) (Fig. 5.1). The next six months of storage (7 – 12 m, which was spring and summer), the RH was low and the temperature was high (49.8% and 19.9°C). The following six months of storage (13 – 18 m, which was fall and winter), the RH was again high, while the temperature was low (57.2% and 12.8°C). The last six months of storage (19 – 24 m, which was spring and summer), the RH decreased, while the temperature increased (46.1% and 20.0°C). The compensation between temperature and RH assisted to maintain the seed quality in WH storage (data shown in section 5.3.3 and 5.3.4).

According to Harrington (1973), the sum of Fahrenheit temperature and RH for successful seed storage should be less than 100. However, the sum of those two in this study was above 100 in all storage conditions, 115, 123, and 140 in WH, RT, and CS, respectively (Table 5.1). In contrast to the rule of 100 (Harrington, 1973), seeds stored in

RT and WH maintained high viability, 95% and 96%, respectively after 24 m of storage (Table 5.3). This might be because of the hard seed coat structure of red clover, which minimizes water and gases exchanges and consequently promotes seed longevity.

5.3.2 Seed Moisture Content

Seed moisture content was significantly different among the three storage conditions in each storage duration (Table 5.2). Regardless of the seed lot used, the initial seed moisture content was approximately 6% (Table 5.3). The moisture contents of seeds stored in WH and RT were remained below 10% throughout the 24-m storage period, whereas the moisture contents of those stored in the CS increased to 22% after 24 m of storage. The moisture content in seed corresponded with the RH of storage environment because of the hygroscopic nature of seeds. For example, in the uncontrolled environment of WH, the seed moisture content fluctuated among the four seasons. It was 10% at the 6 and 18 m of storage duration, which matched the RH of 61.7% and 57.2% (averaged of the 1 – 6 m and the 13 – 18 m of storage, respectively). However, it decreased to 8% at the 12 and 24 m of storage duration, which matched the RH of 49.8% and 46.1% (averaged of the 7 – 12 m and the 19 – 24 m of storage, respectively) (Fig. 5.1 and Fig. 5.2). Similar results were reported by Ellis and Hong (2006), where moisture content in red clover seeds varied with the RH of the storage environment. They also reported that seed moisture content remained below 10% when RH less than or equal to 55%.

The TE-field treatment had a significant effect on seed moisture content (Table 5.2). The interactions among storage condition, duration, and seed lot were also significant. The initial seed moisture contents in the UC and the TE-treated seed lots were at 5.7% and 5.9%, respectively (Fig. 5.2). During storage, the moisture content was slightly lower in UC seed lot than in TE-field treated seed lot in most storage duration, especially, when seed stored in CS environment for 6 m. However, both seed lots had similar response to each storage condition as described above.

5.3.3 Seed Viability

5.3.3.1 Tetrazolium test

The viability of seeds stored for 24 m in WH and RT was significantly better than those stored in CS (Fig. 5.3a). Regardless of the seed lots, treated with TE or untreated, the initial seed viability by TZT was high, 96.6%. After 24 m of storage, the viability of seed stored in the WH and RT maintained high at 96.1% and 94.6%, respectively. In contrast, the viability of seeds stored in the CS declined gradually over time and dropped to 0% at the end of the 24-m storage period, i.e., 92.1%, 22.3%, 7.3%, and 0.0% for 6, 12, 18, and 24 m, respectively. This is mainly due to the adverse effect of high RH (90%) in the CS.

The TE-field treatment did not have significant effect on seed viability by TZT (Table 5.2). However, the interactions among seed storage condition, storage duration, and seed lot (UC and TE) were significant. The effect of TE-field treated seed lot on seed viability was inconsistent in different storage conditions and durations as shown in Fig.

5.5. The initial seed viability of UC and TE was similar at 97.3% and 96.0%, respectively. During storage, the viability was slightly different between UC and TE seed lots in each storage condition and duration. However, both UC and TE maintained high viability throughout the 24-m study period, which was 95.5% and 96.8% in WH conditions and 96.3% and 93.0% in RT conditions, respectively.

5.3.3.2 Standard germination test

Seed viability results by the SGT was similar to those by the TZT. The WH and RT were optimum storage conditions for maintaining high seed viability throughout the storage period (Fig. 5.3b). Regardless of the seed lots, the initial germination percentage was 95.0%. After 24 m of storage, seeds stored in the WH and RT maintained high germination at 95.1% and 94.9%, respectively. On the other hand, seeds stored in the CS were unable to maintain their high germination, which gradually dropped until reaching 0% at the end of 24-m storage period. The germination was 78.8%, 24.4%, 4.0%, and 0.0% for the 6, 12, 18, and 24 m, respectively.

Seed viability by SGT was significantly affected by TE-field treatment without significant interaction with storage conditions and durations (Table 5.2). The initial seed germination percentage was similar at 95.5% and 94.5% for UC and TE, respectively. During storage, the germination was slightly higher in the UC than in the TE under all storage conditions (Fig. 5.5). However, both UC and TE seed lots maintained high germination percentage during the 24-m storage, 95.5 and 94.8% in WH conditions and 96.8% and 93.0% in RT conditions, respectively.

These results showed that the seeds that stored in the WH and RT conditions maintained high viability for two years. This suggests that constant temperature of 20°C with 55% RH of RT was suitable environment to store red clover seed for two years. The fluctuation of ambient environment in the WH, with temperature ranging from 1°C to 29°C and RH ranging from 36% to 79%, did not have adverse effect on seed viability during the 24-m storage period. This might be due to the compensation between RH and temperature, i.e., the RH was low when the temperature was high and vice versa. In addition, neither the high temperature nor the high RH lasted enough to affect seed viability significantly. However, the low temperature (10°C) in the CS was not low enough to offset the negative effect of the high RH (90%) in the storage, which eventually caused the loss of viability in two years. This confirmed the finding of Evans (1957) that RH was the most important factor for red clover seed longevity. This finding agreed with other reports in different crops. For example, seed viability of soybean seed maintained high when seed stored in cold temperature (10°C), but with low RH (below 60%) (Mbofung et al., 2013). In addition, Elias and Copeland (1994) reported that the viability of canola remained high when stored in low temperature of 5°C even with high RH of 75%. The high RH in cold storage did not interfere seed viability because seeds were placed in the plastic Ziploc bags. The bags acted as a gas and moisture barrier. Therefore, the moisture content of seed remained quite stable (Elias and Copeland, 1994). This indicated that seed container is an important factor to prevent negative effect of RH and prolong seed quality in storage. The woven polypropylene bags, which

were used in this study, did not have proper moisture barrier. Therefore, the high RH from the cold storage environment affected the seeds inside the bags and increased the seed moisture content (data presented in section 5.3.2).

Seed deterioration in CS is caused by loss in seed coat integrity. When seed absorbed moisture from the high RH in the CS environment, seeds expanded in size (data not shown). The increase in seed size reduced the density and thickness of the seed coat and then gradually it lost its integrity. The weak seed coat and the high RH favored microflora to grow and consequently affect the viability and the vigor of seeds. This was confirmed by the finding of high amount of microflora during SGT, TZT, CT, and AAT, as well as the high seed leachates content in the EC test of the seeds that stored in CS conditions (data shown in section 5.3.4). Also, seed coat color in deteriorated seed was changed from bright to dim and faded color. This finding was also reported in a previous study in clover (Vaughan and Delouche, 1968).

5.3.4 Seed Vigor

5.3.4.1 Cold test

The vigor of seeds stored for 24 m in WH and RT was significantly higher than those in CS (Fig. 5.4a). Regardless of the seed lots (UT or TE), seed vigor by the CT was high, 92.5%, at the beginning of the study. After 24 m of storage, seed vigor remained high at 92.4% in both WH and RT conditions. In contrast, the vigor of the seeds stored in the CS gradually decreased over time and dropped to 0% at the end of the 24-m storage period. It was 77.1%, 26.9%, 5.3%, and 0.0% for 6, 12, 18, and 24 m of storage,

respectively. Therefore, the WH and RT conditions were the near optimum for both seed lots in maintaining seed vigor as measured by the CT throughout the 24-m storage duration.

The TE-field treatment did not have significant effect on seed vigor by CT (Table 5.2). However, the interactions among storage condition, storage duration, and seed lot (UC and TE) were significant. The effect of TE-field treated seed lot on seed vigor was inconsistent in different storage conditions and durations as shown in Fig. 5.6. The initial seed vigor of UC and TE was similar at 93.5% and 91.5%, respectively. During storage, seed vigor was slightly different between UC and TE seed lots in each storage condition and duration. For example, at 6 m of CS storage, vigor of TE seed lot decreased in a faster rate than those of UC seed lot, i.e., the vigor was 86.3% and 67.5% for UC and TE, respectively. After that, seed vigor decreased to the similar value in both seed lots and dropped to zero at the end of the 24-m study. However, both UC and TE successfully maintained high vigor throughout the 24-m study period, which was 90.8% and 94.0% in WH condition and 92.0% and 92.8% in RT conditions, respectively.

5.3.4.2 Accelerated aging test

Seed vigor as measured by the AAT was significantly different among the three storage conditions in each storage duration (Fig. 5.4b). The average initial seed vigor by AAT was high at 93.3% for both seed lots. The RT environment was the optimum for maintaining high seed vigor of 87.0% throughout the 24-m study period, whereas seeds that stored in the WH maintained high seed vigor of 81.4% after 18 m of storage and

then the vigor dropped to 67.1% at the end of the 24 m of storage period. The vigor of seeds stored in the CS decreased rapidly over time after 6 m of storage, i.e., 49.9%, 3.5%, 0.5%, and 0.0% in 6, 12, 18, and 24 m of storage, respectively.

Seed vigor by AAT was significantly affected by TE-field treatment. Moreover, the interactions among storage condition, duration, and seed lot were significant (Table 5.2). The initial seed vigor was similar at 94.0% and 92.5% for UC and TE, respectively. During storage, the vigor was slightly higher in the UC than in the TE under all storage conditions (Fig. 5.6). For example, the TE seeds lost their vigor faster than the UC after 6 m of storage, i.e., the vigor was 67.3 and 32.5% for the UC and the TE, respectively. The vigor of both UC and TE seed lots was low at 3.0 and 4.0%, respectively after 12 m of CS storage and dropped to 0% after 24 m. However, both UC and TE seed lots successfully remained high vigor, which were 87.5% and 86.5%, respectively, during 24 m of storage only in the RT environment.

5.3.4.3 Electrical conductivity

The EC results were significantly different among the three storage conditions in each storage duration (Table 5.2). Regardless seed lot (treated with TE or untreated control), the average initial EC was 82.0 µS cm^{-1} g^{-1} (Table 5.3). The EC values were slightly increased to 95.9 and 109.5 µS cm^{-1} g^{-1} of the seeds stored for 24 m in RT and WH, respectively. On the other hand, the EC values of the seeds stored in the CS environment for 24 m sharply increased to 333.3 µS cm^{-1} g^{-1} (Fig. 5.4c).

The TE-field treatment had a significant effect on EC (Table 5.2). The interaction between storage duration and seed lot (UC and TE) was also significant. The electrical conductivity was lower in UC, compared to TE at the beginning of seed storage, i.e., 72.4 and 91.7 μS cm^{-1} g^{-1}, respectively. During storage, the EC was mostly lower in UC than in TE (Fig. 5.6). However, the EC of both seed lots had similar response to each storage condition.

The EC test measures the leakage of electrolytes, such as amino acids and inorganic ions from cell membrane. Low quality seeds contain poor membrane structure that allows electrolytes to diffuse to the water. These leakage compounds are necessary for seedlings to emerge speedily. High quality seeds have their nutrients within their membranes. Therefore, seeds with higher EC values showed more electrolyte leakage and lower quality (AOSA, 2009). Seeds stored in CS condition had the highest EC value which related to the lowest seed viability and vigor when compared to those in WH and RT conditions (Fig. 5.3 and 5.4).

In summary, the TE seed lot was slightly lower in viability and vigor than the UC during the storage period. The possible explanation might be due to the smaller seed size of TE seed lot (data shown in chapter 3). The larger seeds produced higher germination and vigor rates than smaller seeds in several crops, such as oats (*Avena sativa* L.) (Willenborg et al., 2005), triticale (xTriticosecale Witm. cv. Presto) (Kaydan and Yagmur, 2008), and lentil (*Lens culinaris* Medik.) (Hojjat, 2011). The water uptake of larger seeds was at higher rate than smaller seeds as it is the case in safflower

(*Carthamus tinctorius* L.) (Farhoudi and Motamedi, 2010). Moreover, the amount of carbohydrates and other nutrients in larger seeds was higher than those in smaller seeds (Gunaga et al., 2011).

5.3.5 Relationship among Seed Quality Tests

All quality tests were significantly correlated as presented in Table 5.4. The relationships of each test were as follows:

5.3.5.1 Tetrazolium and standard germination tests

The results from the TZT were closely related to those from the SGT with a linear regression equation of $Y = 1.0072X + 1.6737$ (where, Y is the viability by TZT and X is the viability by SGT; $R^2 = 0.9818$, $P < 0.001$) (Fig. 5.7a). Therefore, TZT can be a practically alternative viability test for red clover rather than the SGT. Similar correlations between the TZT and the SGT were reported in forbs (*Lespedeza capitata* Michx.) (Riebkes et al., 2015).

5.3.5.2 Cold and accelerated aging tests

The results from the CT were significantly correlated with those from the AAT with a linear regression equation of $Y = 1.0321X - 9.2535$ (where, Y is the vigor by AAT and X is the vigor by CT; $R^2 = 0.9043$, $P < 0.001$) (Fig. 5.7b). Such equation suggests that CT tends to over-evaluate seed vigor compared to AAT. For example, seed vigor evaluation by CT indicated that seed stored in WH and RT maintained high vigor (92.4% in both storage conditions) after 24 m. However, AAT evaluation showed that seed vigor slightly declined and had significant difference between this two storage conditions

(67.1% and 87.0% in WH and RT, respectively) after 24 m of storage (Fig. 5.4a and b). This means that cold temperature (10°C) in the CT was not sufficient to stress the seeds. On the other hand, the high temperature and high humidity in the AAT effectively stressed the seeds and provided meaningful evaluation of seed vigor in red clover. Similar results on the effectiveness of the AAT were reported in red clover (Havstad et al., 2011), and soybean (Mbofung et al., 2013). Moreover, both CT and AAT were effective vigor tests which were closely correlated with field emergence for canola (Elias and Copeland, 1997).

5.3.5.3 The electrical conductivity and other seed quality tests

The EC results significantly correlated with other seed quality determinations. The relationship between viability tests and EC was expressed by the linear regression equations: $Y = 128.95 – 0.37X$ for the SGT and $Y = 131.81 – 0.38X$ for the TZT; where Y is the viability percentage and X is the EC; $P < 0.001$, $R^2 = 0.9687$ and 0.9606 for SGT and TZT, respectively (Fig. 5.8a and Fig. 5.8b). The relationship between vigor tests and EC was expressed by the linear regression equations: $Y = 126.35 – 0.36X$ for the CT and $Y = 123.25 – 0.39X$ for the AAT; where, Y is the vigor percentage and X is the EC; $P < 0.001$, $R^2 = 0.9621$ and 0.941 for CT and AAT, respectively (Fig. 5.8c and Fig. 5.8d). Such equations suggest that seed viability and vigor are maintained their quality of at least 80% when EC is less than 112 $\mu S\ cm^{-1}\ g^{-1}$. Atis et al. (2011) found close relationship between electrolyte leakage value and seed quality, particularly seed vigor and seedling growth rate in red clover. They also found that the leakage was comparable to the seed

color changing from brightness to darkness in seed lots. The yellow colored seed lot had a low EC value of 171.0 µS cm^{-1} g^{-1} with high percentage of seedling emergence of 77%. On the other hand, the brown colored one had high EC values of 222.3 µS cm^{-1} g^{-1} with low percentage of seedling emergence of 30% (Atis et al., 2011). Other studies in soybean found close relationships between EC results and seedling field emergence depending on soil water content (Colete et al., 2004; Vieira et al., 2004). These studies showed that when water availability in soil is limited, laboratory results become difficult to relate to seed field performance. AOSA (2009) reported that EC results can identify seed lots as ranking from high to low vigor without relating them to seedling emergence under field conditions.

5.3.5.4 Seed moisture content and quality tests

Seed moisture content significantly correlated with seed quality. The relationship between viability tests and SMC was expressed by the linear regression equations: $Y = 136.50 – 5.70X$ for the SGT and $Y = 138.03 – 5.64X$ for the TZT; where, Y is the viability percentage and X is the SMC; $P < 0.001$, $R^2 = 0.8327$ and 0.7874 for the SGT and the TZT, respectively (Fig. 5.9a and Fig. 5.9b). The relationship between vigor tests and SMC was a linear regression equation of $Y = 133.57 – 5.52X$ for CT and $Y = 134.29 – 6.24X$ for AAT; where, Y is the vigor percentage and X is the SMC; $P < 0.001$, $R^2 = 0.8239$ and 0.8933 for CT and AAT, respectively (Fig. 5.9c and Fig. 5.9d). Such equations suggest that seed viability and vigor is maintained their quality of at least 80% for 24 m when

SMC is less than 10%. The same level of SMC of less than 10% was also suggested for red clover storage by Taylor and Quesenberry (1996).

5.4 CONCLUSIONS

Red clover seeds successfully maintained average viability of 95% to 96% in RT and WH, respectively throughout the 24-m storage period. However, seeds maintained high vigor of 87% as measured by the AAT only under RT environment. The vigor of seed stored in the WH maintained vigor of above 80% for 18 m and then dropped to below 80% after 24 m of storage. The viability and vigor of seeds stored in CS gradually decreased and completely lost (0%) by the end of the 24- m storage period. This is due to the adverse effect of high RH (90%) in the CS. The SGT and TZT tests were valid tests for measuring seed viability during storage. The AAT was an appropriate test for assessing seed vigor. Although CT was able to determine seed vigor, it had a tendency to over-estimate seed vigor. We suggest using 5°C instead of 10°C for CT in future studies. Seed quality was closely correlated with SMC. Seeds that stored in WH and RT maintained their viability and vigor at or above 80% and the EC below 112 µS cm^{-1} g^{-1} for 24 m when SMC was less than 10%. The TE-field treatment slightly lowered seed viability and vigor compared to untreated control during the 24-m storage period. However, both untreated and field-treated seed lots responded similarly to the storage conditions used in the study. Therefore, red clover seeds from untreated and TE-treated plots can be stored safely in open warehouse conditions in the Willamette Valley, OR for up to 18 m and in the controlled room temperature storage for at least 24 m when seed moisture content is under 10%.

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Table 5.1. Mean temperature (Temp) and relative humidity (RH) and the sum of Temp and RH of three storage conditions, open warehouse with ambient condition, room temperature with controlled temperature at 20°C, and cold storage with controlled temperature at 10°C.

Storage condition	Temp (°C)	RH (%)	Sum of Temp and RH (°F + %RH)
Open warehouse (WH) †	16	54	115
Room temperature (RT)	20	55	123
Cold storage (CS)	10	90	140

† WH: min and max temperature = 1 – 29°C; min and max relative humidity (RH) = 36 – 79%; min and max of Temp in F plus RH = 99 – 135.

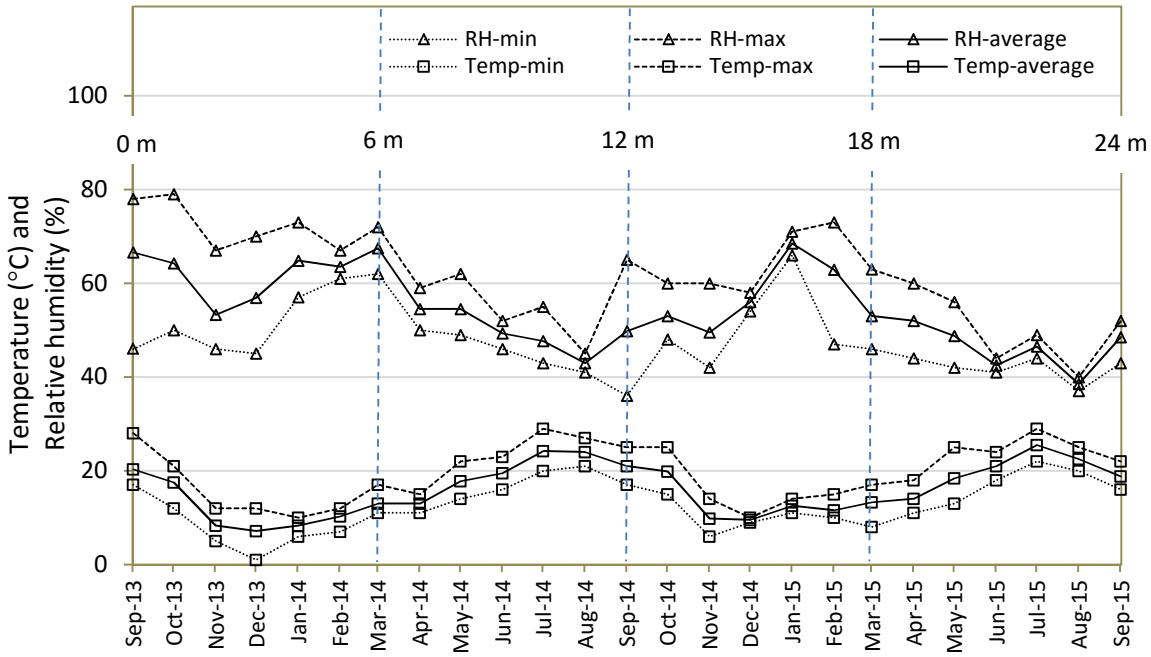

Figure 5.1. Temperature (Temp, °C) and relative humidity (RH, %) of ambient condition in open warehouse during the 24-m seed storage duration (0, 6, 12, 18, and 24 m of storage).

1 – 6 m: RH-average = 61.7%, Temp-average = 10.8°C
7 – 12 m: RH-average = 49.8%, Temp-average = 19.9°C
13 – 18 m: RH-average = 57.2%, Temp-average = 12.8°C
19 – 24 m: RH-average = 46.1%, Temp-average = 20.0°C

Table 5.2. Analysis of variance for the effects of storage conditions, durations, and seed lots on moisture content (SMC), viability, and vigor of red clover seeds stored for 24 m. Viability and Vigor were measured by standard germination (SGT), tetrazolium (TZT), cold (CT), accelerated aging (AAT) and electrical conductivity (EC) tests.

Source of variation	df	SMC	Viability		Vigor		
			SGT	TZT	CT	AAT	EC
Storage condition (C) †	2	***	***	***	***	***	***
Seed lot (S) ‡	1	***	**	ns	ns	***	***
Storage duration (D) §	4	***	***	***	***	***	***
C x S	2	**	ns ¶	*	*	*	ns
C x D	8	***	***	***	***	***	***
S x D	4	**	ns	*	**	***	**
C x S x D	8	***	ns	***	***	***	ns

*, **, *** Significant at the 0.05, 0.01, and 0.001 probability level, respectively.
† Storage condition: open warehouse, room temperature, and cold storage.
‡ Seed lot: seeds harvested from untreated and trinexapac-ethyl treated plots.
§ Storage duration: 0, 6, 12, 18, and 24 m of storage.
¶ ns, nonsignificant.

Table 5.3. Means of seed moisture content (SMC), seed viability by standard germination (SGT) and tetrazolium (TZT), and seed vigor by cold (CT), accelerated aging (AAT) tests, and electrical conductivity (EC) of two red clover seed lots stored at three different storage conditions for 24 months.

Factor	SMC	Viability		Vigor		
		SGT	TZT	CT	AAT	EC
	---------- % ----------					$\mu S\ cm^{-1}\ g^{-1}$
Storage condition						
WH - Open warehouse	8.5 b†	95.0 a	96.8 a	93.8 a	85.6 b	99.4 b
RT - Room temperature (20°C)	6.5 c	94.5 a	96.2 a	92.7 a	91.4 a	87.0 c
CS - Cold storage (10°C)	16.5 a	40.4 b	43.7 b	40.4 b	29.4 c	237.2 a
Seed lot						
UC - Untreated control	10.3 b	77.5 a	79.2 a	76.2 a	71.1 a	136.5 b
TE - TE foliar treatment	10.6 a	75.8 b	78.6 a	75.0 a	66.5 b	145.9 a
Storage duration (months)						
0	5.8 e	95.0 a	96.6 a	92.5 a	93.3 a	82.0 d
6	10.9 d	89.8 b	94.7 b	88.6 b	80.2 b	104.8 c
12	11.1 c	71.3 c	73.0 c	72.5 c	62.4 c	162.4 b
18	12.0 b	63.8 d	66.5 d	63.0 d	56.8 d	177.3 a
24	12.7 a	63.3 d	63.6 e	61.6 d	51.4 e	179.5 a

† Within each column and by factor, means followed by the same letter are not significantly different by Fisher's protected LSD values ($P \leq 0.05$).

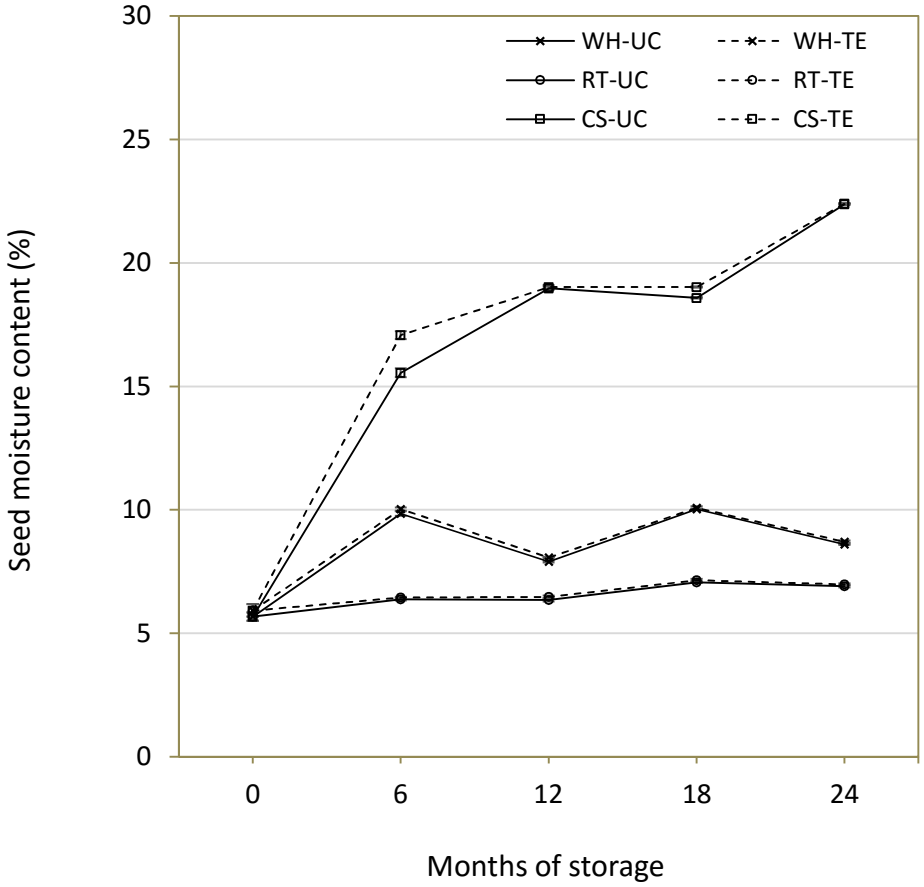

Figure 5.2. Moisture content in red clover seed affected by storage condition and trinexapac-ethyl (TE) field treatment.
(WH = open warehouse with ambient condition, RT = room temperature storage with controlled temperature of 20°C, CS = cold storage with controlled temperature of 10°C, UC = seeds harvested from untreated control plot, and TE = seeds harvested from TE-treated plot).

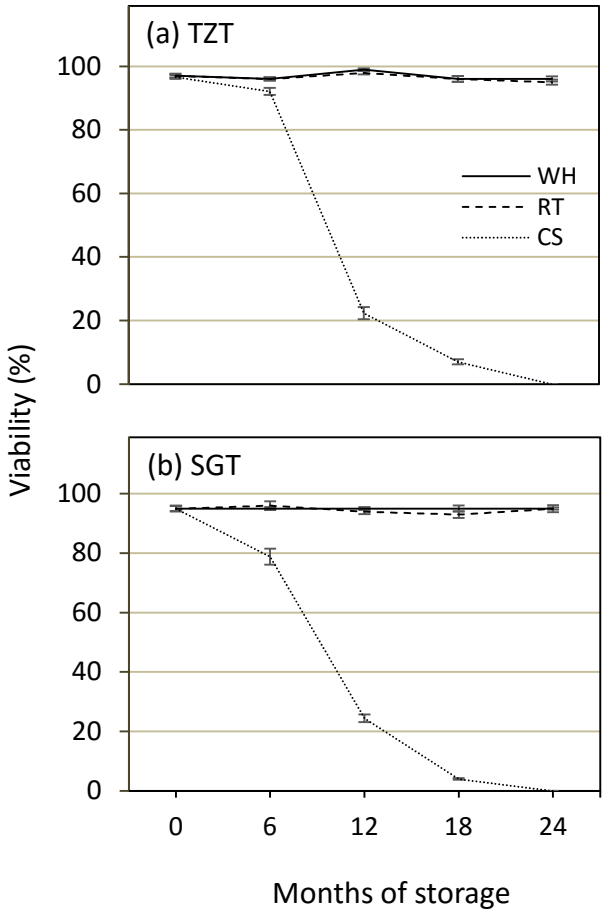

Figure 5.3. Effect of storage condition on viability of red clover seed in 24 months of storage. If error bars regions do not overlap, treatments are significantly different. (WH = open warehouse with ambient condition, RT = room temperature storage with controlled temperature of 20°C, CS = cold storage with controlled temperature of 10°C, UC = seeds harvested from untreated control plot, and TE = seeds harvested from TE-treated plot).

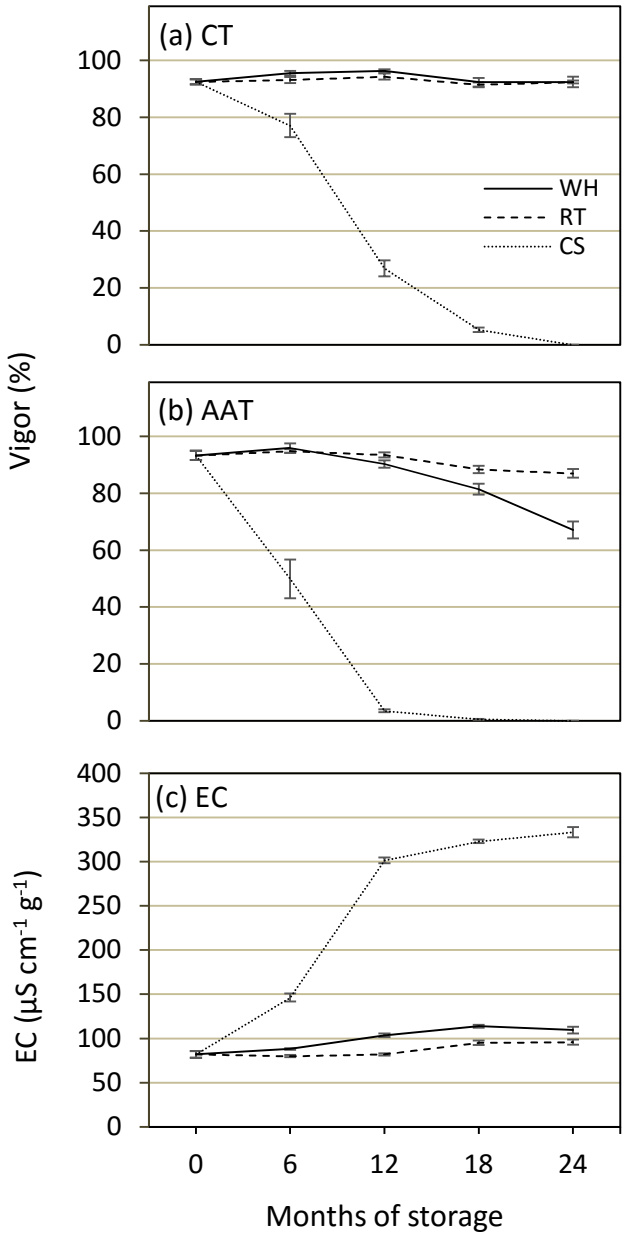

Figure 5.4. Effect of storage condition on vigor of red clover seed in 24 months of storage. If error bars regions do not overlap, treatments are significantly different. (WH = open warehouse with ambient condition, RT = room temperature storage with controlled temperature of 20°C, CS = cold storage with controlled temperature of 10°C, UC = seeds harvested from untreated control plot, and TE = seeds harvested from TE-treated plot).

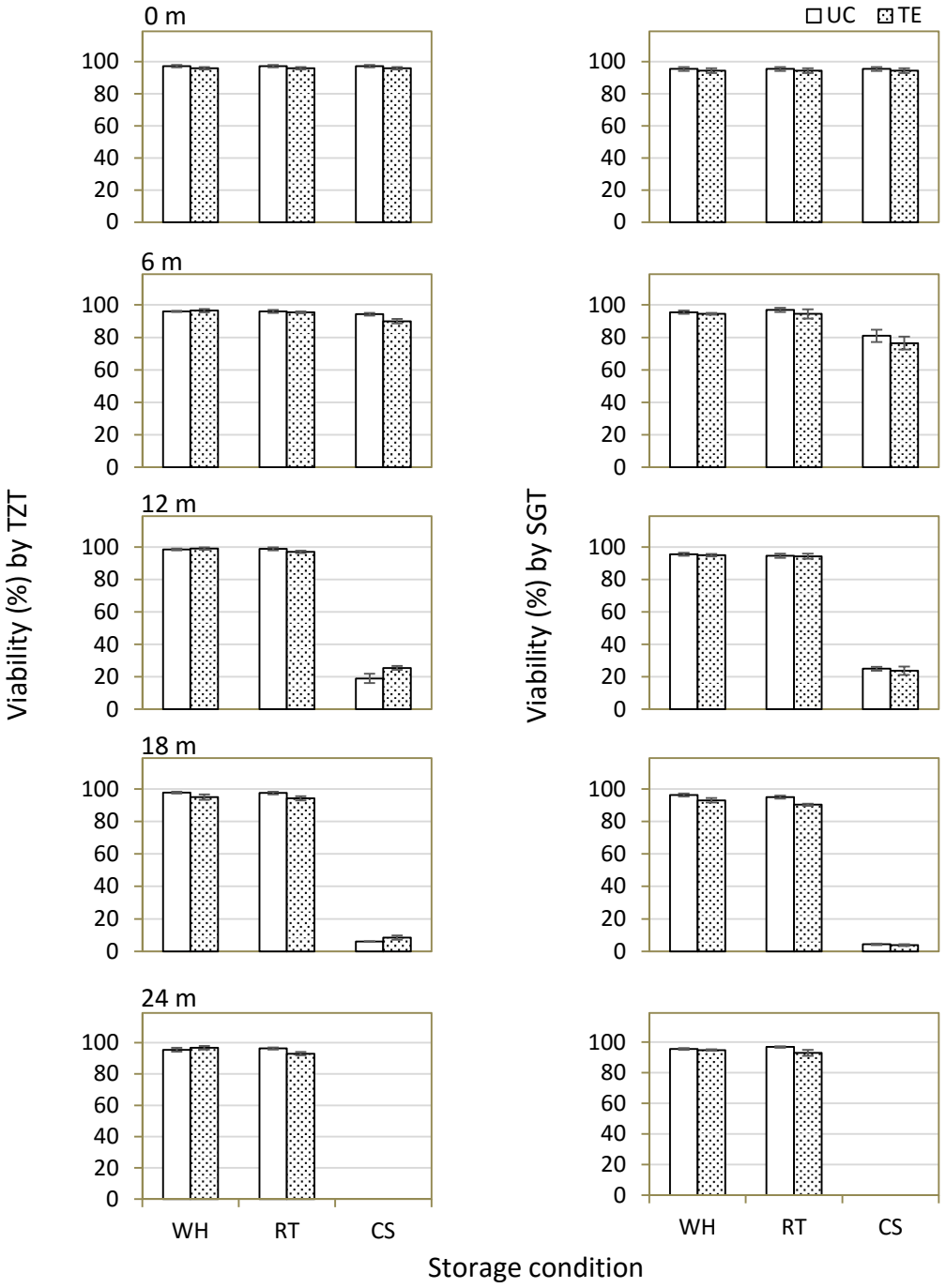

Figure 5.5. Seed viability by standard germination (SGT) and tetrazolium (TZT) tests in red clover seed affected by trinexapac-ethyl (TE) field treatment, storage condition, and storage duration.
If error bars regions do not overlap, treatments are significantly different. (WH = open warehouse with ambient condition, RT = room temperature storage with controlled temperature of 20°C, CS = cold storage with controlled temperature of 10°C, UC = seeds harvested from untreated control plot, and TE = seeds harvested from TE-treated plot).

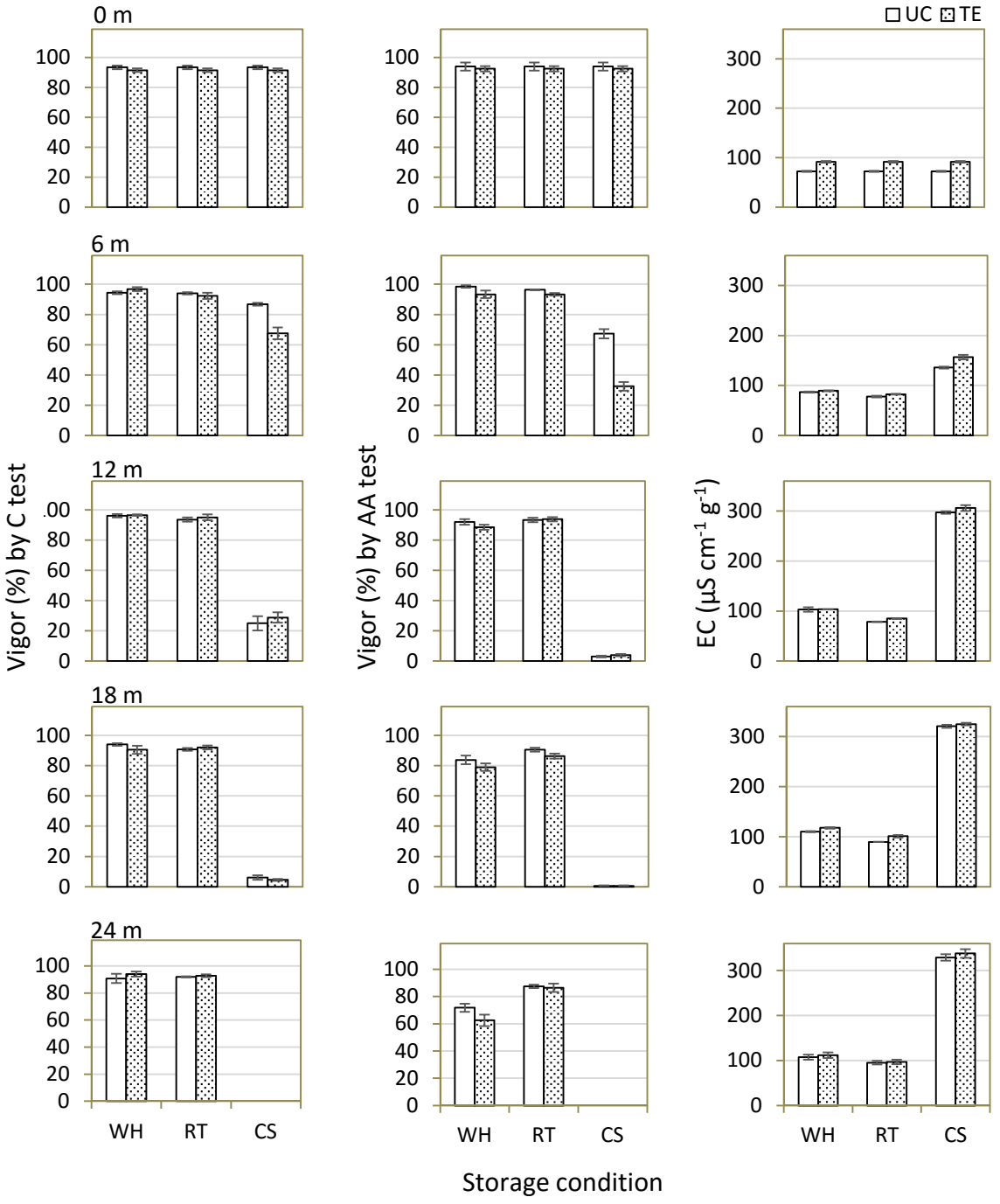

Figure 5.6. Seed vigor by cold (CT), accelerated aging (AAT), and electrical conductivity (EC) tests in red clover seed affected by trinexapac-ethyl (TE) field treatment, storage condition, and storage duration. If error bars regions do not overlap, treatments are significantly different.
(WH = open warehouse storage with ambient condition, RT = room temperature storage with controlled temperature of 20°C, CS = cold storage with controlled temperature of 10°C, UC = seeds harvested from untreated control plot, and TE = seeds harvested from TE-treated plot).

Table 5.4. Correlation coefficients (r) between seed moisture content (SMC) and quality tests: tetrazolium (TZT), standard germination (SGT), cold (CT), accelerated aging (AAT), and electrical conductivity (EC) tests of red clover seeds.

	TZT	SGT	CT	AAT	EC
SMC	-0.887***	-0.913***	-0.908***	-0.945***	0.938***
TZT		0.991***	0.987***	0.935***	-0.980***
SGT			0.989***	0.949***	-0.984***
CT				0.951***	-0.981***
AAT					-0.970***

*** Significant at the 0.001 probability level.

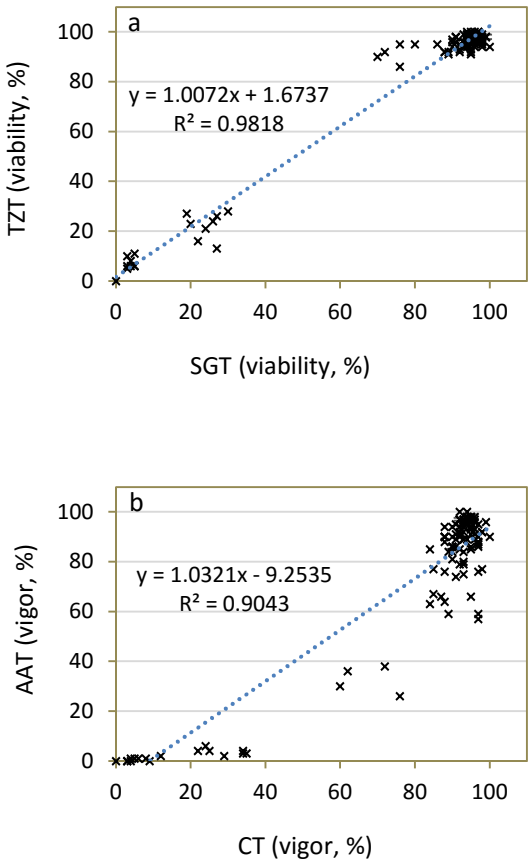

Figure 5.7. (a) Relationship between standard germination (SGT) and tetrazolium (TZT) tests and (b) relationship between cold (CT) and accelerated aging (AAT) tests.

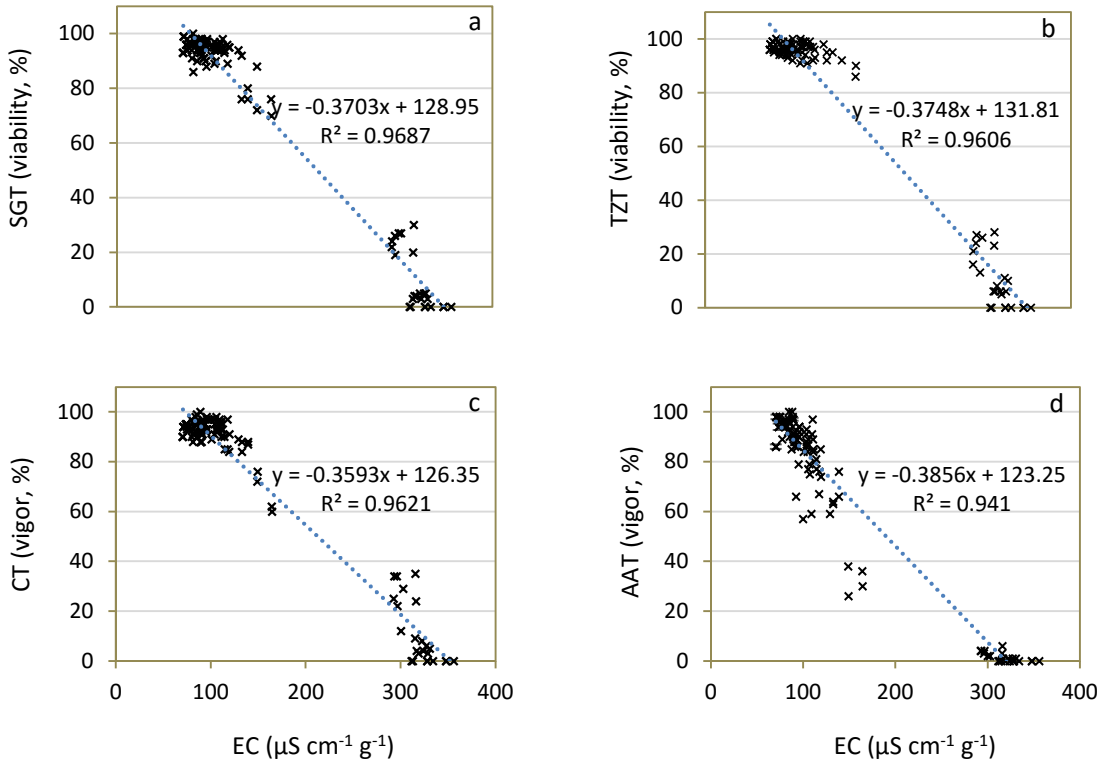

Figure 5.8. Relationship between electrical conductivity (EC) and other seed quality tests; (a) standard germination test (SGT), (b) tetrazolium test (TZT), (c) cold test (CT), and (d) accelerated aging test (AAT).

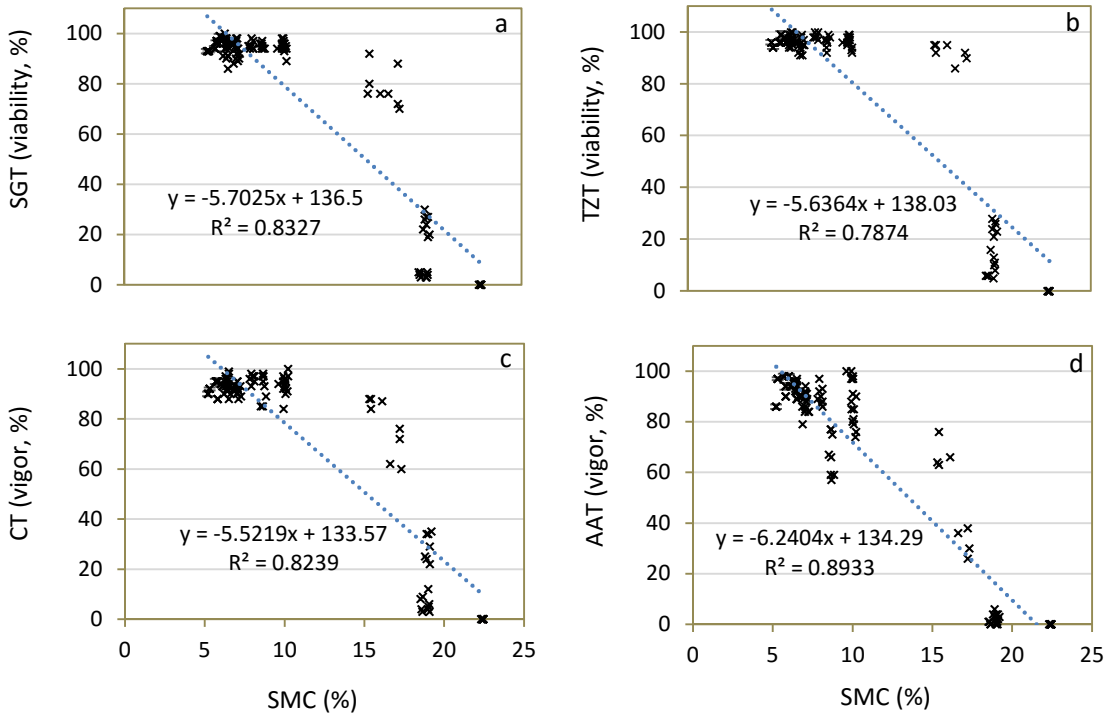

Figure 5.9. Relationship between seed moisture content (SMC) and seed quality tests; (a) standard germination test (SGT), (b) tetrazolium test (TZT), (c) cold test (CT), and (d) accelerated aging test (AAT).

Chapter 6: Conclusions

Neither rate nor timing of TE application had significant effect on attaining PM or HM of red clover compared to untreated control. Irrigation treatment, however, delayed PM and HM by approximately four days. Seed quality, viability and vigor, reached maximum levels at HM and was not significantly different among TE and irrigation treatments. For maximum seed yield, harvesting after PM and not beyond HM can help to avoid the negative effects of unfavorable weather condition, e.g., rainfall and wind, to reduce seed loss by shattering. Swathing the crop can be done approximately three to four weeks after anthesis and threshing time is one week later.

Irrigation and TE independently increased seed yield in red clover; however, the interaction between these two factors was not significant. The irrigation increased seed yield in the first and second years by an average of 10% due to the greater seed weight. However, TE increased seed yield by up to 18% only when applied at stem elongation stage in the second-year stand under study conditions due to the greater number of heads per stem. Seed quality from all treatments were similar with high percentage of viability and vigor, which was slightly correlated with seed weight and number of stem per m^2, respectively. However, none of them significantly affected seed quality. The study revealed that seed yield can be increased by: 1) a single irrigation application during first flowering stage (BBCH 55) in both years; and 2) TE application at a rate of 280 g a.i. ha^{-1} at the stem elongation stage (BBCH 32) in the second-year stand of red clover.

The ABA content was rapidly reduced by 56% 3 WAA and gradually dropped by an additional 29% 4 WAA. Further drop by 15% occurred 5 WAA. However, GA_3 content stayed unchanged from seed formation until HM. Neither irrigation nor TE application had significant effect on the endogenous production of GA_3 and ABA during seed development and maturation. The ABA:GA_3 ratio was high at the early stage of seed development (6.7), but seed germination was low (24%). When seeds reached HM, the ABA:GA_3 ratio dropped to 2.2 and seed germination increased to 93%. These results suggest that physiological dormancy is not a substantial concern in red clover seeds.

Red clover seeds successfully maintained average viability of 95% to 96% in RT and WH, respectively throughout the 24-m storage period. However, seeds maintained high vigor of 87% as measured by the AAT only under RT environment. The vigor of seed stored in the WH maintained vigor of above 80% for 18 m and then dropped to below 80% after 24 m of storage. The viability and vigor of seeds stored in CS gradually decreased and was completely lost (0%) by the end of the 24- m storage period. This is due to the adverse effect of high RH (90%) in the CS. The SGT and TZT tests were valid tests for measuring seed viability during storage. The AAT was an appropriate test for assessing seed vigor. Although CT was able to determine seed vigor, it had a tendency to over-estimate seed vigor. We suggest using 5°C instead of 10°C for CT in future studies. Seed quality was closely correlated with SMC. Seeds that stored in WH and RT maintained their viability and vigor at or above 80% and the EC below 112 µS cm^{-1} g^{-1} for 24 m when SMC was less than 10%. The TE-field treatment slightly lowered seed

viability and vigor compared to untreated control during the 24-m storage period. However, both untreated and field-treated seed lots responded similarly to the storage conditions used in the study. Therefore, red clover seeds from untreated and TE-treated plots can be stored safely in open warehouse condition in the Willamette Valley, OR for up to 18 m and in the controlled room temperature storage for at least 24 m when seed moisture content is under 10%.

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