COMPARISONS OF SEED WEIGHT AND SEEDLING CHARACTERISTICS OF DIPLOID AND AUTOTETRAPLOID RED CLOVER

By

HIDETO FURUYA

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This thesis is dedicated to my grandmother, Morimoto Matsuyo (1907-), who has farmed all of her life.

with respect,

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This research was designed to improve red clover establishment in Florida. I would be very grateful if these research results could provide an improvement of forage production worldwide as well as contribute knowledge to academia.

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COMPARISONS OF SEED WEIGHT AND SEEDLING CHARACTERISTICS OF DIPLOID AND AUTOTETRAPLOID RED CLOVER

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Hideto Furuya

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Seedling establishment is a critical aspect associated with the profitability of annual forage production in Florida. The objective of this research was to evaluate the effect of chromosome doubling on seed production and seedling characteristics of red clover (*Trifolium pratense* L.), a cool-season forage legume adapted to winter-spring conditions in north Florida. Autotetraploid (4*x*) populations were produced by nitrous oxide treatment within the diploid (2*x*) cv. 'Cherokee.' Four clones of 2*x* and 4*x* seedlings from each of eight crosses were grown in the field to increase seed with the 2*x* and 4*x* populations physically isolated. Seeds were harvested from individual clones, and seed number and weight of each genotype were determined at each ploidy level. The mean seed number per plant for the 2*x* and 4*x* populations was 806 and 26, respectively. Mean seed weight of the 4*x* was approximately 1.5 times that of the 2*x*. There was a ploidy level x

cross interaction effect, indicating that superior seed-producing genotypes at the 2*x* level were different from those at the 4*x* level. Thereafter, seeds from all genotypes within each ploidy level were composited into two populations. The 2*x* and 4*x* seeds were germinated in growth pouches at 12, 20, and 28°C in the dark to compare seedling growth of 2*x* and 4*x* populations. The seedling characteristics measured were total length, hypocotyl length, root length, crown diameter, hook diameter, and middle diameter of the seedling between the hook and crown. The characteristics were measured at four dates in each temperature. The 4*x* had greater means for all seedling characters at any temperature. Especially, the 4*x* hypocotyl mean was longer than the 2*x*. The increases in diameter may indicate greater emerging forces of the 4*x*. The effect of seed weight on seedling growth at both ploidy levels was tested at a constant temperature of 20°C using regression analysis. Seed weight was shown to influence all the growth characters measured. The influence of seed weight was generally the same regardless of ploidy level. Thus, the greater seedling response means of 4*x* were primarily due to heavier individual seed weights. The effect of various chemical additions and combinations (Hoagland solution, indole-3-acetic acid, kinetin, and sucrose) in the germination medium and presoaking treatments was studied to determine their effect on seedling growth at both ploidy levels. Among the chemicals tested, only sucrose had a positive effect on root development. These experiments indicated that the 4*x* seeds were heavier and the 4*x* seedlings were longer in length and thicker in diameter. This would suggest that, under field conditions, the use of 4*x* seeds would probably result in superior establishment. However, the 4*x* seed fertility in the first generation was low limiting practical use unless greater fertility can be obtained in successive generations.

CHAPTER 1
INTRODUCTION

General

Approximately 11.5 million acres of Florida are occupied by grasslands, 43.5% of which are grazed forest lands, 30.4% planted in pasture and 26.1% in native range. The importance of the grasslands is shown by the fact that these grasslands account for 95% of beef cattle nutrition, 60% for goats, 40% for horses, and 10-15% for dairy. It is estimated that the forage contributes approximately $380 million a year to animal livestock value and the value of hay in Florida (L.E. Sollenberger, personal communication, 2001). As new species and cultivars were introduced, the forage system has changed from native ranges to planted pasture, and the livestock number doubled between 1940 and 1980 (Mislevy et al., 1999).

Forage crops are primarily used for pasture, but also for stored feed. Grazing is more convenient and costs less than half the amount of stored feed, which has additional costs associated with hay and silage harvesting and feeding. One goal of forage production in a grazing program is to extend the growing season of the crop. Since the price of stored feed is increasing, producers should maximize the production of grazed forage. Depending on the size of farm, producers should consider various combinations of forage management and production (Ball et al., 1996).

Influence of Climate in Southeastern USA

Compared to central USA, southern USA is warmer and has reasonably distributed rainfall, but occasional droughts restrict the growth of plants. There are frequent thunderstorms during summer. The humid atmosphere prevents rapid loss of heat, and there is little air movement in this region, resulting in conditions favorable for disease development. The frequent rainfall during summer makes haymaking difficult, and quick drying grasses are favored in summer production. Winter rainfall is caused by weather frontal activity. Sudden polar air movements drop temperature occasionally, causing frost or cold damage. Severe cold winter temperatures are a major factor determining the survival and adaptation of warm-season perennial or tropical grasses. The general forage scheme in the southern USA is warm-season perennial grasses supplemented by cool-season annual grasses and legumes (Ball et al., 1996).

However, there is a critical shortage of feed from late fall through early spring in north and northwest Florida, and in winter in south Florida. This shortage is primarily due to the unpredictable weather patterns described above. Good cool-season forage establishment and production can be readily accomplished during years with fall seasons having moderate to cool temperature and adequate rainfall, but in years when fall seasons are hot and dry, inadequate establishment of the cool-season forages can result. Occasional and yearly variable frosts may also impair the establishment and production of cool-season annual forages (Mislevy et al., 1999).

There are numerous solutions to the forage deficit problem of subtropical Florida. Extending the growing season of warm-season forages, later in fall and earlier in spring,

can help limit the shortage of feed during winter time. Some new warm-season grass cultivars such as 'Floralta' limpograss [*Hemarthria altissima* (Poir.) Stapf and C.E. Hubb.] (Quesenberry et al., 1987) are able to produce forage under short days and cool temperature. Winter production of small grains, cool-season legumes, and use of fall produced peanut hay, are also options. Production and purchase of hay or deferring grazing can increase the animal feed (Ball et al., 1996; Mislevy et al., 1999).

Various attempts have been made to reduce the cool-season forage production problems by evaluation of different perennial forage species, but most perennial temperate species do not tolerate hot summer weather and diseases. Use of corn (*Zea mays* L.) and forage sorghum (*Sorghum bicolor* L. Moench) for silage production are possible alternatives, but their production may be limited by the drought during late spring and early summer. Increasing fuel costs discourage hay import as well as use of expensive nitrogen fertilizer for grasses. There are also water use restrictions created by increasing urban demand that has priority over irrigation for hay production. The high cost of irrigation does not justify its return (Mislevy et al., 1999). Therefore, needs exist for the development and use of more persistent cool season legumes to provide feed during the winter season.

Red Clover

The plant used in this research is red clover (*Trifolium pretense* L.), which is a self-incompatible, cross-pollinated species. It is a cool-season, short-lived perennial legume that is believed to have originated in southeastern Europe and Asia Minor. This species belongs to the genus *Trifolium*, which has approximately 230 other species.

Clovers *per se* have not only been used as legume crops, but also used as charms, religious symbols, good luck symbols, emblems, food, medicine, and decoration (Taylor, 1985). They certainly have some intrinsic value to humans. James Whitcomb Riley wrote a poem of clover:

> *...And so I love clover - it seems like a part*
> *Of the sacerdest sorrows and joys of my hart;*
> *And wharever it blossoms, oh, thare let me bow*
> *And thank the good God as I'm thankin' Him now;*
> *And I pray to Him still fer the stren'th when I die,*
> *To go out in the clover and tell it good-bye,*
> *And lovin'ly nestle my face in its bloom*
> *While my soul slips away on a breth of purfume.* (Manlove, 1982)

Forage Uses

Compared to grasses, clovers in general have higher nutritive value such as more crude protein, digestibility, minerals, and vitamins. When mixed with grasses such as tall fescue (*Festuca arundinacea* Schreb), they can reduce endophyte toxicity (Lacefield and Ball, 2000). The animals that consume red clover are primarily beef, dairy, and sheep. However, red clover can also cause antiquality problems such as bloat, isoflavones, and slaframine if not managed properly (Taylor and Quesenberry, 1996).

Mode of Pollination

Because of the self-incompatible system, red clover depends on bees for cross pollination. Honeybees (*Apis mellifera* L.) and bumblebees (*Bombus* spp.) are known to be the principal pollinators. The latter are more effective in the pollination, but they are not present in a large number in seed production fields. Five honeybee colonies ha^{-1} generally provide good seed production, but the long corolla tube of the flower reduces

the effectiveness especially in the tetraploid form (Rincker and Rampton, 1985). For seed production, the planting pattern should be carefully designed, otherwise the pollinators may pollinate other attracting plants nearby (Smith et al., 1985).

Nitrogen Fixation

The true clovers form mutual relationships with *Rhizobium* spp. In this symbiosis, the host plant forms nodules on roots, which are filled with *Rhizobium* bacteroids. These bacteroids obtain energy and nutrients for growth from the host plants, and, in turn, fix or reduce atmospheric nitrogen (N_2) into an organic form that is available to the plants. Environmental effects such as temperature and water stress also influence the correct specific interaction (Leonard and Dodson, 1933). In general, the host range of *Rhizobium* is limited to legume plants, even though within the legumes, there exists host-species and *Rhizobium* strain specificity. In the evolution of the symbiotic relationship with the host, *Rhizobium* has lost the ability to fix N_2 independently (Dilworth and Parker, 1969). The process of N_2 fixation has been considered the second most important biochemical reaction on earth after photosynthesis. As a result of N_2 fixation, legumes depend less on soil N as well as N fertilizers. The pasture and pulse legumes provide about 85% of N_2 fixed in agricultural soils. The legume plants are a major source of human and animal proteins, and are very important world crops (Vance and Johnson, 1981).

Red Clover in Florida

Red clover and other cool-season forage legumes are not well adapted to Florida or the southeastern USA in general. This is due to drought, heat, soil acidity, low soil fertility, pests, and other production requirements (Ball et al., 1996). Even though red

clover is a short-lived perennial plant in temperate regions, it grows primarily as an annual plant in Florida (Chambliss and Quesenberry, 2000). Therefore, it is desirable to obtain more adapted cultivars of this species in this region.

'Cherokee' red clover was developed at the University of Florida and released in 1991 (Quesenberry et al., 1993). Prior to that time, the red clover cultivars used in Florida were developed in the Midwest USA, and had winter dormancy; therefore, they had slow growth in spring (Chambliss and Quesenberry, 2000). Cherokee was developed through the use of recurrent selection for early spring growth, vigorous early flowering, regrowth vigor, early dry matter yield, and adaptation to the southeastern USA. Field selection in an area with high root knot nematode (RKN) (*Meloidogyne* spp.) population also resulted in increased resistance to RKN (Quesenberry et al., 1993). Comparisons of dry matter yields of Cherokee with other cool-season legumes as well as other red clover cultivars in spring indicated that it generally has superior first harvest and total seasonal yields (Chambliss and Quesenberry, 2000).

Red clover, in general, can be grazed, made into green chop, or used as hay. It is an upright bunch type clover without stolons or rhizomes. It has leafy stems, which arise from a thick crown. The root system of this plant is a taproot with numerous adventitious branches. Soil pH should range between 6 and 6.5. A moderate amount of water is required for this plant, but red clover will not tolerate flooding conditions for a extended period of time. Higher soil organic matter or clay content is desirable to keep the soil moist (Chambliss and Quesenberry, 2000).

As a result of the limited winter growing season in Florida as well as its general

sandy soils and high temperatures, water shortages can be a problem for red clover production. In addition, scarce rainfall in the fall seeding time can also be a critical problem in the establishment of cool-season forage crops. Thus, the cool-season plants need to have rapid seedling growth. Especially root elongation is helpful for effective water uptake from the water stored deeper in the soil.

The size of seeds has an effect on early growth and development. This is because heavier or larger seeds generally have more nutrition that is necessary for the development of seedling growth (Black, 1959). This effect would be more pronounced in small-seeded crops, and the seeds are generally sown at shallow depths. At this level, however, the seeds are subjected to quickly drying soil conditions, compared with deeper levels. Therefore, it is desirable to have heavier seed weights in small-seeded crops. Moon (1993) reported increased seed weight of red clover following five cycles of half-sib family and four cycles of mass selection methods. The former resulted in a 22.5% increase in seed weight, while the latter resulted in a 9.2% increase. However, these changes did not lead to differences in dry matter yield of 4-wk-old seedlings.

Polyploidy

Polyploidy is a multiple set of basic genomes. The change in ploidy levels indicates a change of the number of basic genomes (x) from diploid ($2x$) to tetraploid ($4x$) or higher. The number of chromosomes in a gamete is defined as n; thus, normal sporophytic tissue is $2n$. Diploid red clover has 14 somatic chromosomes ($2n=2x=14$), and tetraploid red clover has 28 chromosomes ($2n=4x=28$). Polyploids can be allopolyploid or autopolyploid. The allopolyploid is one that has two or more different basic genomes,

resulting from hybridization of genetically distant parents followed by chromosome doubling. On the other hand, an autopolyploid has multiple copies of the same basic genome because of chromosomes doubling within the same species (Schulz-Schaeffer, 1980).

In general, autotetraploids have greater vegetative volume, larger seed weight, and adaptability to wider ecological region, but lower reproductive fertility than their diploid counterparts. At the cellular level, the cytoplasm and nucleus of polyploids are larger than those of normal plant. Chemical composition of the plants may also change. As the ploidy level increases, the concentration of some chemical contents may increase (Swaminathan, 1970; Poehlman and Sleper, 1995; Li, 1976; Schulz-Schaeffer, 1980). In addition, Stebbins (1947) noted that tetraploids often flower and fruit later than diploids. Levan (1942) suggested that the slower growth of polyploids was caused by a slower mitotic rhythm.

Generally, autopolyploids have reduced reproductive fertility compared to their diploid counterparts. Stebbins (1947) listed three causes of reduced fertility: first, "irregular chromosomal distribution caused by unequal separation of multivalents [such as trivalents and quadrivalents]," second, "irregular distribution caused by meiotic abnormalities of a physiological nature, presumably controlled genetically," and finally, "genetic physiological sterility of an unexplained nature, but not associated with meiotic irregularity" (Swaminathan, 1970; Schulz-Schaeffer, 1980).

Polyploidy is considered important in the evolutionary trends of plants. Around 30% to 50% of all angiosperms are estimated to be polyploids, 70% of grasses, and 23%

of legumes (Poehlman and Sleper, 1995). Moore et al. (1998) mentioned that polyploids also tolerate colder temperatures, so the polyploid species are found in higher latitudes. They can survive in the harsh environment where selection pressure is more intense. This characteristic allows them to develop into permanent populations. Moore et al. (1998) stated that allopolyploid was an immediate cause of speciation; that is, creation of a new species. Despite the fact that autopolyploids produce fewer seeds, autopolyploidy can also be a force in speciation.

Swaminathan (1970) summarized the ways polyploids contribute to evolutionary processes among species. The genetic redundancy buffers small amounts of mutations as well as adaptation without interfering in reproduction rate, and allows wider hybridization, thus, increasing genetic variability. It would also influence gene actions and interaction. More frequent bivalent pairings in later generations help keep the new ploidy level species in existence, while maintaining higher chromosome numbers.

Followed by diploidization, the polyploidy can even promote greater number of chromosomes in plants (Swaminathan, 1970). One species of fern *Ophioglossum reticulatum* has as many as 1260 somatic chromosomes, but it behaves as a diploid (Abraham and Ninan, 1954). Polyploidy is rare in gymnosperms and some woody angiosperms, but their chromosome number is high. This may suggest they may have gone through a polyploidization process. However, the recent evolutionary trend was not favorable for them. The “changes in physiological and developmental rhythm often arising from polyploidy seem to be of negative selective value” (Swaminathan, 1970).

However, the evolutionary trend is not always from lower to higher ploidy level. It can be reversed (Hougas and Peloquin, 1958). Doubling chromosome numbers does not always lead to large size and vigorous growth. Since the increase in size seems to have a limitation after a certain increase in ploidy levels, there might be an optimal ploidy level in each species. Welsh (1981) points out that there exists "a delicate balance in most plants for numbers of chromosomes within each cell." Thus, it is necessary to test and evaluate the effects of altering chromosome numbers of each species.

As for the effect of doubling chromosomes on the legume and *Rhizobium trifolii* symbiosis, Weir (1961) compared the infection of three strains of *Rhizobium* on autotetraploid and diploid red clover. He found that the nodule production on diploid plants was 6 d faster than that on tetraploids. In both ploidy levels, 100% nodulation was achieved 7 wk after the inoculation, but the number of nodules on diploids was greater than that of tetraploids. The author indicated that the difference in the total number of nodules between diploids and tetraploids was due to the faster rate of nodulation on diploids between 22 and 30 d after planting. Also, the size of nodules on tetraploids was larger than that of diploids. Wipf and Cooper (1940) found "disomatic cells" at the site of infection in the normal diploid plants. Thus, the disomatic cells in tetraploid plants could be octaploid. In one particular case, differences between nodulation on the diploid and autotetraploid were pronounced (Weir, 1961).

Methods of Chromosome Doubling

The methods of chromosome doubling include: decapitation followed by callus formation with or without indoleacetic acid, twin seedlings, cold and heat shocks,

colchicine, chloral hydrate, ether, chloroform, acenaphthene, phenylurethane, ethyl-mercury-chloride, sulfanilamide, nitrous oxide (N_2O), and others chemicals (Allard, 1966; Briggs and Knowles, 1967). The formation of polyploids may also be genetically controlled. If a plant has a homozygous recessive gene for asynapsis, it may produce diploid (2*n*) gametes, instead of haploids, leading to autotetraploids (Allard, 1966; Briggs and Knowles, 1967). In nature, the most frequent method of formation of polyploids was speculated to be the union of unreduced gametes (Swaminathan, 1970).

The use of N_2O for chromosome doubling results in higher percentage of tetraploidy in *Trifolium* compared to the use of colchicine. The use of colchicine resulted in 9% chromosome doubling (Neubauer and Thomas, 1966). However, the use of N_2O increased doubling up to 100% (Berthaut, 1968). Previously, Taylor et al. (1976) produced tetraploid red clover with N_2O. N_2O was applied 24 h after pollination and withdrawn after the subsequent 24 h. They obtained tetraploid plants, ranging from 50 to 100% among the crosses tested. Their results indicated the superiority of N_2O use in tetraploid induction on red clover.

Research Justification and Objectives

There are three justifications for this research. First, the economic importance of red clover is primarily the vegetative part for forage, so reduced fertility associated with tetraploidy is less of a concern than with grain crops. Second, the basic chromosome number of red clover is low. The use of polyploid breeding in species with low chromosome numbers is generally more successful (Poehlman and Sleper, 1995). In fact, 16% of the species in the genus *Trifolium* are polyploid (Cleveland, 1985). Taylor et al.

(1979) concluded that *Trifolium* species with higher chromosome numbers are cross pollinators and perennial. This may suggest that red clover can possibly function as a polyploid species in the genus. Finally, red clover is cross fertile (Levan, 1942; Bingefors and Ellerstrom, 1964). This may lead to greater hybrid vigor at the multiple loci of tetraploids than diploids.

The objective of this research was to examine the effects of chromosome doubling in red clover seedling development to determine if the autotetraploid seedlings have the potential for better establishment and consequently leading to the increased winter forage production.

CHAPTER 2
THE EFFECT OF PLOIDY LEVEL ON SEED NUMBER AND WEIGHT

Introduction

Autotetraploid plants generally have thicker leaves and stems, increased winter hardiness, slower growth, and increased seed weight but reduced seed number compared to their diploid counterparts. The reduced fertility and lower seed numbers may be related to pairing problems and unbalanced chromosome numbers in the gametes (Schulz-Scharffer, 1980). The reduced seed numbers could be also caused by the changes in morphological characteristics of the autotetraploid plants. Pollinating insects may not be capable of obtaining pollen because of the longer corolla tubes. However, the increases in seed volume and weight due to the gigantism from chromosome doubling, may overcome the problem of low seed production (Swaminathan, 1970) or it could be possible to find favorable environments for seed production of the tetraploids (Bingefors and Ellerstrom, 1964).

Literature Review

Bingefors and Ellerstrom (1964) conducted a cytological experiment to produce tetraploid red clover (*Trifolium pratense* L.) using a colchicine solution applied to the seeds of a local diploid red clover cultivar, Ultuna. This diploid cultivar and resulting tetraploid cultivar, Ulva, were compared for their seed production in different environments for 8 yr. Their results indicated that the tetraploids produced fewer seeds

than diploids. They suggested possible causes. Ulva (4*x*) produced a lower number of flower heads per unit area; 25-30% lower than Ultuna (2*x*). Lower seed set could be attributed to Ulva (4*x*) having about 0.74 mm longer corolla tube than Ultuna (2*x*), resulting in poorer pollination by bees. Ulva (4*x*) had a narrower environmental range such as a shorter flowering period, which may have contributed lower seed production. Valle et al. (1960) also indicated that environmental factors, such as rainfall pattern, temperature, relative humidity, and appropriate pollinators, can influence seed production. As for seed weight, the tetraploid plants produced heavier seeds. The average weight of 1,000 tetraploid seeds was 2.75 g, whereas that of diploids was 1.78 g (Bingefors and Ellerstrom, 1964).

In a case of rye *(Secale cereale* L.), Pfahler et al. (1987) found in their 4-yr study that tetraploid cultivars had lower grain yield (1,080 kg ha^{-1}) than that of comparative diploids (1,850kg ha^{-1}), lower seed filling per spikelet (62.4%) compared to the diploids (74.3%), but higher mean seed weight (21.1 mg $seed^{-1}$) than diploids (18.8 mg $seed^{-1}$). In all cases, they found highly significant cultivar differences, suggesting the selection of more fertile diploid lines for the induction of chromosome doubling could lead to more fertile tetraploid lines.

Selection to improve autotetraploid fertility may be possible. Gilles and Randolph (1951) selected for improved fertility in tetraploid corn (*Zea mays L.*) for 10 cycles. They found lower incidences of multivalents in the last generation and more frequent incidences of bivalents. Swaminathan and Sulbha (1959) also obtained a similar correlation in *Brassica campestris* var. Toria after 19 generations. This tendency of bivalent pairing in

autotetraploids is called diploidization (Schulz-Schaeffer, 1980) or preferential pairing in a case of complete intervarietal chromosome pairing, which could lead to higher and more stable heterosis (Briggs and Knowles, 1967).

The objective of this experiment was to compare the effects of doubling the chromosome number of red clover on seed number and mean seed weight in the generation immediately after doubling. The hypothesis is that seed number will be lower for these autotetraploids due to cytogenetic and morphological factors. However, the seed weight would probably higher for the tetraploids than the diploids.

Materials and Methods

Production of Tetraploid Plants

All experiments were conducted using plants (FRU1, FRU2, FRU4, FRU5, FRU6, FRU7, A155, and J041) representing advanced selection cycles of the diploid red clover cv. 'Cherokee.' The first six were high yielding lines derived from field selection. The last two were clones resistant to root knot nematodes *(Meloidogyne* spp.*).*

These clones were crossed without emasculation, and after 24 h they were placed into a nitrous oxide (N_2O) tank and maintained at 0.62 MPa for the next 24 h. N_2O is known to disturb the spindle fiber formation during the first embryonic division of the zygote. Each chromosome is not pulled to the opposite poles, and remains in the middle of the cell. In the next cell cycle when N_2O is removed, normal mitosis should occur except the cell contains double the chromosome number. From this N_2O treatment, some tetraploid and some diploid plants were obtained from the same cross. Plants produced from the above treatment were classified as diploid or tetraploid based on size and

morphology of dry pollen (Taylor et al., 1976). Full-sib diploid and tetraploid plants from the same cross on the same plant were used to establish corresponding diploid and tetraploid populations.

Field Study

A diploid and a tetraploid plant resulting from each of eight crosses were evaluated. Four ramets of each of the eight diploid and tetraploid plants were produced by crown bud cuttings. The apical meristems were cut and treated with Rhizopon@ and placed into a wet vermiculite tray to induce root formation. Each rooted ramet was transferred to a pot to stabilize growth in the soil. Then, both tetraploid and diploid plants were transplanted into the field at the University of Florida in May 2000; the different ploidy groups were separated by approximately 2 km to avoid interpollination between ploidy levels. Each field had four blocks, and the ramets were randomly distributed in each of the four blocks. Naturally-occurring bees were allowed to cross-pollinate the plants within and among the four blocks. The flower heads were harvested from an individual ramet from each replication as they matured between June and August 2000.

Seeds were threshed by hand rubbing, and severely shriveled seeds were discarded. After cleaning the seeds, the seed number was counted. The total seed weight of each individual plant was measured. The mean seed weight was calculated by the total seed weight divided by seed number.

The experimental design was a randomized block with four replications. As a result of the heterogeneity of variance, seed number was transformed to base 10 logarithms. Data were analyzed by analysis of variance as a 2 x 8 factorial. The minimum differences

for significance among means were compared using Duncan's Multiple Range Test for the maximum number of means to be compared (Harter, 1960).

Results

Seed Number

There were effects of ploidy level ($p \leq 0.001$) and the interaction between ploidy level and cross ($p \leq 0.05$), but no effect of cross ($p \geq 0.05$) (Table 2-1). Overall crosses, the mean numbers of seeds plant^{-1} among diploid plants was 806, whereas that of tetraploid plants was 26 (Table 2-2). Therefore, the tetraploid lines produced only about 3% as much seed as the diploids. As an interaction effect was detected, cross 4 x Al55 had lower seed number than all the other crosses within the diploid level. On the other hand, Al55 x 2 and 1 x Al55 had the lowest seed number produced within the tetraploid level (Table 2-2).

Mean Seed Weight

There were significant effects ($p \leq 0.001$) of cross, ploidy level, and the interaction on mean seed weight (Table 2-1). The mean seed weight was higher for tetraploids than diploids in all crosses (Table 2-3). The weight increases associated with the ploidy level increase were not uniform. The cross with the greatest mean seed weight was 5 x Al55 at both ploidy levels, but the lowest mean seed weight was from 4 x Al55 at the diploid level and 4 x J041 at the tetraploid level (Table 2-3).

Discussion and Conclusions

From the results, the seed number of tetraploids was substantially lower than that of the diploids and was dependent upon the genotype of the cross. However, it should be remembered that the plant materials tested in this experiment were the first generation

after the N_2O treatment, and that no selection had been practiced for tetraploid fertility or balanced chromosome pairing. Additional generations of selection for fertility or diploidization would improve seed set. Maximum fertility of autopolyploids was generally attained after several generations of interpollination after doubling (Gilles and Randolph, 1951; Swaminathan and Sulbha, 1959; Briggs and Knowles, 1967).

Although the induction of autotetraploidy resulted in reduced fertility expressed as lower seed number, some crosses appeared to be superior, suggesting a genetic component for fertility. Also, good seed-producing diploid crosses did not necessarily produce high fertility tetraploids. Thus, identification of high seed-producing diploid lines would not necessarily result in high seed-producing tetraploids. Evaluation of tetraploid fertility from other genotypes is needed in future research.

Overall, tetraploid seeds were heavier than diploid seeds. This could possibly be the result of greater availability of photosynthates per seed throughout the growing season for the tetraploids, compared to the need of the diploid seeds to divide assimilate among many seeds. However, there was no correlation between seed number and seed weight. So, the increase in the seed weight probably was due to the increased cell volume associated with autotetraploidy. Even though the fertility of tetraploids will probably never exceed that of diploids, the increased mean seed weight might overcome this problem by increasing the total seed yield per unit area, as reported by Valle et al. (1960).

The mean seed weights at both ploidy levels obtained in this experiment were considerably less than those reported by other authors (Bingefors and Ellerstrom, 1964; Anderson, 1971). This was probably because this red clover seed was produced in the

summer season in Florida, which is generally adverse for optimal seed production. The plants should have been planted as early as in February rather than in May to optimize seed production in this subtropical region. This change would have probably produced more vigorous growth, resulting in greater seed weight and numbers. The performance and adaptability of the tetraploids especially for seed production should be evaluated at different locations and years to determine the extent of the genotype effect.

Table 2-1. Mean squares and significance levels from the analyses of variance of seed number plant^{-1} (transformed) and seed weight.

Source of variation	df	Seed number	Seed weight
Treatment	15	3.85***	0.61***
Ploidy level (PL)	1	44.38***	7.11***
Cross (CR)	7	0.76	0.18***
PL x CR	7	1.16*	0.11***
Error	39	0.42	0.02

*, *** F value significance at the 5 and 0.1% levels, respectively.

Table 2-2. Mean seed number plant^{-1} from each cross at each ploidy level. The log 10 transformed mean is given after each mean in parentheses for statistical comparisons.

Cross	Ploidy level		Cross means
	2x	4x	
A155 x 2	621 (2.70)†	3 (0.35)	312 (1.52)
A155 x 6	1658 (3.19)	51 (1.65)	855 (2.42)
1 x A155	1062 (2.95)	2 (0.39)	532 (1.67)
4 x A155	41 (1.67)	99 (1.47)	70 (1.57)
5x A155	871 (2.28)	2 (0.78)	437 (1.53)
4 x J041	1453 (3.06)	8 (0.89)	731 (1.98)
6 x J041	471 (2.53)	6 (0.66)	239 (1.60)
J041 x 7	273 (2.33)	33 (1.20)	153 (1.77)
Ploidy level mean	806 (2.59)	26 (0.92)	

† Minimum differences for significance among the means in the parenthesis were: ploidy level means= F value significant at the 0.1% level; cross means= F value not significant and; any combination of ploidy level and cross means= 1.12 and 1.48 at the 5 and 1% levels, respectively.

Table 2-3. Mean seed weight from each cross at each ploidy level.

Cross	Ploidy level		Cross mean
	2x	4x	
	----------mg----------		
A155 x 2	1.53 †	2.17	1.85
A155 x 6	1.54	2.42	1.98
1 x A155	1.57	2.16	1.86
4 x A155	1.22	2.23	1.72
5 x A155	1.74	2.60	2.17
4 x J041	1.53	1.88	1.71
6 x J041	1.59	2.09	1.84
J041 x 7	1.54	2.04	1.79
Ploidy level mean	1.53	2.20	

†Minimum differences for significance among the means were: ploidy level means= F value significant at the 0.1% level; cross means= 0.17 and 0.22 mg at the 5 and 1% levels, respectively and; any combination of ploidy level and cross means= 0.25 and 0.33 mg at the 5 and 1% levels, respectively.

CHAPTER 3
THE EFFECT OF TEMPERATURE ON DIPLOID AND TETRAPLOID RED CLOVER SEEDLING GROWTH

Introduction

To improve seedling establishment, it is desirable to have rapid seedling and, more importantly, root growth so that the root can remain in contact with moist soil while sown at the soil surface. At the same time, greater shoot growth can enhance competition with weeds, and thus maximizing growth of the developing seedling by photosynthesis. During the period of establishment with grasses or seeded in a pure stand, the plants are subject to intra- or inter-species competition. These plants compete for light, water, and soil minerals. Those that intercept the light first would have an advantage in receiving light and producing chemical energy for further growth of both shoots and roots (Kendall and Stringer, 1985).

Literature Review

Seedling Characteristics of Clovers

Seedling growth begins with germination of the seed. Germination is defined as the imbibition of water, resulting in "the rupture of the testa by an extruding radicle" (Black, 1959). However, many plants have mechanisms to prevent germination as a means to ensure that proper environmental conditions exist for proper development after germination. Seed coat hardness is one of them. Nevertheless, it can be broken by cold and

subsequent alternating temperature, microbial action, abrasive materials, sulfuric acid, radio frequency electrical treatment, and other factors (Kendall and Stringer, 1985).

The type of germination and seedling development in red clover is called epigeal. In this type, the radicle emerges from the seed, the first root absorbs water and nutrients are transported to the hypocotyl. The hypocotyl elongates toward the soil surface with cotyledons suspended by the hook (Moore et al., 1998). In the dark, seedling development is entirely dependent on the cotyledons for energy. However, on exposure to light, the cotyledons are capable of photosynthesis (Kendall and Stringer, 1985).

Seedling growth is divided into three phases: heterotrophic, transitional, and autotrophic. During the heterotrophic phase, a plant is dependent on nutrient reserves in the endosperm and cotyledons for energy, since it is not capable of photosynthesis. This phase begins from germination and ends at the emergence of cotyledons from the soil. Planting depth is important in this phase because the plant has to expend energy to elongate the hypocotyl. During the transitional phase, the embryo obtains energy from both the cotyledon and newly-emerging leaves. Finally, the autotrophic phase starts when the plant becomes independent; that is, it can produce its own energy from photosynthesis. Through these phases, seed weight, temperature, and seeding depth, are related to competitiveness (Kendall and Stringer, 1985).

Temperature Effect on Seedling Growth

In diploid subterranean clover (*Trifolium subterraneum* L.), the effects of temperature (7, 14, 21, and 28˚C) and depth of sowing (1.3, 2.5, 3.8, and 5.1 cm) on seedling growth were examined by Black (1955), using pots. He found no differences in

total plant weight among seeds sown at any depth at the same temperature on any day before emergence. The proportions of cotyledon, hypocotyl, and root, were the same regardless of the sowing depth. However, there were differences in days of emergence because of the depth at each temperature. At higher temperatures, the seedlings were able to emerge from the soil quicker than at lower temperature, even though they were sown more deeply. The seeds planted at 7˚C never emerged.

It was found that the emerged cotyledon weights decreased as the depth of sowing increased. The transfer rate of nutrient from cotyledons was calculated from the loss of cotyledon weight and the gains in root or hypocotyl weight. The stored energy in the cotyledons is used for both respiration and growth of hypocotyl and root, but the respiration cost was ignored because he assumed it had a relatively small effect. It was found that regardless of the depth of seed sowing, 21˚C germination temperature had the greatest remaining cotyledon weights at emergence. That is, 21˚C was the optimum temperature for subterranean clover seedling growth (Black, 1955).

Temperature and depth effects were both important as well as the interaction effect for seedling emergence. The interaction was due to the lack of parallelism of the response to depth for temperatures of 14 and 21˚C. The rate of decrease in cotyledon weight was independent of the depth of sowing. Thus, there were no differences on any day between plants sown at different depths in the relative portions of cotyledon and hypocotyl (Black, 1955).

Sullivan and Pfahler (1986) studied temperature effects (12, 20, and 28˚C) on seedling growth in five diploid and corresponding tetraploid rye (*Secale cereale* L.)

populations. They found highly significant effects of temperature, ploidy, and genetic background on shoot and root growth. There was also a temperature x genetic background interaction, and temperature x genetic background x ploidy interaction effects on shoot length. There was a temperature x ploidy interaction on root length. At both ploidy levels, an increase in temperature resulted in increases in shoot growth and primary root growth. The tetraploids had shoot growth of 12.0, 20.0, and 28.1 mm d^{-1}, whereas those of diploids were 11.1, 18.4, and 27.0 mm d^{-1}, at 12, 20, and 28˚C, respectively. The primary root growth was always greater for the tetraploids than diploids by 16.7 vs. 15.0 mm d^{-1} at 12˚C, 26.9 vs. 25.8 mm d^{-1} at 20˚C, and 39.7 vs. 37.4 mm d^{-1} at 28˚C, respectively. On the other hand, there were highly significant effects of ploidy, temperature, and temperature x genetic background interaction on primary root cross-sectional area. Increasing temperature resulted in a decrease in primary root cross-sectional area at both ploidy levels, 0.12 mm^2 for diploid and 0.17 mm^2 for tetraploid at 12˚C, 0.10 and 0.15 mm^2 at 20˚C, and 0.09 and 0.13 mm^2 at 28˚C, respectively. Since autotetraploids have a heavier seed weight, they should have advantages in seedling competition compared to diploids (Swaminathan, 1970).

In this experiment, the seedling development of each ploidy level ($2n=2x=14$ and $2n=4x=28$) of red clover (*Trifolium pratense* L.) was measured over a range of temperatures. The hypothesis was that the increase in ploidy level increased rate and length of seedling growth. Also, as temperature increases, the rate of growth would increase within the ploidy level, since an increase in temperature would stimulate enzymatic or respiratory activities.

Materials and Methods

Because of the shortage of tetraploid seeds, the diploid and tetraploid seeds of various crosses (Chapter 2) were bulked together at each ploidy level for this and subsequent experiments. The same crosses were used if at all possible but a number of tetraploid seeds were added from crosses in which no diploid seeds were available. The crosses and estimated seed number are shown in Table 3-1. As a result, there were two populations tested: one diploid (2*x*) and the other tetraploid (4*x*). To increase the germination percentage, both diploid and tetraploid seeds were scarified by immersion in 95% sulfuric acid solution for 5 min with thorough stirring. After the acid was removed, the seeds were rinsed 5 times with distilled water.

Plastic pouches (Northrup King Co., Minneapolis, MN, Seed Pack Growth Pouch, U. S. Patent 3241264) which control moisture level uniformly in and among the pouches, were used in this experiment to measure seedling development. To enhance germination uniformity, all seeds were soaked in distilled water for 24 h at 5°C. Thereafter, the seeds were germinated and grown in darkness. Each pouch contained 20 seeds. There were two replications for each date. The pouches were inserted vertically into the slits of growth cans. The replicated pouches were separated from each other within the same can. The cans were stored in a temperature chamber at each constant temperature throughout the experiment. The whole experiment was repeated twice.

According to the Association of Official Seed Analysts (1995), testing for laboratory germination for red clover is conducted at a temperature of 20˚C. However, to evaluate the effect of different temperatures on growth, the temperature ranges of 12, 20,

and 28˚C were chosen based on the preliminary information (data not shown). Preliminary experiments were also conducted to determine the number of days required for seedling growth under dark conditions at the above germination temperatures. The seedling characteristics measured were total seedling length (TL), hypocotyl length (HL), root length (RL), crown diameter (CD), hook diameter (HD), and middle point diameter between crown and hook (MD). The measurements were made to the nearest mm. Diameter measurements were made in mm converted to μm, using a microprojector at a magnification of 50X. The dates of measurements were on 2, 4, 6, and 8 d at 28°C, 3, 6, 9, and 12 d at 20°C, and 7, 14, 21, and 28 d at 12°C after germination initiation or after the seeds were placed in the pouches. Day effect was included to examine the growth rate over time.

The experimental units were dates of measurement and ploidy levels at each temperature. The sampling units were the seeds at each temperature. From each pouch, ten seedlings were measured. The experimental design was 2 x 4 factorial at each temperature. The data were analyzed by analysis of variance. The minimum differences for significance among means were compared using Duncan's Multiple Range Test for the maximum number of means to be compared (Harter, 1960).

Results

Evaluation at 28˚C

Length measurement

There were effects of ploidy level on TL and HL ($p \leq 0.001$) and on RL ($p \leq 0.05$) (Table 3-2). The means of the tetraploids were always greater than those of the diploids

for any character (Table 3-3). Day effects were also observed for all characters ($p \leq 0.001$) (Table 3-2). That is, differences in the means among the days were detected. However, days 6 and 8 were shown to be not significantly different for any character. The elongation of TL and HL appeared to stop after about day 6. In addition, day 4 was not significantly different from day 6 or day 8 for RL. The root elongation appeared to stop after about day 4 (Table 3-3).

The ploidy x day interaction effect was not significant except for HL ($p \leq 0.05$) (Table 3-2). Initially, the tetraploids had greater TL means than diploids at day 2, and the differences between ploidy levels were maintained throughout the rest of the period (Table 3-3). Therefore, their growth rates were parallel. As for HL, the mean differences between the diploids and tetraploids widened from 4, 5, 8, to 9 mm, at days 2, 4, 6, and 8, respectively (Table 3-3). Therefore, the interaction effect was from the progressive increase of HL by days.

Diameter measurement

There were effects of ploidy levels for CD, MD, and HD ($p \leq 0.001$) (Table 3-4). The tetraploids always had greater means than those of diploids (Table 3-5). The day effect was only found for HD ($p \leq 0.001$), but not for CD or MD. The interaction effect was only found for MD, but not on CD and HD (Table 3-4).

Evaluation at 20˚C

Length measurement

There were significant effects of ploidy level on TL, HL, and RL ($p \leq 0.001$) (Table 3-6). The tetraploids always had greater means than those of diploids (Table 3-7). Day

effects were also found for all the responses ($p \leq 0.001$) (Table 3-6). The day effects indicated that the mean lengths of TL, HL, and RL, were different among the given days. For all characters, day 12 had the greatest means, but they were not significantly different from those of day 9 (Table 3-7).

The effect of interaction between ploidy level and day was only expressed for TL ($p \leq 0.05$) (Table 3-6). Although the TL means increased as days increased, those of the diploids stopped increasing at day 9, whereas the means of tetraploids continued to increase (Table 3-4). Therefore, the differences in TL means between ploidy levels at each day increased.

Diameter measurement

There were effects of ploidy level on CD, MD, and HD ($p \leq 0.001$) (Table 3-8). The tetraploids had larger means than those of diploids (Table 3-9). Unlike at 28˚C, the day effect was expressed in MD ($p \leq 0.01$), but not in CD and HD (Table 3-8). In general, the means of MD decreased as days increased. The MD of earlier days were significantly greater than those of later days ($p \leq 0.05$) (Table 3-9). No interaction effects were observed (Table 3-8). At both ploidy levels, the means of CD and HD did not change significantly across days (Table 3-9).

Evaluation at 12˚C

Length measurement

There were effects of ploidy level on TL, HL, and RL ($p \leq 0.001$) (Table 3-10). The tetraploids always had greater means than those of diploids (Table 3-11). Day effects were also found for all the responses ($p \leq 0.001$) (Table 3-10). The day effects indicated that the

mean lengths of TL, HL, and RL, were different among the given days. The means increased as the days progressed until day 21 for all responses, but the means of day 28 were not different from that of day 21 for TL and RL. In HL, the mean continued to increase until the end of the measurement period (Table 3-11).

The interaction effect between ploidy level and day was only expressed for HL ($p \leq 0.05$), but not for TL and RL (Table 3-10). In HL, the increase of tetraploids between day 7 and day 14 were significantly greater from that of diploids (Table 3-11).

Diameter measurement

There were effects of ploidy level on CD, MD, and HD ($p \leq 0.001$) (Table 3-12). The tetraploids had the greater means than those of diploids (Table 3-13). Similar to the results at 20˚C, the day effect was expressed for MD ($p \leq 0.001$), but not in CD and HD (Table 3-12). The means of MD decreased as the days progressed (Table 3-13). Also similar to the results at 20˚C, no interaction effects were expressed in any of the characters (Table 3-12).

Discussion and Conclusions

The tetraploid seedlings always had greater length and diameter than those of diploids at all germination temperatures. Also, as temperature increased, the rate of seedling growth increased at both ploidy levels. These results agreed with Black (1955) who found that as temperature increased, emergence was improved within the range of temperatures. Even though the growing environment in this experiment was different from his, when the diploid and tetraploid seeds were grown within this temperature range, the

tetraploid materials are expected to show greater seedling vigor and emergence than that of diploids.

The ploidy level effects were pronounced in all response variables measured. The tetraploid seedlings had longer hypocotyls and longer roots than the diploids. However, it was observed during the experiment that at lower temperatures, the seedlings tended to achieve longer hypocotyl than those at higher temperatures within the range of temperature, though the reason is unknown. This may be because of the assimilate priority of the cotyledon reserve to the hypocotyl during the period of the allocation or because of negative gravitropic growth.

Even though significant differences in diameter measurement were found, the differences between the ploidy levels were generally greater than those among days. The day effect on HD at 28°C (Table 3-4) seemed to be random in nature. However, the day effects on MD (Table 3-8 and 3-14) suggest the elongation of red clover seedlings is due to cell elongation rather than cell division. The relative role of cell division and elongation in hypocotyl length was discussed with the conclusion that cell elongation was the major factor (Jones and Moll, 1983). The hypocotyl elongation of lettuce (*Lactuca sativa* L.), which also has the epigeal germination pattern, was shown to be caused by cell elongation. Therefore, a major part of the increase in hypocotyl length in the tetraploids was probably the result of the larger cell size of the tetraploids compared to that of diploids.

In general, when a seedling must emerge through a compacted soil, a plant experiences a thickening of the seedling or root. This retards the growth, but it helps the seedling push up through the compacted soil (Mayer and Poljakoff-Mayber, 1989). The

tetraploid seedlings were shown to have both of the positive characteristics, faster growth and larger diameter for greater emergence ability. The latter characteristic should be especially important by the increased HD, since the hook area of the hypocotyl protects the cotyledon as well as meristematic region during the penetration toward the soil surface.

The diploid and tetraploid red clover populations used in this study were genetically similar, and this experiment showed the clear ploidy differences in the expression of the seedling growth. Overall, the lack of interaction indicated a parallel growth of diploid and tetraploid seedlings. The initially-greater means of the tetraploids may imply a more rapid germination of tetraploid seeds compared to that of the diploids. Variations in length at later evaluation dates were found probably because of the heterozygosity of red clover, even though there was less variation in the initial period.

Table 3-1. The crosses and the estimated seed numbers composited to develop the diploid (2*x*) and tetraploid (4*x*) populations used in this and subsequent studies.

Cross	2*x*	4*x*
155 x 1	-	32
155 x 2	2419	13
155 x 6	18545	997
1 x 155	7583	7
2 x 155	-	52
3 x 155	-	36
4 x 155	144	456
5 x 155	3108	6
6 x 155	-	983
7 x 155	-	233
4 x J041	5203	32
6 x J041	3412	48
J041 x 7	971	537
J054 x 3	-	420
MI119 x 4	-	197

Table 3-2. Mean squares and significance levels from the analyses of variance of total seedling length (TL), hypocotyl length (HL), and root length (RL) at 28˚C.

Source of variance	df	Length character		
		TL	HL	RL
Treatment	7	8975***	5857***	350***
Ploidy level (PL)	1	5208***	3699***	129*
Day (D)	3	19022***	12326***	724***
PL x D	3	183	108*	49
Error	312	70	34	32

*, *** F value significant at the 5 and 0.1% levels, respectively.

Table 3-3. Means of total seedling length (TL), hypocotyl length (HL), and root length (RL) at each ploidy level (PL) at 28˚C on each of four days after germination initiation.

Character	PL	Day after germination initiation 2	4	6	8	PL mean
		----------mm----------				
TL†	2*x*	43	68	73	73	64
	4*x*	49	73	84	84	72
	Day mean	46	70	78	78	
HL‡	2*x*	19	38	43	43	36
	4*x*	23	43	51	52	43
	Day mean	21	41	47	47	
RL§	2*x*	24	30	29	31	28
	4*x*	25	29	33	31	30
	Day mean	25	29	31	31	

†Minimum differences for significance among the means were: ploidy level means = F value significant at the 0.1% level; day means = 3 and 4 mm at the 5 and 1% levels, respectively and; any combination of ploidy level and day means = 4 and 6 mm at the 5 and 1% levels, respectively.

‡Minimum differences for significance among the means were: ploidy level means = F value significant at the 0.1% level; day means = 2 and 3 mm at the 5 and 1% levels, respectively and; any combination of ploidy level and day means = 3 and 4 mm at the 5 and 1% levels, respectively.

§Minimum differences for significance among the means were: ploidy level means = F value significant at the 5% level; day means F value =2 and 3 mm at the 5 and 1% levels, respectively and; any combination of ploidy level and day means = 3 and 4 mm at the 5 and 1% levels, respectively.

Table 3-4. Mean squares and significance levels from the analyses of variance of crown diameter (CD), middle diameter (MD), and hook diameter (HD) at 28˚C.

Source of variance	df	Diameter character		
		CD	MD	HD
Treatment	7	191966***	200351***	174962***
Ploidy level (PL)	1	1292861***	1357205***	1128125***
Day (D)	3	14825	6182	25088***
PL x D	3	2141	8902*	7115
Error	312	5853	3020	2936

*, *** F value significant at the 5 and 0.1% levels, respectively.

Table 3-5. Means of crown diameter (CD), middle diameter (MD), and hook diameter (HD) at each ploidy level (PL) at 28˚C on each of four days after germination initiation.

Character	PL	Day after germination initiation				PL mean
		2	4	6	8	
		----------μm----------				
CD†	2*x*	760	781	787	776	776
	4*x*	889	901	928	894	903
Day mean		825	841	857	835	
MD‡	2*x*	665	663	640	651	655
	4*x*	796	778	800	767	785
Day mean		730	721	720	709	
HD§	2*x*	537	573	566	567	561
	4*x*	656	678	712	673	679
Day mean		590	625	639	620	

†Minimum differences for significance among the means were: ploidy level means=F value significant at the 0.1% level; day means = F value not significant and; any combination of ploidy level and day means = 39 and 51 μm at the 5 and 1% levels, respectively.
‡Minimum differences for significance among the means were: ploidy level means=F value significant at the 0.1% level; day means = F value not significant and; any combination of ploidy level and day means = 28 and 37 μm at the 5 and 1 % levels, respectively.
§Minimum differences for significance among the means were: ploidy level means=F value significant at the 0.1% level; day means = 18 and 24 μm at the 5 and 1% levels, respectively and; any combination of ploidy level and day means = 28 and 36 μm at the 5 and 1% levels, respectively.

Table 3-6. Mean squares and significance levels from the analyses of variance of total seedling length (TL), hypocotyl length (HL), and root length (RL) at 20˚C.

Source of variance	df	Length character		
		TL	HL	RL
Treatment	7	7393***	5213***	260***
Ploidy level (PL)	1	3768***	1144***	760***
Day (D)	3	15810***	11722***	316***
PL x D	3	184*	60	38
Error	312	60	27	26

*, *** F value significant at the 5 and 0.1% levels, respectively.

Table 3-7. Means of total seedling length (TL), hypocotyl length (HL), and root length (RL) at each ploidy level (PL) at 20˚C on each of four days after germination initiation.

Character	PL	Day after germination initiation				PL mean
		3	6	9	12	
		----------mm----------				
TL†	2*x*	44	64	71	71	63
	4*x*	47	72	78	81	70
	Day mean	46	68	75	76	
HL‡	2*x*	22	40	46	46	39
	4*x*	24	45	50	51	43
	Day mean	23	43	48	49	
RL§	2*x*	22	23	25	25	24
	4*x*	23	27	28	30	27
	Day mean	23	25	27	27	

†Minimum differences for significance among the means were: ploidy level means = F value significant at the 0.1% level; day means = 3 and 4 mm at the 5 and 1% levels, respectively and; any combination of ploidy level and day means = 4 and 5 mm at the 5 and 1% levels, respectively.

‡Minimum differences for significance among the means were: ploidy level means = F value significant at the 0.1% level; day means = 2 and 3 mm at the 5 and 1% levels, respectively and; any combination of ploidy level and day means = 3 and 4 mm at the 5 and 1% levels, respectively.

§Minimum differences for significance among the means were: ploidy level means = F value significant at the 5% level; day means F value =2 and 3 mm at the 5 and 1% levels, respectively and; any combination of ploidy level and day means = 3 and 4 mm at the 5 and 1% levels, respectively.

Table 3-8. Mean squares and significance levels from the analyses of variance of crown diameter (CD), middle diameter (MD), and hook diameter (HD) at 20˚C.

Source of variance	df	Diameter character		
		CD	MD	HD
Treatment	7	193500***	247368***	166092***
Ploidy level (PL)	1	1333861***	1682000***	1139031***
Day (D)	3	5998	12083**	6255
PL x D	3	881	4443	1615
Error	312	6051	2927	3123

, * F value significant at the 1 and 0.1% levels, respectively.

Table 3-9. Means of crown diameter (CD), middle diameter (MD), and hook diameter (HD) at each ploidy level (PL) at 20˚C on each of four days after germination initiation.

Character	PL	Day after germination initiation 3	6	9	12	PL mean
		----------μm----------				
CD†	2*x*	782	765	761	782	772
	4*x*	912	898	895	901	901
	Day mean	847	831	828	841	
MD‡	2*x*	685	669	646	653	663
	4*x*	808	823	802	799	808
	Day mean	746	746	724	726	
HD§	2*x*	583	577	568	556	571
	4*x*	708	704	690	698	700
	Day mean	645	640	629	627	

†Minimum differences for significance among the means were: ploidy level means=F value significant at the 0.1% level; day means = F value not significant and; any combination of ploidy level and day means = 40 and 52 μm at the 5 and 1% levels, respectively.
‡Minimum differences for significance among the means were: ploidy level means=F value significant at the 0.1% level; day means = 18 and 24 μm at the 5 and 1% levels, respectively and; any combination of ploidy level and day means = 28 and 36 μm at the 5 and 1% levels, respectively.
§Minimum differences for significance among the means were: ploidy level means=F value significant at the 0.1% level; day means = F value not significant and; any combination of ploidy level and day means = 29 and 37 μm at the 5 and 1% levels, respectively.

Table 3-10. Mean squares and significance levels from the analyses of variance of total seedling length (TL), hypocotyl length (HL), and root length (RL) at 12˚C.

Source of variance	df	Length character		
		TL	HL	RL
Treatment	7	7499***	5849***	149***
Ploidy level (PL)	1	4183***	1975***	410***
Day (D)	3	15947***	12854***	184***
PL x D	3	156	136*	28
Error	312	62	46	15

*, *** F value significant at the 5 and 0.1% levels, respectively.

Table 3-11. Means of total seedling length (TL), hypocotyl length (HL), and root length (RL) at each ploidy level (PL) at 12˚C on each of four days after germination initiation.

Character	PL	Day after germination initiation 7	14	21	28	PL mean
		----------mm----------				
TL†	2*x*	41	60	67	68	59
	4*x*	44	67	75	78	66
	Day mean	42	63	71	73	
HL‡	2*x*	26	42	48	51	42
	4*x*	27	48	55	58	47
	Day mean	26	45	51	54	
RL§	2*x*	15	18	19	17	17
	4*x*	18	19	21	21	19
	Day mean	16	18	20	19	

†Minimum differences for significance among the means were: ploidy level means = F value significant at the 0.1% level; day means = 3 and 4 mm at the 5 and 1% levels, respectively and; any combination of ploidy level and day means = 4 and 5 mm at the 5 and 1% levels, respectively.
‡Minimum differences for significance among the means were: ploidy level means = F value significant at the 0.1% level; day means = 2 and 3 mm at the 5 and 1% levels, respectively and; any combination of ploidy level and day means = 3 and 5 mm at the 5 and 1% levels, respectively.
§Minimum differences for significance among the means were: ploidy level means = F value significant at the 5% level; day means F value =1 and 2 mm at the 5 and 1% levels, respectively and; any combination of ploidy level and day means = 2 and 3 mm at the 5 and 1% levels, respectively.

Table 3-12. Mean squares and significance levels from the analyses of variance of crown diameter (CD), middle diameter (MD), and hook diameter (HD) at 12˚C.

Source of variance	df	Diameter character		
		CD	MD	HD
Treatment	7	182617***	262203***	175773***
Ploidy level (PL)	1	1247501***	1690711***	1212781***
Day (D)	3	2165	42581***	3551
PL x D	3	8108	5655	2325
Error	312	7618	3819	4531

*, *** F value significant at the 5 and 0.1% levels, respectively.

Table 3-13 Means of crown diameter (CD), middle diameter (MD), and hook diameter (HD) at each ploidy level (PL) at 12˚C on each of four days after germination initiation.

Character	PL	Day after germination initiation 7	14	21	28	PL mean
		----------μm----------				
CD†	2*x*	775	753	785	762	768
	4*x*	896	895	883	900	893
	Day mean	835	824	834	831	
MD‡	2*x*	677	636	639	612	641
	4*x*	814	792	766	774	786
	Day mean	745	714	702	692	
HD§	2*x*	557	547	563	549	554
	4*x*	677	665	679	688	677
	Day mean	617	606	621	619	

†Minimum differences for significance among the means were: ploidy level means=F value significant at the 0.1% level; day means = F value not significant and; any combination of ploidy level and day means = 45 and 58 μm at the 5 and 1% levels, respectively.
‡Minimum differences for significance among the means were: ploidy level means=F value significant at the 0.1% level; day means = 21 and 27 μm at the 5 and 1% levels, respectively and; any combination of ploidy level and day means = 32 and 41 μm at the 5 and 1% levels, respectively.
§Minimum differences for significance among the means were: ploidy level means=F value significant at the 0.1% level; day means = F value not significant and; any combination of ploidy level and day means = 35 and 45 μm at the 5 and 1% levels, respectively.

CHAPTER 4
THE EFFECT OF SEED WEIGHT ON SEEDLING GROWTH OF DIPLOID AND TETRAPLOID RED CLOVER

Introduction

The subtropical climate of the southeastern USA, as well as unpredictable patterns of autumn weather, often cause a problem in establishment of forage grasses and legumes. Drought conditions of this area are a primary restriction of seedling growth and establishment (Mislevy et al, 1999; Ball et al. 1996). Seed weight is known to influence seedling growth as well as subsequent early vegetative growth. Black (1959) summarized some agronomic characteristics of legume seeds. There seems to be a positive relationship between seed weight and seedling growth. Large-seeded species or heavier seeds had greater emergence as well as they were able to emerge from deeper planting depths under adequate moisture availability.

Literature Review

Seed weight is correlated with the ability of the seed to push the cotyledons through the soil surface, thus, allowing normal seedling development to progress. Williams (1956) measured the strength of emerging hypocotyls of subterranean clover (*Trifolium subterraneum* L.), crimson clover (*T. incarnatum* L.), rose clover (*T. Hirtum* All.), and alfalfa (*Medicago sativa* L.). Among the species, he found that the large-seeded subterranean clover had the highest force, and that small-seeded alfalfa was lowest. The

correlation coefficient found was 0.99. He also found a correlation ($r = 0.84$) between the emergence force and weight of hydrolyzable carbohydrates in the seeds.

Black (1957) showed the effect of seed weight on early seedling weight, using three strains of subterranean clover grown in pots. He found a positive relationship between seed or embryo weight and seedling growth, independent of strains. One strain of this species had greater early dry matter production than the others, but it was found that the strain had the greater seed weight on average. The same weight of seeds from different strains had similar dry weight production at the early stages. The only physiological differences found were the number and area of the leaves. The heavier seed strain resulted in larger but fewer leaves. The total leaf area and the relative growth rate remained similar among the strains.

Black (1956) examined the effect of seed weight on emergence from different depths, 1.3, 3.2, and 5.1 cm in subterranean clover. When seeds of the same weight were used, depth of planting had no effect on the weight of seedlings. The weight of cotyledons of emerging seedlings was shown to decrease, as the length of hypocotyls increased. However, the lower weight subterranean clover seeds had delayed emergence from the soil at the 3.2 cm depth, compared to other larger size seeds, and this size seeds failed to emerge from a 5.1 cm depth. The effects of seed weight and planting depth were significant, and no interaction effect was found. The maximum hypocotyl elongation was also measured at each seed weight, by placing the seedling in the dark after emergence. The smallest seeds (mean of 3.0 mg) had the shortest elongation of 37 mm, and the mean seed weights of 5.0 and 8.0 mg had 52 and 67 mm elongation, respectively.

Black (1956) also examined the effect of seed size on initial leaf area during 30 d. There was a positive relationship between them, but no interaction effect. The growth rate was concluded to be the same regardless of seed weight. As for planting depth, there was no effect on leaf production. It was shown that the cotyledon areas were independent of cotyledon weight. That is, the cotyledon weights decreased as hypocotyls grew, but the cotyledon areas remained identical to the initial areas. Since the cotyledons function as a storage as well as the first photosynthetic tissue, there is an advantage to having a larger cotyledon area for initial photosynthate production. Therefore, for the species that have epigeal germination, it is advantageous to having larger seed weight or size to emerge from deeper soils in addition to having better early vegetative growth.

Anderson (1971) compared the seedling growth of diploid and tetraploid red clover (*T. pratense* L.) growing in pots at the first harvest (32 d from sowing). The lowest mean temperature was 9°C (fluctuating 2 to 17°C), and the highest mean temperature was 21°C (13 to 27°C) during the experimental period. There was a highly significant ploidy effect. The tetraploid seeds (mean = 3.4 mg $seed^{-1}$) had more dry matter production than diploids (mean = 2.1 mg $seed^{-1}$) at the first harvest (25 mg vs 16 mg, respectively). At the second harvest (39 d from sowing), tetraploids had dry matter of 77 mg compared to 48 mg for the diploids. The relative shoot growth rate was not significantly different. He also observed longer petiole length of tetraploids than diploids, but a smaller leaf number for the tetraploids.

Anderson (1971) also separated two sizes of seed populations at each ploidy level, though the smaller tetraploid mean was heavier (2.71 mg) than larger diploid population

(2.01 mg). Within each ploidy level, the heavier population produced more dry matter at the first (28 days from sowing) and second (38 days) harvests. Yet, the relative shoot growth rate was not different. The relationship between seed weight and seedling growth between ploidy levels seemed to follow that described by Black (1959) at the normal diploid level.

Since the weight of red clover seeds was increased from doubling chromosome number, the tetraploids should have greater seedling growth than that of diploids. If the relationship between seed weight and seedling growth was verified, there would be greater potential to sow the heavier seeds, or tetraploid seeds, deeper to avoid desiccation at or closer to the soil surface as well as to remain in contact with a receding soil moisture front, thus increasing water use efficiency. The objective of this research was to evaluate the effect of seed weight at each ploidy level on seedling growth. The hypothesis was that an increase in seedling growth occurs as the seed weight increases regardless of ploidy levels.

Methods and Materials

The seedling growth procedure was described in Chapter 3, but in this study the seeds were not presoaked. Four hundred seeds at each ploidy level were individually weighed to 0.01 mg, maintaining the identities of seeds with their corresponding weight throughout the experiment. The temperature in the growth chamber was maintained at 20˚C. The growth measurements at 4, 7, 10, and 13 d were recorded. The seedling characteristics measured included total seedling length (TL), hypocotyl length (HL), root length (RL), crown diameter (CD), hook diameter (HD), and middle point diameter

between crown and hook (MD). At each day of measurement, the seedlings from 100 weighted seeds at both ploidy levels were measured. The seed weight distributions within the ploidy level were not significantly different among days. During the experiment, obviously abnormal seedlings were discarded. A regression of seed weight against all the response variables described above at various dates was conducted to determine whether slope (*b*-value) at each ploidy level was different from zero. The slope differences between ploidy levels were compared using Student's *t*-test. Each response variable at both ploidy levels on each day was compared using the F-test.

Results

The total seed weight distributions at both ploidy levels are shown in Table 4-1. The mean tetraploid seed weight (2.28 mg) was greater than that of diploids (1.54 mg) by approximately 1.5 times. The seed weight distributions were overlapping. The seed weight distributions at both ploidy levels were normal as shown by Shapiro and Francia (1972) (Table 4-1). Therefore, the seed weight distributions of the samples in any population were statistically equal.

The linear regression equations were shown in Table 4-2 for length characters and in Table 4-3 for diameter characters. All the *b*-values were positive and significantly different from zero. Therefore, an increase in seed weight would lead to an increase in the values of any character at both ploidy levels. The differences in *b*-values between ploidy levels at the same dates for each character were also tested using the *t*-test, shown in the same tables. Overall, there were few significant differences in *b*-values between the ploidy levels with occasional exceptions. When differences were detected, the values of diploids

tended to be greater than those of tetraploids. For the length characters, no additional growth was obtained after 10 d (Table 4-2).

Discussions and Conclusion

From the results, all the responses were found to be related to seed weight regardless of the ploidy levels. Increasing seed weights led to an increase in the values of all the characteristics at both ploidy levels. This would explain the greater mean growth measurements of tetraploids than diploids, since the mean seed weight of the former was greater than the latter. Therefore, the hypothesis that an increase in seed weight within each ploidy level results in the increase in seedling growth, was supported by these results. Selection to increase population seed weight would probably improve seedling growth at both ploidy levels.

The finding of no significant differences in *b*-values between ploidy levels indicated that each increase in seed weight led to the same increase in growth. However, the mean seed weight or the weight distributions were not the same, the mean tetraploid seedlings had greater means values of any characteristics measured. This result also supported the advantage of the heavier seeds in small-seeded species, at least in the early growth or seedling stage.

The *b*-values of diploids were occasionally greater than those of tetraploids. However, since the weight distribution or mean weight of tetraploids were larger than those of the diploids, the differences in seed weight offset the expected higher rate of increase of diploids.

The advantage of heavier seeds within each ploidy levels was also supported in this study. This experiment indicated that the induction of autotetraploidy resulted in heavier seeds compared to the diploid. It would be more efficient than selection for increasing seed weight at a diploid level. Since tetraploids are generally associated with a greater cell volume, the length and diameter of the tetraploid seedlings possibly could elongate to a greater extent, and the tetraploid seedlings had greater length and diameter than the diploids. Thus, the heavier or tetraploid seeds should result in better seedling growth and establishment of red clover.

Table 4-1. The seed weight mean, standard error (SE), standard deviation (SD), maximum and minimum values, normality test, and total sample number of diploid (2*x*) and tetraploid (4*x*) populations.

	2*x*	4*x*
Seed weight mean (mg)†	1.54	2.28
SE	0.01	0.02
SD	0.23	0.29
Maximum (mg)	2.15	3.05
Minimum (mg)	0.83	1.49
Normality test (W values)‡	0.9949	0.9965
Total sample number	363	381

† The seed weight means of diploids and tetraploids were significantly different at 1% level.
‡ The W values were not statistically significant indicating normal distribution in both populations (Shapiro and Francia, 1972).

Table 4-2. Linear regression analyses [L (mm) = a (mm) + b (mm mg^{-1}) SW (mg), where seed weight (SW) is the independent variable] between SW and the various characters at each date and ploidy level. The data reported are the mean, regression equation, and standard error (SE) of the *b*-value for total seedling length (TL), hypocotyl length (HL) and root length (RL) at each ploidy level (PL) on each day.

Character	Date	PL†	Mean ‡	Regression equation§	SE of *b*	*t*-value¶
			--mm--			
TL	4	2*x*	53*	TL = 27 + 16 SW***	± 3	0.09
		4*x*	56	TL = 18 + 16 SW***	± 3	
	7	2*x*	70***	TL = 34 + 24 SW***	± 4	0.52
		4*x*	78	TL = 28 + 22 SW***	± 4	
	10	2*x*	76***	TL = 35 + 26 SW***	± 4	3.79***
		4*x*	82	TL = 51 + 13 SW***	± 3	
	13	2*x*	75***	TL = 38 + 24 SW***	± 4	2.01*
		4*x*	83	TL = 44 + 17 SW***	± 3	
HL	4	2*x*	36	HL = 20 + 10 SW**	± 2	0.53
		4*x*	36	HL = 15 + 9 SW***	± 2	
	7	2*x*	52**	HL = 29 + 15 SW***	± 3	1.27
		4*x*	55	HL = 29 + 11 SW***	± 3	
	10	2*x*	56***	HL = 28 + 18 SW***	± 3	3.11**
		4*x*	60	HL = 38 + 9 SW***	± 3	
	13	2*x*	56***	HL = 33 + 15 SW***	± 3	1.61
		4*x*	60	HL = 36 + 11 SW***	± 2	
RL	4	2*x*	17***	RL = 8 + 6 SW***	± 1	0.92
		4*x*	20	RL = 3 + 7 SW***	± 2	
	7	2*x*	18***	RL = 5 + 9 SW***	± 2	0.82
		4*x*	23	RL = -1 +10 SW***	± 2	
	10	2*x*	20*	RL = 7 + 8 SW***	± 2	2.21*
		4*x*	22	RL = 13 + 4 SW*	± 2	
	13	2*x*	19***	RL = 5 + 9 SW***	± 2	1.31
		4*x*	23	RL = 9 + 6 SW***	± 2	

†For all characters at each date at each ploidy level, the number of pairs measured (n) were: date 4=89 and 93, date 7=91 and 95, date 10=92 and 97, and date 13=91 and 96 for diploid and tetraploid, respectively.

‡Ploidy means at each day and for each character were tested using a F-test.

*, **, *** F-value significant at 5, 1, and 0. % levels, respectively.

§*, **, *** *a*- and *b*-values significantly different from zero at 5, 1, and 0.1% levels, respectively.

¶ The differences between the *b*-values of each ploidy level at each day and for each character were tested using a *t*-test.

*, **, *** *t*-value significantly different at 5, 1, and 0.1% levels, respectively.

Table 4-3. Linear regression analyses [L (μm) = a (μm) + b (μm mg^{-1}) SW (mg), where seed weight (SW) is the independent variable] between SW and the various characters at each date and ploidy level. The data reported are the mean, regression equation, and standard error (SE) of the *b*-value for crown diameter (CD), middle diameter (MD), and hook diameter (HD) at each ploidy level (PL) on each day.

Character	Date	PL†	Mean ‡	Regression equation§	SE of *b*	*t*-value¶
			--μm--			
CD	4	2*x*	876***	CD = 644 +148 SW***	± 33	2.40*
		4*x*	1000	CD = 832 + 73 SW*	± 30	
	7	2*x*	862***	CD = 673 +124 SW**	± 44	0.07
		4*x*	989	CD = 713 +121 SW***	± 31	
	10	2*x*	862***	CD = 543 +208 SW***	± 35	1.95
		4*x*	974	CD = 634 +148 SW***	± 27	
	13	2*x*	867***	CD = 685 +118 SW***	± 32	0.12
		4*x*	981	CD = 706 +122 SW***	± 31	
MD	4	2*x*	700***	MD = 563 + 88 SW***	± 19	1.66
		4*x*	858	MD = 732 + 55 SW*	± 21	
	7	2*x*	662***	MD = 505 +103 SW***	± 20	0.78
		4*x*	817	MD = 615 + 88 SW***	± 19	
	10	2*x*	653***	MD = 502 + 99 SW***	± 22	1.82
		4*x*	807	MD = 494 +137 SW***	± 20	
	13	2*x*	657***	MD = 453 +133 SW***	± 18	2.23*
		4*x*	815	MD = 612 + 90 SW***	± 20	
HD	4	2*x*	545***	HD = 418 + 81 SW***	± 21	1.15
		4*x*	686	HD = 448 +104 SW***	± 18	
	7	2*x*	532***	HD = 391 + 92 SW***	± 23	0.69
		4*x*	651	HD = 473 + 78 SW***	± 20	
	10	2*x*	524***	HD = 407 + 76 SW***	± 20	2.72**
		4*x*	658	HD = 356 +132 SW***	± 21	
	13	2*x*	522***	HD = 391 + 85 SW***	± 18	0.35
		4*x*	655	HD = 479 + 78 SW***	± 21	

†For all characters at each date at each ploidy level, the number of pairs measured (n) were: date 4=89 and 93, date 7=91 and 95, date 10=92 and 97, and date 13=91 and 96 for diploid and tetraploid, respectively.

‡ Ploidy means at each day and for each character were tested using a F-test.

*** F-value significant at 0.1% level.

§*, **, *** *a*- and *b*-values significantly different from zero at 5, 1, and 0.1% levels, respectively.

¶ The differences between the *b*-values of each ploidy level at each day and for each character were tested using a *t*-test.

*, ** *t*-value significantly different at 5 and 1% levels, respectively.

CHAPTER 5
THE EFFECTS OF VARIOUS CHEMICALS ON DIPLOID-TETRAPLOID RED CLOVER SEEDLING GROWTH

Introduction

Tetraploid seedlings have been shown to grow faster and more vigorously than diploids; apparently, the heavier mean seed weight of tetraploids was primarily responsible for the increased growth (Chapter 3 and 4). Approximately 90% of ungerminated seed weight in dicotyledons is the cotyledon, the primary energy source during germination before photosynthesis is initiated in the seedling (Black, 1955). Heavier seed weight can supply more energy for seedling growth.

Various chemicals are reported to influence seedling growth during germination in a number of species (McWilliam et al., 1970; Copeland, 1976; Stevenson and Cleland, 1981). Auxin is known to cause cell elongation by increasing cell wall extensitivity, hydraulic conductivity, water potential gradient across the plasma membrane, and turgor pressure (Cleland, 1987). Cytokinin is known to increase amylolytic activity of cotyledons (Bewley and Black, 1985), enhance cellular division and expansion of cotyledons, and increase sugar content (Moore et al., 1998). The application of sucrose can also increase seedling growth because the stored energy in the cotyledons is metabolized into highly mobile sucrose and transported to newly-forming tissues (Copeland, 1976). Other sugars and ions are known to be other osmoregulators maintaining the turgor pressure in the cells of some species (Stevenson and Cleland, 1981). Hoagland solution which contains all the

elements essential for plant development, seemed to increase the seedling growth (Hoagland and Arnon, 1938). Much of the research with these compounds has been with excised seedling components: that is, hypocotyls and cotyledons. Literature on the application of these chemicals to intact seedlings is limited. However, these chemicals may influence seedling growth when seeds are associated with chromosome doubling or increasing seed weight. Some chemical treatments may have a positive influence on diploid seedling growth to make it comparable to that of tetraploids. Even though there are many other chemicals that may influence the seedling growth, only the influence of chemicals described above were tested due to the limited amount of time and the number of seeds.

The objective of this study was to test the effects of the chemicals added to the germination medium to determine the seedling responses in both diploid and tetraploid red clover (*Trifolium pratense* L.). In addition, the effect of presoaking seeds in various chemicals and then drying of the seeds at both ploidy levels before placing on germination media was tested to determine the practical uses of presoaking the seeds, or to determine possible chemical treatments that can be applied to seeds and then dried before sowing. In this way, desirable chemical treatments could be used to improve seedling establishment under field conditions.

Materials and Methods

The diploid (2*x*) and tetraploid (4*x*) populations and their genotype composition were described previously (Table 3-1). Six chemical treatments were tested: 1) no chemical added (control); 2) 2 ppm indole-3-acetic acid (IAA) ($C_{10}H_8NO_2Na$, FW 197.2); 3) 3 ppm kinetin (K) (6-Benzylaminopurine- $C_{12}H_{11}N_5$•HCl, FW 261.7); 4) 2 ppm IAA +

3 ppm K; 5) 2% sucrose (S) ($C_{12}H_{22}O_{11}$ FW 342.3); and 6) 2 ppm IAA + 3 ppm K + 2% S. These six treatments were added to either deionized water or half the recommended concentration of Hoagland's No. 2 Basal Salt Mixture (Sigma H-2395) (Hoagland and Arnon, 1938). The concentrations were determined either from preliminary studies, in the case of Hoagland solution, and/or information obtained from the literature showing a positive influence in the species studied (McWilliam et al., 1970; Copeland, 1976; Stevenson and Cleland, 1981).

The seeds at each ploidy level were soaked in each of 12 treatments, six chemical treatments and each of two Hoagland solution treatments (- and +; - indicates no addition of Hoagland solution and + indicates the addition in Tables 5-2, 5-3, 5-4, 5-5, and 5-6), for 48 h at 5˚C. After the soaking treatment at 5˚C, one half of the seeds were immediately germinated in growth pouches at 20˚C in the dark with the same solution in which they had been soaked. This group is referred to as 'media' treatment in Tables 5-2, 5-3, 5-4, 5-5, and 5-6. Each growth pouch with the corresponding 25 mL Hoagland solution and chemical solution, contained 20 seeds. The osmotic potentials of the various germination solutions were not measured. The other half of the seeds were dried for 24 h at room temperature. These seeds were then soaked in deionized water for 48 h at 5˚C and germinated in deionized water at 20˚C in the dark. This second group is referred to as 'presoaked' treatment in Tables 5-2, 5-3, 5-4, 5-5, and 5-6. This seed was then germinated at 20˚C in deionized water in the dark. There were two replications (growth pouches) for each treatment. The pouches were inserted vertically into the slits of growth cans. The replicated pouches were separated from each other within the same can. Measurements

were made at 10 d after the seeds were placed in the pouches. The whole experiment was repeated twice.

The experimental units were the means of each ploidy level, chemical, Hoagland solution, and presoaking treatment. Thus, there were 96 experimental units. The sampling units were the eight randomly selected normal seedlings. The seedling characteristics measured were hypocotyl length (HL), root length (RL), crown diameter (CD), hook diameter (HD), and middle point diameter between crown and hook (MD). The measurements were made to the nearest mm for length characters. Diameter measurements were made in mm converted to μm, using a microprojector at a magnification of 50X. The treatments were all factor combinations of two ploidy levels x two soaking treatments x two Hoagland solutions x six chemicals arranged in factorial design. The minimum differences for significance presented in the tables were obtained using Duncan's Multiple Range Test for the maximum number of means to be compared (Harter, 1960).

Results

The analyses of variance for all seedling characteristics are shown in Table 5-1. Generally, all the main effects were significant for most characters. The ploidy level effect was always found for all characteristics, and the means of tetraploids were almost always greater than those of diploids.

Hypocotyl Length

For the media treatment, the use of Hoagland solution slightly increased HL in control and in combination with IAA and sucrose at the diploid level (Table 5-2). The

increases were comparable to the means of tetraploids without Hoagland solution. On the other hand, tetraploids did not respond to the Hoagland solution effect except in combination with sucrose. Applications of IAA and sucrose alone did not lead to an increase in HL. The applications of K, IAA + K, and IAA + K + S resulted in considerable decreases in HL at both ploidy levels. There were no significant effects of Hoagland solution in these cases.

For the presoaking treatment, there were generally decreases in elongation of HL in control, IAA, and sucrose treatment at both ploidy levels. There were generally no significant differences in K, IAA + K and IAA + K + S treatment. The Hoagland solution effect was not present in the presoaking treatment.

Root Length

There were generally no increases from Hoagland treatment at either ploidy levels in either media or presoaking treatment (Table 5-3). The additions of IAA, K, IAA + K, and IAA + K + S resulted in significant decreases in RL in both the media and presoaked treatments at both ploidy levels. Only sucrose addition produced an increase in RL in the media treatment at both ploidy levels. However, a significant interaction between sucrose and Hoagland solution was observed. The combination of Hoagland solution and sucrose offset the increase of sucrose alone. In the presoaked treatment, the addition of sucrose had no effect over the control.

The presoaking treatment resulted in an increase in RL of the control, compared to non-presoaking at both ploidy levels. After presoaking, the effect of sucrose application on RL growth was not significantly different from control, but was lower before presoaking.

This treatment increased RL in the IAA and IAA + K + S applications as well as combination with Hoagland solution. The RL in the IAA application after presoaking was comparable to that of control (Table 5-3).

Crown Diameter

The Hoagland solution generally had no effect on CD (Table 5-4). Applications of IAA, K, IAA + K, and IAA + K + S resulted in increases in CD at both ploidy levels, but there was no effect of sucrose. Comparing the presoaking treatment with media treatment, presoaking with IAA, K, IAA + K, and IAA + K + S resulted in significant decreases in CD. However, the CD from K, IAA + K, and IAA + K + S applications were still greater than that of control without Hoagland solution (Table 5-4).

Middle Diameter

The Hoagland solution generally had no effect on MD at both ploidy levels (Table 5-5). Applications of K, IAA + K, and IAA + K + S resulted in significant increases, but IAA and sucrose had no effect. The presoaking treatment generally had no effect, but it decreased MD when K was applied. The MD from K, IAA + K, and IAA + K + S applications were greater than that of the control after presoaking (Table 5-5).

Hook Diameter

There were generally no Hoagland solution and presoaking treatment effects on HD at either ploidy level (Table 5-6). The applications of K, IAA + K and IAA + K + S resulted in greater increases in HD.

Discussion and Conclusions

From these results, greater HL was obtained from addition of Hoagland solution and from the Hoagland solution in combination with IAA and sucrose at the diploid level. Significant RL growth increase at both ploidy levels was observed from sucrose application only. IAA actually resulted in decreases in RL. The use of K and combinations of K with other chemicals resulted in decreases in length characters. The measurement was made only at one day, when the maximum growth was achieved at each ploidy level. Thus, the effects of these chemicals on growth rate within this growing period were not determined in this experiment.

From this experiment, it would be reasonable to establish the general relationship between seedling length and diameter. The negative relationship between HL and diameter found in this research supports the statement by Stuart et al. (1977) that the hypocotyl elongation was due to cell elongation not cell division in lettuce (*Lactuca sativa* L.), which also has the epigeal germination pattern as red clover. Since the cell volume is fixed, elongation probably results in decreased diameter. This may be the case in red clover.

The reductions from IAA application on RL and from K on both HL and RL and their associated combinations would indicate sensitivity of red clover to these concentrations. Cleland (1987) also pointed out the contradictory evidences of the effects of growth regulators because the tests were conducted using either intact plants or isolated growth regulator-responsive tissues. Since these growth regulators are involved in cell growth and/or elongation and already present, the additions of these growth regulators might be redundant. Obviously, the negative effects indicated that addition of the growth

regulators was inhibitory. Further research to determine the optimal concentrations of various growth regulators and chemicals should be pursued in future programs.

The previous experiment (Chapter 4) indicated that the increase in seedling growth of tetraploids was because of heavier seed weights. The tetraploids were expected to have larger cotyledons: therefore, more stored energy in seeds. Therefore, the addition of sucrose was expected to increase seedling growth. From a different point of view, Bewley and Black (1985) noted the feedback mechanisms of end products so that the addition of any nutrient would not lead to an increase in seedling growth. Nevertheless, it was surprising to find the increase in seedling growth because of sucrose application was found only in RL, but not HL at both ploidy levels. A practical use of sucrose in seedling establishment should be explored further, and the measurement at various dates should be made in the future research.

On the other hand, Hoagland solution application showed a slight increase in HL elongation at the diploid level. Hoagland solution contains all the essential chemical elements necessary for plant growth (Hoagland and Arnon, 1938). Therefore, it was not known which elements or combinations had the primary impact on increasing HL. In an experiment by McWilliam et al. (1970), seedling growth of subterranean clover (*Trifolium subterraneum* L.) and Australian phalaris (*Phalaris tuberosa* L.) was examined using the Hoagland solution. Their results were similar to this experiment. In their longitudinal study, the seedlings increased RL also, but the proportional growth of the shoot was greater than the root at later stages of measurement.

One of the important findings of this experiment was the interaction of sucrose and Hoagland solution. The effect of sucrose on RL growth was offset by Hoagland at both ploidy levels. The reason is unknown, but the result showed the increases in both HL and RL were not obtained, when sucrose and Hoagland solution were applied together. The applications of both solutions would not be recommended.

Despite the fact that the sucrose application increased the RL growth of both diploid and tetraploid red clover, Hoagland solution application only increased HL in the diploid seedlings. This may indicate that the tetraploids attained maximum hypocotyl extension. Further increases in seed weight may not lead to an increase in HL (Black, 1959).

The presoaking treatment resulted in reduction of HL and slight increase in RL. Copeland (1976) listed some advantages and disadvantage of presoaking. The uptake of solutions was evidenced by the dramatic reductions of seedling length from IAA or K. Nevertheless, in the normal conditions, the leaching from the seeds would have caused the reduction in growth. Therefore, this practice would not be favored.

Table 5-1. Mean squares and significance levels from the analyses of variance of hypocotyl length (HL), root length (RL), crown diameter (CD), middle diameter (MD), and hook diameter (HD).

Source of variance	df	HL	RL	CD	MD	HD
Treatment	47	2457***	2772***	1096134***	380533***	374831***
Ploidy level (PL)	1	5061***	2638***	4108538***	7095938***	7284771***
Presoaking (PS)	1	4524***	9836***	13615024***	149626***	13
Hoagland (H)	1	455***	1058***	37604	36038*	14138
Chemicals (C)	5	18219***	18836***	4813718***	1975830***	1948727**
PL x PS	1	63	44	11704	4134	27846*
PL x H	1	179*	6	28017	10004	24544
PS x H	1	124	1242***	153600***	69876***	45719*
PL x C	5	90*	162***	54658***	6498	14314
PS x C	5	2399***	2696**	1648469**	85172***	49199***
H x C	5	108***	809***	49124***	10190	3118
PL x PS x H	1	29	25	43350	51	2763
PL x PS x C	5	40	40	44957**	4289	11294
PL x H x C	5	33	13	28382*	2540	1993
PS x H x C	5	89*	504***	23135	9959	9004
PL x PS x H x C	5	33	25	40630**	9402	5804
Error	1488	35	24	11423	6178	7172

*, **, and *** F value significant at the 5, 1, and 0.1 % levels, respectively.

Table 5-2. Means of hypocotyl length (HL) in various chemical treatments at both ploidy levels and between media and presoaked treatments 10 days after germination initiation. HS = Hoagland solution. (-) indicates no HS added. (+) indicates HS added.

		Media treatment		Presoaked treatment	
Treatment	HS	2*x*	4*x*	2*x*	4*x*
		----------mm----------			
0	-	47†	51	38	44
	+	52	52	39	41
IAA	-	45	48	39	41
	+	49	51	40	44
K	-	26	31	30	33
	+	26	30	30	34
IAA + K	-	27	29	29	32
	+	28	27	28	32
S	-	46	52	38	45
	+	50	55	41	43
IAA+K+S	-	28	33	27	33
	+	29	33	30	35

† Minimum differences for significance among the means were 3 and 4 mm at the 5 and 1% levels, respectively.

Table 5-3. Means of root length (RL) in various chemical treatments at both ploidy levels and between media and presoaked treatments 10 days after germination initiation. HS = Hoagland solution. (-) indicates no HS added. (+) indicates HS added.

		Media treatment		Presoaked treatment	
Treatment	HS	2*x*	4*x*	2*x*	4*x*
		----------mm----------			
0	-	23†	26	26	29
	+	22	26	26	30
IAA	-	13	14	27	29
	+	9	12	28	30
K	-	11	13	12	13
	+	13	13	12	13
IAA + K	-	5	7	12	13
	+	5	5	12	13
S	-	35	42	27	30
	+	19	26	26	29
IAA+K+S	-	6	11	12	13
	+	6	7	12	15

† Minimum differences for significance among the means were 3 and 4 mm at the 5 and 1% levels, respectively.

Table 5-4. Means of crown diameter (CD) in various chemical treatments at both ploidy levels and between media and presoaked treatments 10 days after germination initiation. HS = Hoagland solution. (-) indicates no HS added. (+) indicates HS added.

		Media treatment		Presoaked treatment	
Treatment	HS	2*x*	4*x*	2*x*	4*x*
		---------- µm----------			
0	-	816†	904	779	912
	+	800	904	773	877
IAA	-	1151	1288	794	873
	+	1068	1354	741	853
K	-	1017	1101	943	1011
	+	1042	1076	915	1021
IAA + K	-	1224	1329	925	1042
	+	1274	1388	964	1063
S	-	757	880	763	863
	+	821	910	749	852
IAA+K+S	-	1219	1221	941	1044
	+	1244	1385	953	1005

† Minimum differences for significance among the means were 66 and 67 µm at the 5 and 1% levels, respectively.

Table 5-5. Means of middle diameter (MD) in various chemical treatments at both ploidy levels and between media and presoaked treatments 10 days after germination initiation. HS = Hoagland solution. (-) indicates no HS added. (+) indicates HS added.

		Media treatment		Presoaked treatment	
Treatment	HS	2*x*	4*x*	2*x*	4*x*
		----------µm----------			
0	-	686†	831	685	816
	+	696	853	689	832
IAA	-	684	842	697	807
	+	701	859	661	812
K	-	871	1030	829	947
	+	890	1020	796	932
IAA + K	-	859	1002	814	963
	+	880	1026	851	985
S	-	649	771	680	802
	+	678	814	667	810
IAA+K+S	-	819	896	844	982
	+	829	971	834	951

† Minimum differences for significance among the means were 48 and 49 µm at the 5 and 1% levels, respectively.

Table 5-6. Means of hook diameter (HD) in various chemical treatments at both ploidy levels and between media and presoaked treatments 10 days after germination initiation. HS = Hoagland solution. (-) indicates no HS added. (+) indicates HS added.

		Media treatment		Presoaked treatment	
Treatment	HS	2*x*	4*x*	2*x*	4*x*
		----------µm----------			
0	-	591†	705	601	738
	+	573	719	618	731
IAA	-	611	756	606	719
	+	592	751	573	721
K	-	718	875	732	884
	+	728	874	687	871
IAA + K	-	795	913	749	898
	+	776	923	726	896
S	-	564	658	600	737
	+	580	686	586	718
IAA+K+S	-	756	838	746	902
	+	750	885	709	872

† Minimum differences for significance among the means were 52 and 53 µm at the 5 and 1% levels, respectively.

CHAPTER 6
SUMMARY AND CONCLUSIONS

Eight crosses of red clover (*Trifolium pratense* L.) cv. 'Cherokee' were exposed to a N_2O atmosphere to induce autotetraploidy in the zygotes 24 h after pollination. Comparisons of the seed number and weight of the diploid and the autotetraploid plants obtained in each cross were tested in the field during the 2000 summer season. Over all crosses, the fertility (seed number) of the first generation of tetraploid plants was only 3% that of diploid plants. Crosses were found to be a significant factor controlling seed number or fertility within each ploidy level. Therefore, the induction of autotetraploidy in diverse crosses would be important to find more fertile lines at this ploidy level in future research. Since the seed number or fertility of the diploid and tetraploid plants was tested in the summer season which is not optimum for seed production in red clover and, more importantly, the plants represented the first generation after autotetraploidy induction, maximum seed yields and numbers were not expected. However, in successive generations of interpollination among the tetraploids, fertility should improve through the process of diploidization which improves pairing regularity and gamete fertility. Also, the tetraploid plants may require slightly different pollen vectors. Because the tetraploid flowers have longer corolla tubes, bumblebees (*Bombus* spp.) rather than honeybees (*Apis mellifera* L.) may be more effective pollinators of the tetraploid plants.

Over all crosses, the mean seed weight of the diploids and tetraploids was 1.53 and 2.20 mg, respectively. A highly significant ploidy level x cross interaction was found,

indicating that the tetraploid seed weight in the crosses was not associated with diploid seed weight. The autotetraploids have been shown to have greater mean seed weights and larger vegetative parts than their diploid ancestors presumably because of the increased cell volume associated with chromosome doubling.

Because of the shortage of tetraploid seeds, the diploid and tetraploid seeds were bulked within each ploidy level. Therefore, in additional studies, one diploid and one tetraploid population were used. The procedure involved seedling growth in pouches to compare seedling characteristics at four dates at three constant germination temperatures (12, 20, and 28˚C) in the dark. The response variables measured were total length (hypocotyl length + root length), hypocotyl length, root length, crown diameter, hook diameter, and middle point diameter of the seedling between hook and crown. The tetraploid seedlings had greater length and diameter than those of diploids at all temperatures evaluated. The growth pattern over dates indicated that the tetraploids were longer at the beginning of germination and this difference was maintained over dates. The greater diameter of tetraploids may indicate a greater emerging ability of the tetraploids in general. As the temperature increased, the days to achieve the maximum length were reduced. The critical temperature at a time of sowing should range between 20 and 28˚C.

The effect of seed weight on seedling characteristics within each ploidy level was evaluated using regression analysis. Increasing seed weight resulted in increases in the values of all the response variables measured. The comparisons of *b*-values between diploids and tetraploids were made for each response variable at each day of measurement. Generally, no differences were detected between the *b*-values of the two ploidy levels,

indicating that seed weight had the same effect on the responses at both ploidy levels. Since tetraploids are generally associated with a greater cell volume, the greater length and diameter of the tetraploid seedlings possibly conferred on them the ability to elongate to a greater extent than the diploids. Thus, the use of heavier or tetraploid seeds should result in better seedling growth and establishment of red clover.

The additions of Hoagland solution, indole-3-acetic acid (IAA), kinetin, sucrose, and some combinations in the germination media as well as a presoaking treatment were made to determine the effect on seedling characteristics at both ploidy levels. The addition of sucrose had no effect on hypocotyl length, but greatly increased root length at both ploidy levels. Hoagland solution addition slightly increased hypocotyl length of the diploid seedling, but not the tetraploids. IAA, kinetin, and their combinations resulted in reduction of root and/or hypocotyl length in both the diploid and tetraploid seedlings. The presoaking treatment was not favored in this research due to decreased hypocotyl length. These aspects should be further explored to determine if certain growth regulators and /or chemicals might improve germination characteristics and enhance seedling establishment.

This research primarily compared the seedling stage of diploid and tetraploid red clover, and the tetraploid seedlings were shown to have greater and faster elongation than those of diploids. However, greater seedling growth is not necessarily related to the final yield (Black, 1959). Therefore, other important traits in red clover production such as forage quality, pest resistant and so forth, need to be evaluated in the future before releasing a tetraploid cultivar.

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BIOGRAPHICAL SKETCH

Hideto Furuya, the second son of Toshihiko and Tomoe Furuya, was born on December 13, 1977, in Konosu city in Saitama prefecture, which is very close to Tokyo in Japan. After graduating from Josai Kawagoe High School in 1996, he went to Georgetown College, Georgetown, KY, for one year (1996-1997). During his brief attendance at Georgetown, he received the Outstanding Freshman Mathematics Student Award. From 1997-1999, he attended the University of Kentucky, majoring in agronomy with minors in agricultural economics and sociology graduating in December 1999 with a B.S. degree (*Cum Laude*). He was awarded the George Roberts Memorial Scholarship, and became a member of Gamma Sigma Delta, University of Kentucky Chapter. Graduate study in the Agronomy Department at the University of Florida was begun in January 2000. A research assistantship associated with the small grain breeding and genetics was granted Spring Term 2001. The Master of Science degree with an agronomy major (plant breeding and genetics specialty) and a horticultural sciences minor will be awarded in August 2001.

Since 1997, he was actively involved in Aikido. At this time, he is at the second Kyu level. On Thursday, 4 January 2001, his photograph appeared in the local & state section of the Gainesville Sun (local daily newspaper) showing him diligently practicing this difficult martial art.