GENETIC CONTROL OF ALFALFA SEED QUALITY CHARACTERISTICS

by

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A Thesis

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ABSTRACT

Gjuric, Radisa. Ph.D., The University of Manitoba, March, 1995.
Genetic control of alfalfa seed quality characteristics
Major professor: Dr. S. Ray Smith, Jr., Department of Plant Science.

There is limited information on the genetic control on alfalfa (Medicago sativa L.) seed quality characteristics and the effects of genotype and environment on these traits. The major objectives of this research were: i) to study the effect of cultivar and environment on alfalfa seed quality characteristics; ii) to estimate the components of genetic variance involved in the inheritance of alfalfa seed size and to evaluate the factors affecting response to selection for seed size in alfalfa; and iii) to determine if RAPD markers can be used to estimate outcrossing/selfing rates in autotetraploid alfalfa through a proposed method labelled 'RAPD nulliplex loci analysis'. A series of field experiments were conducted in southern Manitoba during 1992-1994 to study the effect of genotype and environment on alfalfa seed quality. Plant material for the research on the genetic control of seed size and RAPD nulliplex analysis was grown under controlled environmental conditions. A broad based alfalfa germplasm 'BIC-7WH' was used as a reference population and seed size was measured both on the parental plants involved in the mating design and their progeny plants. The proposed method of RAPD nulliplex loci analysis included: (i) RAPD marker selection based on the polymorphism between a given seed parent and its open pollinated progeny and (ii) screening individual open

pollination progeny of that seed parent for the presence of these markers. The results from the field experiments showed that cultivar ranking for seed yield was consistent over an wide range of seed yields obtained under different environmental conditions. All seed quality characteristics were strongly influenced by the environment, and there was a cultivar response for seed weight and percentage hard seed. This research demonstrated that digital imaging of alfalfa seed has potential for developing alternative measurements of seed quality. Results from the genetic study showed that the seed parent had the greatest influence on the seed size and that the genetic expression for seed size should not be measured on the parental plants involved in a crossing design, but seed size should be measured on the progeny plants. Seed size was shown to be controlled by both additive and non-additive components of genetic variance and appeared to be a highly heritable trait. RAPD nulliplex loci analysis allowed accurate identification of F_1 and S_1 progeny and estimation of outcrossing rates in autotetraploid alfalfa. In conclusion, this research indicates that there is a genetic effect for most of the alfalfa seed quality characteristics, but these effects are often masked by a combination of the environmental conditions and seed parent effect.

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FORWARD

This thesis is written in manuscript style. Four manuscripts are presented, each including abstract, introduction, material and methods, results and discussion. A general introduction and literature review precede the manuscripts. A general discussion, summary, and a literature citation sections terminate the thesis. The format of the manuscripts generally follows the format required by 'Crop Science' with the exception of the tables, due to their size. The appendices at the end of the thesis contain data collected in these studies but not included in the manuscripts. The first manuscript (Chapter III) 'Alfalfa seed size, shape and colour quantified through digital image analysis' coauthored with Drs. Ray Smith, Jr. and Felicitas Katepa-Mupondwa, was submitted to the Canadian Journal of Plant Science to be published as a note. The second, third and fourth manuscripts (Chapters IV, V and VI) will also be submitted to refereed journals.

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I. INTRODUCTION

Alfalfa (*Medicago sativa* L.) seed production has grown rapidly during the last 20 years in Western Canada with current estimates of over 60,000 planted hectares (Fairey and Lefkovich, 1992). Further increases are limited because cold winter conditions restrict alfalfa seed production almost exclusively to winterhardy, fall dormant cultivars. An ongoing project at the Univ. of Manitoba has focused on developing an establishment year alfalfa seed production system (Smith, 1992). Such system would allow seed production of moderately fall-dormant and non-dormant alfalfa cultivars. The introduction of these cultivars for seed production in Western Canada requires a thorough understanding of the effect of genotype and environment on alfalfa seed quality.

Currently, there is only a limited literature base on the effect of genotype and environment on alfalfa seed quality. Previous research indicated that germination and hard seed content are both strongly influenced by environmental conditions during seed maturation (Bass *et al.*, 1988). Alfalfa seed produced at more northern latitudes generally contains a higher percentage hard seed. Also, more fall dormant alfalfa cultivars tend to produce smaller seed with a higher percentage hard seed (Fairey and Lefkovich, 1991).

The relationship between seed size and subsequent seedling vigour has generated considerable research interest in almost all crop species (Black, 1959). It is generally accepted that in alfalfa seed size is positively associated

with seedling vigour, but that relationship diminishes with plant age (Erickson, 1946; Carnahan, 1963). The availability of large seeded alfalfa cultivars would be advantageous in regions with short growing seasons by providing faster establishment and better options for short-term crop rotation systems. At present, research on the genetic control of alfalfa seed size is limited, and there are no published reports on selection response for seed size in alfalfa.

Alfalfa is partially self-incompatible, therefore the majority of seed results from outcrossing and is referred to as F_1 seed (Viands *et al.*, 1988). Self-pollination rates depend on the genotype and environment and selfing is followed by a strong inbreeding depression. There have been attempts to utilize several pollination control systems in developing alfalfa hybrids including: self-incompatibility (Tysdal and Kiesselbach, 1944), cytoplasmic male sterility (Barnes *et al.*, 1972), and combinations of male- or self-sterile seed parents and female sterile pollen parents (Brown and Bingham, 1984). Each of these methods is limited in that none provide 100% pollination control. Therefore, an efficient method to estimate outcrossing/selfing rates is needed to facilitate the development of hybrid alfalfa cultivars.

The research reported in this thesis was conducted under field and growthroom conditions. The specific objectives of this research were as follows:

1) to study the effect of cultivar and environment on the following alfalfa seed quality characteristics: seed weight/size, seed colour, percentage germination, hard seed and non-viable seed;

2) to demonstrate the appropriateness of measuring alfalfa seed characteristics through computer Digital Image Analysis and to describe the methodology of data acquisition;

3) to estimate the components of genetic variance involved in the inheritance of alfalfa seed size;

4) to determine the most efficient selection method to increase alfalfa seed size;

5) to determine if RAPD markers can be used to estimate outcrossing/selfing rates in autotetraploid alfalfa through a proposed method termed 'RAPD nulliplex loci analysis'.

II. LITERATURE REVIEW

2.1. Origin and distribution of alfalfa

Alfalfa *(Medicago sativa* L.) is currently cultivated on more than 32 million hectares worldwide (Michaud *et al.*, 1988) and is considered the most important forage crop in the world. Alfalfa originated near Iran, but related forms and species were found scattered over central Asia. Alfalfa was first introduced into Canada in 1871, in the province of Ontario. In Western Canada, alfalfa was rarely grown until the introduction and further selection of extremely winterhardy types. The first winterhardy cultivar 'Grimm' was introduced from Minnesota in 1908 and subsequent selection led to the release of 'Grimm 666' from the Univ. of Saskatchewan in 1926 (Barnes *et al.*, 1988). Currently, alfalfa is grown on an estimated 4-5 million ha in Canada (Goplen *et al.*, 1982).

In recent years, alfalfa seed production has became an increasingly important enterprise in the western Canadian provinces of Manitoba, Saskatchewan, and Alberta, worth approximately 25 million dollars annually (Fairey and Lefkovich, 1992; Smith, 1992). Traditionally, alfalfa seed production in Western Canada involves establishing the crop during the first year and harvesting seed during the subsequent years. Therefore, seed production has been restricted almost exclusively to winterhardy, fall dormant cultivars (Fairey and Lefkovich, 1991, 1992).

The alfalfa industry uses a 1 to 9 rating system describing fall dormancy, with "1" designating those cultivars that produce the least amount of growth during the fall and "9" those cultivars that produce the most growth (Barnes *et al.*, 1991). Fall dormancy is influenced by a number of environmental factors, primarily photoperiod and temperature. Fall dormancy has been associated with winter survival, such that cultivars that express early fall dormancy usually have good winter survival potential.

2.2. Seed quality characteristics of alfalfa

High-quality seed of alfalfa (*Medicago sativa* L.) has been described as plump, usually bright yellow to olive green in colour, with high percentage viability and a relatively low percentage hard seed (Stewart, 1926; Bass *et al.*, 1988). Procedures for testing and labelling alfalfa seed for purity, noxious-weed seed content, and germination were outlined by the Canada Seeds Act and Seeds Regulation (1989), and the rules of International Seed Testing Association (1979) and the Association of Official Seed Analysts (AOSA) (1981). The AOSA rules specify that alfalfa seed be "planted" between blotters, in rolled or folded paper towels, or in sand or soil at 20^{0}C, under constant dark. The number of normal seedlings are to be first counted on day 4, and on day 7 the number of normal and abnormal seedlings, and hard seeds are counted. Abnormal seedlings usually develop from mechanically damaged seed.

In recent years, computer Digital Image Analysis (DIA) systems have been used for studying various plant organs including seed (reviewed by Price and Osborne, 1990). Several studies suggested a possible application of DIA in assessment of seed quality. Draper and Travis (1986) used DIA to study seed shape of several crop and weed species. Digital image analysis is also being investigated as an effective method for cereal grain classification (Sapirstein, 1987), as well as to improve the cereal grain grading system using quantified grain colour (Neuman *et al.*, 1989). DeKoeyer (1992) used DIA to evaluate the changes in oat kernel morphology following five cycles of recurrent selection for grain yield.

2.2.1. Hard seed and germination in alfalfa

Seed that usually contain viable embryos, but with palisade layers impermeable to moisture are referred to as hard seed (Fairey and Lefkovich, 1991). The testa or seed coat of an alfalfa seed is comprised of three layers of cells; the outer palisade layer, the intermediate hour-glass layer and the inner parenchymatous layer (Teuber and Brick, 1988). Lute (1928) demonstrated that the thickened outer wall of the palisade cells, not the cuticle, constitutes the moisture barrier. She also identified the lens, the weakest point in the palisade layer, as the point of entry for water into the alfalfa seed during the imbibition phase of germination. The impermeability of the palisade layer in legumes was

found to be a result of deposition of suberin in the outer walls of the palisade or malpighian cells and their subsequent shrinkage (Hyde, 1954).

The occurrence of hard seed in alfalfa is determined by genetic and environmental factors during and after seed maturation. Alfalfa seed development can be conveniently divided into three stages: 1) growth stage, up to 22 days after pollination (DAP); 2) stage of reserve accumulation, from 23 to 40 DAP; and 3) the ripening stage, from 41 to 75 DAP. Maximum dry weight is attained at approximately 40 DAP. Seed harvested at 40 DAP has a very high germination rate (95%) and no hard seed. Hard seededness begins to develop at about 55 DAP and percentage hard seed increases with ripening (Kowithayakorn and Hill, 1982).

Other related species (small-grained legumes) show similar developmental patterns. Black medic (*Medicago lupulina* L.) seed passes through the same phases of development, but the switch between germinable and hard seeds occurs between 39 and 40 DAP. Black medic seed detached from the plant between 18 and 29 DAP show various amounts of viability and no hard seed, while seeds detached between 30 and 38 DAP are all viable with no hard seed development (Sidhu and Cavers, 1977).

Hard seed can also develop during the storage period immediately following harvest (Bass *et al.*, 1988). Kowithayakorn and Hill (1982) reported that alfalfa seed harvested at different maturity levels and stored for three

months exhibited greatly increased percentage hard seed, compared to the hard seed content at harvest.

It has been assumed that temperature during, and immediately after, seed maturation plays a major role in hard seed development (Bass *et al.*,1988). Alfalfa grown in the warm growing season in southern California usually has a hard seed content of less than 20%, while seed produced in the cooler regions of the Pacific northwest contains between 40-50% hard seed. A recent study in Western Canada (Fairey and Lefkovich, 1991) showed that percentage hard seed in the most northern growing areas in the Peace River region of Alberta and BC ranged from 31 to 51%, while the percentage hard seed elsewhere in Western Canada varied from 22 to 38%.

Several studies reported a relationship between seed colour and seed quality. Light green and bright yellow coloured alfalfa seed were more responsive to heat treatment, as a method to decrease percentage hard seed, than was brown coloured seed (Staker, 1925). Stewart (1926) compared seven colour separates of alfalfa seed for germination and seedling vigour. The "true colour" (bright yellow with a tinge of olive green) seed had the highest percentage hard seed, and dark brown seed had the lowest seedling vigour. In a subsequent study Stewart and Carlson (1932) used the same colour grading scale and compared the alfalfa seed colour separates for laboratory and field emergence. They reported the lowest emergence among the 'shrivelled brown' seed. Similarly, Nel and Burgers (1968) reported lower emergence rates for

brown seed, although seed colour separates did not differ in the germination percentage.

Alfalfa seed colour is also controlled by genetic factors. Red endosperm is a simply inherited trait that modify alfalfa seed coloration (Sockness and Barnes, 1987). Black seed trait is another simply inherited trait, which is frequently used in genetic studies (Hunt *et al.*, 1976). Both red endosperm and black seed were associated with reduced seed weight.

A comprehensive study on the relationships among hard seed content, seed maturity, scarification and storage was conducted by Lute (1942). Mature seed contained a higher percentage hard seed (up to 90%) than immature seed (12-40%). All mature hard seed was viable after scarification, while immature hard seed had a variable percentage of viable seed. Seed stored for a period of 13 years showed a greatly reduced percentage hard seed. Seed previously classified as hard seed did not lose viability after the storage period, but there was an overall loss of viability due to death of previously permeable seed.

Fairey and Lefkovich (1991) investigated genetic differences in hard seed content from pedigreed alfalfa seed production in Western Canada and reported that *falcata* type cultivars had the highest percentage hard seed (34-38%) and *media* and *flemish* types showed a lower percentage hard seed (21.6-33.9%). These alfalfa types describe some of the germplasm sources of current North American alfalfa cultivars (Barnes *et al.* 1977). Other researchers also indicated that cultivars with higher percentage of *falcata* germplasm in their

genetic background generally exhibited a high percentage hard seed (Lute, 1928; Watson, 1948). There have been several attempts to relate germination rates with winterhardiness. Although non-winterhardy cultivars showed a tendency to germinate faster than winterhardy cultivars, the results were inconclusive (Larson and Smith, 1963; Heinrichs, 1967).

The mechanism that triggers hard seed development in alfalfa is not known, therefore it is difficult to explain why certain genotypes and environments produce more hard seed than others. Abscisic acid (ABA), a known inhibitor of germination, can occur at a relatively high concentration in legume seed (Black and Bewley, 1994). The presence of ABA in alfalfa seed also has been demonstrated (Xu *et al.*, 1990). ABA has been identified as a common mediator of various stresses to plants. Increased ABA concentration has been found in freezing tolerant (winterhardy, fall dormant) alfalfa genotypes in comparison to less winterhardy genotypes (Luo *et al.*, 1992.).

Hard seededness has ecological advantages in spreading germination over time, but the opinions on the agricultural benefit of this trait are varied. In some growing areas in the U.S. percentage hard seed in a seed lot should not exceed 10%, but in other areas levels between 20 and 30% are acceptable (Nelson, 1968). The EEC official germination standards require that alfalfa seed lots have less than 40% hard seed (Pedron, 1978). In the U.S., the Department of Agriculture regulations require that percentage hard seed be stated on all seed labels (Hall *et al.*, 1993). According to Bass *et al.* (1988)

hard seed have little value when planted because their delayed germination seldom contribute to stand improvement.

Hard seed content can be reduced by storage, blending or scarification. A number of scarification techniques have been developed, but with all of these techniques the reduction in hard seed content is partially offset by a higher percentage abnormal seedlings and deterioration of seed vigour and viability. Research by Hall *et al.* (1993) indicated that after scarification to break the hard seed coat the increase in stand density was not proportional to the increase in germination percentage. A possible explanation was that some seed made permeable by scarification did not contain viable embryos or scarification injured some already permeable seed. Other methods to reduce hard seed content are also available, including: combination of high and low temperatures; moisture and high pressure; high-frequency electrical energy; infrared rays; radio-frequency; and gas-plasma (reviewed by Ballard *et al.*, 1976; Bass *et al.*, 1988).

2.2.2. Seed size in alfalfa

2.2.2.1. Relationship between seed size and seedling vigour

Black (1959) summarized data for most agricultural plants and concluded that early growth and development was related to seed size, but harvestable yield was often not related to seed size. In alfalfa, a high correlation between

seed size and seedling vigour has been demonstrated (Beveridge and Wilsie, 1959), and the advantage of larger seed increased with seeding depth (Erickson, 1946). Contradictory research results suggested that there was only a weak relationship between seed size and seedling vigour (Nel and Burgers, 1968), and that this relationship diminished with plant age (Smith, 1961). Carnahan (1963) reported a positive correlation between alfalfa seed weight and unifoliate leaf area, and unifoliate leaf area and seedling height at 4 weeks, but there was no direct correlation between seed size and seedling height.

2.2.2.2. Environmental effect on seed size

Plant response mechanisms to environmental stress during seed development are diverse and complex. Stresses such as water deficits, high and low temperatures, nutrient deprivation, and shading can occur at any time during seed development. Often more than one stress may be experienced by the plant during any given time period. Bewley and Black (1994) summarized the literature on the effect of the environment on seed development and reported that results were often contradictory and that very few generalizations could be made.

There has been very little research on the effect of the environment on seed size in alfalfa, but seed size was considered to be under the environmental control to a lesser extent than hard seed content (Bass *et al.*, 1988). From research conducted over a four year period Rincker *et al.* (1988)

observed that seed weight within cultivars was influenced by the environment. Recent research results reported that seed weight was influenced by the temperature during pollination (Katepa-Mupondwa *et al.*, 1995). The average seed weight was larger at 18°C than at 27°C, however there was a significant seed parent x temperature interaction. Pedersen *et al.* (1956) reported that seed weight was a function of total flowering and the amount of pollination. Their results showed a reduced seed weight with complete pollination as compared to seed weight with one-third pollination.

2.2.2.3. Genotype effect, inheritance of seed size

Pedersen and Barnes (1973) postulated that since alfalfa seed has essentially no endosperm, seed size should reflect only embryo size and therefore could serve as an indication of seed hybridity. They reported a positive relationship between seed size and seed hybridity. In their results, hybrid seed (F_1) were approximately 5% larger than sibbed seed, and sibbed seed were approximately 5% larger than selfed (S_1) seed. Dunbier and Bingham (1975) suggested that heterozygosity was important in conditioning the maximum performance for seed weight. They found the highest seed weight in populations with the highest proportion of tri- and tetra-allelic loci. In contrast to these results, Bowley (1980) found that seed weight was greater in S_1 than in F_1 seed. He suggested that reduced fertilization caused by self-

incompatibility may have reduced the number of S_1 seed resulting in less within-pod competition.

More recently, a model of genetic control of seed weight was established by Peterson and Barnes (1982). Their results suggested that seed weight was controlled primarily by additive gene action, and that seedling vigour was controlled primarily by non-additive gene action. They also reported a significant maternal effect on seed weight. Subsequent research (Katepa-Mupondwa *et al.*, 1995) also showed that the influence of the pollen parent on seed weight was less consistent than the influence of the seed parent. Expected response to selection for seed weight was assumed to be higher when selection was performed among seed parent families than among pollen parent families.

There have been no reports on selection response for seed size in alfalfa. Hesterman *et al.* (1981) did report genotypic response in the fact that less fall dormant cultivars tended to produce larger seeds than more fall dormant cultivars. After three cycles of selection for large seed in birdsfoot trefoil (*Lotus corniculatus* L.) the average gain per cycle ranged between 6.25-20% (Draper and Wilsie, 1965). Only general combining ability showed significant effect on birdsfoot trefoil seed size.

2.3. Estimation of outcrossing/selfing rates in alfalfa

Improvement in the genetic yield potential of alfalfa has been relatively small in comparison to the majority of other crop species. Hill *et al.* (1988) reported that there was only a 3% increase in the genetic yield potential of alfalfa between 1956 and 1974. This small improvement was attributed in part to the inability of synthetic cultivars to maintain the heterotic effect over successive generations of seed increase (Bowley and McKersie, 1992). Preliminary research has indicated that the development of alfalfa hybrids would have several advantages over synthetic cultivars including: (1) greater avoidance of inbreeding; (2) reduced natural selection when seed production is outside the area of adaptation; and (3) full utilization of non-additive gene action (Busbice *et al.* 1972)

The development of successful hybrid alfalfa cultivars requires an efficient pollination control system. Self-pollination in alfalfa leads to substantial reduction in fertilization compared to cross-pollination. Despite this limitation, most alfalfa plants are at least partially self-compatible (Viands *et al.*, 1988). Self-incompatibility in alfalfa was described as partial (Cooper and Brink, 1940) or weak (Busbice *et al.*, 1975). Pollen tube-ovule interactions that occur within the ovarian cavity were identified as a principal self-compatibility mechanism in alfalfa (Viands *et al.*, 1988). In a review of angiosperm incompatibility systems, alfalfa was listed under the category of late acting self-incompatibility with the ovary inhibiting pollen tube growth before it reaches the ovule (Seavey and Bawa, 1986).

A number of methods have been proposed for controlling pollination in hybrid alfalfa seed production including: (1) self-sterility/incompatibility (Tysdal and Kiesselbach, 1944), (2) cytoplasmic male sterility (CMS) (Barnes *et al.*, 1972), and (3) male- or self-sterile seed parents and female-sterile pollen parents (Brown and Bingham, 1984). Since environmental conditions are extremely important for the stability of the CMS system (Viands *et al.* 1988) and the self-incompatibility system (Barnes *et al.*, 1972), there are major concerns as to whether either system would provide complete control over pollination in alfalfa.

In a more recent study, Campbell and Bauchan (1990) tested the stability of self-incompatibility and cross-compatibility in a series of partially self-incompatible alfalfa clones that had been selected as potential parents for hybrid seed production. They reported that it was possible to select alfalfa genotypes with high levels of self-incompatibility and concluded that the self-incompatibility system had potential for hybrid alfalfa seed production.

Simply inherited phenotypic traits provide one method to distinguish outcrossing from selfed progeny (Barnes and Hanson, 1967). Flower colour was used in most studies which estimated outcrossing rates in alfalfa (reviewed by Steiner *et al.*, 1992), despite the fact that pollinators preferentially visit certain colour flowers and therefore bias selfing rates (Knapp and Teuber, 1993). Charlesworth (1988) indicated another deficiency of these markers in that progeny populations must be grown to the stage of maturity that will allow

the marker to be scored. Furthermore, since S_1 progeny are less viable only a fraction of these progeny will grow to an appropriate maturity stage for effective scoring.

Another method to determine outcrossing/selfing rates was proposed by Pedersen and Barnes (1973). They demonstrated a strong relationship between seed size and outcrossing rate in alfalfa and proposed that the percentage of hybrid (F_1) seed could be estimated through seed size. Subsequently, Charlesworth (1988) also described a method for estimating outcrossing rates in natural plant populations based on seed size or seed viability. He found that these traits were useful because they could be scored relatively soon after pollination. However, Charlesworth's method required production of certain quantities of seed from both controlled cross- and self-pollination, which would be impractical for larger scale applications. In addition, other researchers have reported an inconsistent relationship between seed size and the presence of S_1 seed (Bowley, 1980; Gjuric and Smith, unpublished data), therefore raising questions about the validity of estimating outcrossing rates based on seed size.

In recent years, molecular markers have proven to be a useful method for determining outcrossing rates in plants. Knapp and Teuber (1993) used allozyme markers and 'TETRAT' software (Ritland, 1990) to estimate outcrossing rates in populations selected for an "easy-to-trip" trait. Newer molecular markers including RFLPs (random fragment length polymorphism)

and RAPDs (random amplified polymorphic DNA) have been used to map the alfalfa genome (Brummer *et al.*, 1994), but mapping was performed on diploid populations and therefore was not directly transferable to cultivated tetraploid alfalfa. Neither RFLPs or RAPDs have been used to estimate outcrossing rates.

RAPD markers (Williams *et al.*, 1990) are obtained by PCR amplification of random DNA segments from single arbitrary primers. Since RAPDs require minimum target DNA quantity and tolerate crude extraction their use is technically relatively simple (Waugh and Powell, 1992; Yu *et al.*, 1993). The limitation to the use of RAPDs is that they are dominant markers. Only two phenotypes can be distinguished, dominant (+) and null (-), and allelic variants cannot be detected. In an autotetraploid species like alfalfa four genotypes on a single locus (A...) will have dominant (+) phenotype and only one (aaaa) genotype will be (-). Dominant Mendelian inheritance of RAPDs was reported in diploid alfalfa (Echt *et al.*, 1991), and dominant tetrasomic inheritance was reported in autotetraploid potato (Quiros *et al.*, 1993). More recently, Yu and Pauls (1993) analyzed the segregation of RAPDs in tetraploid alfalfa. Their results indicated that random chromosome segregation was likely the predominant, but not exclusive mode of inheritance in alfalfa. The level of double reduction, typical for chromatid segregation, was very low (1 out of 121). They also reported the first molecular linkage groups in tetraploid alfalfa.

III. ALFALFA SEED SIZE, SHAPE AND COLOUR QUANTIFIED THROUGH DIGITAL IMAGE ANALYSIS

3.1. ABSTRACT

Digital Image Analysis (DIA) provides rapid and accurate measurements of specific images and has been used in cereal grain classification. This study was conducted to demonstrate the appropriateness of DIA measurements in alfalfa seed with several applications, i.e. seed quality assessments and breeding for seed size. The colour and size of alfalfa seed are determined by maturation, environmental conditions during seed development and storage conditions immediately after harvest. All of these factors affect seed quality (i.e. percentage hard seed and seed vigour), therefore the characteristics of the seed image are associated with seed quality. Digital Image Analysis (DIA) shows a great potential for indirect assessment of alfalfa seed quality by providing a rapid and accurate measurement of the seed image characteristics.

3.2. INTRODUCTION

Computer digital image analysis (DIA) provides a technique to rapidly and accurately measure and conduct analysis on specific images (e.g. seeds). Digital image analysis has been applied in cereal grain classification and studying kernel morphology, including attempts to improve the cereal grain

grading system using DIA quantified grain colour (Neuman *et al.*, 1989). In alfalfa (*Medicago sativa* L.), as with many other crops, there is no seed grading system based on the visual characteristics. Currently, alfalfa seed quality analysis relies on laborious traditional methods to determine seed weight, germination, percentage hard seed and seed viability.

Alfalfa seed size essentially reflects only embryo size, and has been positively related to seedling vigour and subsequent forage yield. However, there has been no concerted effort by breeders to increase alfalfa seed size through traditional selection procedures. Also, large seed size has been generally associated with a lower percentage hard seed (Black, 1959). Alfalfa seed colour has also been related to seed quality, specifically seed maturation and percentage hard seed (Staker, 1925; Stewart, 1926). Nel and Burgers (1968) reported that brown alfalfa seed had lower emergence rates than other seed colour classes, although seed viability did not differ over the range of seed colour.

Seeds that contain viable embryos, but have palisade layers that are impermeable to water are referred to as hard seed. The occurrence of hard seed in alfalfa is determined by both genetic and environmental factors during and after seed maturation. Alfalfa seed development can be conveniently divided into three stages: 1) the growth stage, up to 22 days after pollination (DAP); 2) the reserve accumulation stage, from 23 to 40 DAP; and 3) the ripening stage, from 41 to 75 DAP. Maximum dry weight is attained at

approximately 40 DAP. Seed harvested at 40 DAP has very high germination (95%) and no hard seed. Hard seededness begins to develop at about 55 DAP and percentage hard seed increases with ripening (Kowithayakorn and Hill, 1982).

Other related species (i.e. small-grained legumes) show similar developmental patterns. Black medic (*Medicago lupulina* L.) seed passes through the same phases of development, but the switch between germinable and hard seed occurs between 39 and 40 DAP. Black medic seed detached from the plant between 18 and 29 DAP show various amounts of viability and no hard seed, while seeds detached between 30 and 38 DAP are all viable with no hard seed development (Sidhu and Cavers, 1977). Hard seed can also develop during the storage period immediately following harvest. Alfalfa seed lots that were harvested at different maturity levels and stored for three months exhibited increased percentage hard seed, but percentage hard seed was still lowest in the seed lots harvested at 40 DAP (Kowithayakorn and Hill, 1982).

The onset of colour change in grass and legume seed occurs prior to seed maturity during the second stage of seed development. Alfalfa pod colour begins to change 34 DAP, which is only a few days before maximum dry matter accumulation (Kowithayakorn and Hill, 1982). Sidhu and Cavers (1977) studied the relationship between seed maturity and percentage of hard seed in black medic, using seed colour, appearance, and moisture content as indicators of maturity stage.

The variation in legume seed colour is due to conditions under which ripening takes place. Green seed indicates immaturity, whereas brown seed indicates weathering, sunlight, or frost injury during later development stages. Staker (1925) measured the hard seed content of different colour fractions of alfalfa seed and reported that colour was related to percentage hard seed. He defined 'true colour' of alfalfa seed as bright yellow with a tinge of olive green. Seed lots with the highest percentage of hard seed were in the yellow to dark brown colour range and those with the lowest percentage of hard seed were in the dark green and light brown colour ranges.

Analysis of digitized seed images can be used to precisely characterize a large number of individual alfalfa seed for size, shape and colour. Such analysis is rapid and accurate and provides advantages over traditional analysis. For example, the traditional analysis of seed weight cannot detect the variability among individual seeds, and this would be an essential selection parameter in a breeding program for seed size. The objective of this work was to demonstrate the appropriateness of measuring alfalfa seed characteristics through DIA, and to describe the methodology of data acquisition.

3.3. MATERIALS AND METHODS

3.3.1. Digital image analysis

The image processing system used in this research was designed around a DT-2871 (HSI) true colour frame grabber (Data Translation Inc.,

Marlboro, MA). Seed samples were placed in random orientation on a light stand with both substage and overhead illumination. Camera magnification was kept constant over experiments, and pixel-to-mm^2 conversion was conducted using lined millimetre graph paper and a control seed sample. A Kodak™ grey card (18% reflectance) and kodak colour patches (Kodak Color Dataguide, 1975) were used to control illumination conditions. The measurement software ImageX (Dr. L. Lamari; Dept. Plant Science, Univ. of Manitoba) was used to automatically identify and measure seeds for area, circularity factor (ratio between area of the smallest circle surrounding the object and area of the object), and colour characteristics: intensity, saturation and hue (on a 0-255 scale). A control seed sample was screened at regular intervals, and over the range of screening dates variability was 9.6, 1.4 and 1.1% for intensity, saturation and hue, respectively. The intensity measurements showed the largest variability because they were affected by external illumination factors (e.g. voltage fluctuation, bulb aging, etc..).

3.3.2. Plant material

In the first experiment, a random seed sample of approximately 5g was drawn from a selected seed lot of 'Algonquin' which showed variability for seed of colour. Seeds were visually separated into three general colour fractions: brown, yellow and green. After screening through DIA on petri plates, seed from each fraction was run through a standard germination test in nine (six for

green fractions) replications (50 seed per rep, between blotters, constant dark, 20°C).

In the second experiment, four seed lots were obtained from an alfalfa seed production study in 1991. They included the cultivars 'CUF 101' (non-dormant) and Algonquin (dormant) with seed selected from two establishment methods (direct seeding at 3.36 kg/ha and transplanting 9 week old seedlings at 30 cm spacing). The two establishment methods provided differences in plant developmental stage, timing of seed maturation and plant density. Seed was harvested on 10 Oct. 1991 which allowed sufficient time for maturation. The equipment used for seed harvesting and threshing was adjusted to avoid casual scarification during seed processing. Each seed (300 per lot) was placed in a separate well of a standard (96 well) Eliza dish and screened through the DIA. Seed were then covered with filter paper discs that fit into the wells, watered with 75 µL water, and placed in a germination cabinet at 20°C for 10 days. Germination counts were taken every other day and water was added as required. This procedure made it logistically practical to collect image and germination data on an individual seed basis. Seed were grouped into four germination classes based on the time required for germination (0-1 day; 2-3; 4-10; and +10) and compared for the characteristics obtained from DIA. Seed that germinated in 0-10 days was defined as germinable and those seed that failed to germinate in 10 days was defined as hard seed. Statistical analysis of the arc sin transformed data was performed using SAS (Statistical Analysis

System, SAS Institute, 1988) GLM procedure. Mean separation analysis used Fishers's protected Least Significant Difference procedure.

3.4. RESULTS AND DISCUSSION

Germination tests on the seed from experiment 1 showed that seed classified as yellow (fully mature, mean hue 17.2) had the highest hard seed content of 74.1%. A portion of the green seed fraction (immature seed, mean hue 24.2) had matured sufficiently to became viable seed, but not to develop a hard seed coat. This green seed fraction had a lower percentage of hard seed (62.0%) compared to the yellow fraction. The brown seed fraction (mean hue 13.6) also showed a lower percentage hard seed (59.0%). Brown seed usually results from exposure to unfavourable environmental conditions, such as: repeated dehydration-rehydration, frost, etc..

Germination analysis of individual seeds from experiment 2 showed high percentage hard seed values over all cultivar and establishment treatments. CUF 101 had larger seed than Algonquin (Table 3.4.1) for both establishment treatments. Hard seed were always significantly smaller than germinated seed, with the exception of the Algonquin-seeded treatment which produced the highest percentage hard seed. The variability in hard seed content was most likely a result of weathering (rehydration / dehydration) of already mature seed.

Table 3.4.1. Average seed size and hue values for hard and germinable seed. Four seed lots: CUF 101 and Algonquin, established by both direct seeding (seeded) and transplanting 9 week old seedlings (trans).

Seed lot	Class hard/germ.	Seed size --area mm^2 †		Seed colour --hue 0-255‡--	
CUF 101-trans	hard	2.48	*b*§	18.47	*a*
	germ	2.60	*a*	17.70	*b*
CUF 101-seeded	hard	2.53	*b*	18.74	*a*
	germ	2.60	*a*	17.69	*b*
Algonq.-trans	hard	2.23	*b*	19.55	*a*
	germ	2.27	*a*	18.68	*b*
Algonq.-seeded	hard	2.23	*a*	19.39	*a*
	germ	2.23	*a*	18.39	*b*

† Seed size measured through DIA as the area of the seed image.
‡ Hue measured through DIA on a 0-255 scale.
§ Means followed by the same letter are not significantly different based on Fisher's protected LSD ($P \leq 0.05$). Mean comparison within seed lots.

Seed harvested from all seed production treatments had sufficient time for maturation, regardless of the cultivar and establishment practice. Consequently, there was a narrow range for seed colour values and bias toward the lower hue values (brown colour) with only a small percentage of immature or green seed. Hue values for the hard seed were significantly higher than any germinated seed class, but were still within the yellow colour range (Table 3.4.1). The average intensity and saturation values (data not presented) were also higher for hard seed than for germinated seed.

These results indicate that hard seed content decreased as the colour visually deviated from the 'true colour'. Therefore, the next step was to

determine if this relationship was present in seed lots with much lower variation. In other words, can the DIA identify hard seed where visual colour discrimination with the naked eye was impossible and all seed fall within the yellow range? Seed from experiment 2 was classified into 5 arbitrary hue classes (hue values: 12-16, 16-18, 18-20, 20-22, and 22+) and compared for the percentage hard seed. Due to uniformity in colour of seed lots, very few seed fell outside the 18-22 range. Generally, percentage hard seed decreased as the hue values decreased, but chi-square values in a contingency 4 X 5 table showed that only the CUF-transplanted seed lot had significantly different percentage hard seed among hue classes. CUF-transplanted also had the largest number of seed outside the 18-22 range.

At the University of Manitoba, seed characterization by DIA has been applied to develop a selection procedure in alfalfa for seed size. Digital image analysis not only provides rapid screening and accurate measurements of seed size within individual plants, but it also allows for automatic exclusion of immature and sprouting seed by presetting thresholds for shape and colour values. Currently, with manual positioning of seed, approximately 15,000 individual seed per hour can be analyzed.

During the past two years seed samples were collected from producer fields across Manitoba. Preliminary results showed that alfalfa seed productions consistently differed in variability for seed colour due to maturation differences. If these differences are cultivar dependant, then this information

could be used to determine cultivar adaptability, particularly in areas of short growing season.

In conclusion, DIA rapidly and accurately measures several seed characteristics, including size, shape and colour. Digital imaging of alfalfa seed detects the slightest variability in seed size and colour, and this variability is related to alfalfa seed quality.

IV. THE EFFECT OF CULTIVAR AND ENVIRONMENT ON ALFALFA SEED QUALITY CHARACTERISTICS

4.1. ABSTRACT

Development of an establishment year alfalfa seed production system will allow introduction of non-winterhardy cultivars in Western Canada, but may have a negative effect on seed quality as these cultivars are removed from the area of their adaptation. The objective of this research was to study the effect of cultivar and environment on the following alfalfa seed quality characteristics: seed weight/size, seed colour, percentage germination, hard and non-viable seed. Nine alfalfa cultivars covering the whole range of fall dormancy classes were included in a series of field experiments at three locations in Manitoba over the period 1992-1994. Differential success in establishment and winter survival resulted in seed being harvested in only seven location-years. Seed harvested from these experiments were tested for seed quality using standard germination tests and by using a computer Digital Image Analysis (DIA). Measurements of plant seasonal development showed that the most fall dormant cultivars, 'Rangelander' and 'Algonquin', had slower early spring growth and development, but these differences diminished later in the season. Over a range of location-years the highest seed yields were consistently recorded for 'Arrow', 'Algonquin', 'Cimmaron VR', 'Rangelander' and 'Florida 77', respectively. All seed quality characteristics were under a strong influence

of the environment. Cultivars had significant effect on seed weight and percentage hard seed. The most fall dormant cultivar, Rangelander, consistently developed the highest percentage hard seed and the lowest seed weight. The measurements of seed characteristics through DIA (size and colour) were strongly correlated with measurements of seed quality through standard tests and showed a potential for development of alternative or supplementary methods to measure seed quality.

4.2. INTRODUCTION

Alfalfa (*Medicago sativa* L.) seed production has grown into an important enterprise in Western Canada with current estimates of over 60,000 ha. Further increases are limited because cold winter conditions restrict alfalfa seed production almost exclusively to winterhardy, fall dormant cultivars. Introduction of moderately fall dormant and non-dormant cultivars for seed production in Western Canada will require a thorough understanding of the effect of genotype and environment on alfalfa seed quality.

High-quality seed of alfalfa has been described as plump, usually bright yellow to olive green in colour, with a high percentage viability and a relatively low percentage hard seed (Stewart, 1926; Bass *et al.*, 1988). Seed that usually contain viable embryos, but with palisade layers impermeable to moisture are referred to as hard seed (Fairey and Lefkovich, 1991). The testa or seed coat

of alfalfa is composed of three layers of cells; the outer palisade layer, the intermediate hour-glass layer and the inner parenchymatous layer (Teuber and Brick, 1988). The thickened outer wall of the palisade cells constitutes the moisture barrier (Lute, 1928). This moisture barrier is formed by deposition of suberin in the outer walls of the palisade or malpighian cells and their subsequent shrinkage (Hyde, 1954).

The occurrence of hard seed in alfalfa is determined by genetic and environmental factors during and after seed maturation. A study of alfalfa seed development showed that hard seededness began to develop approximately 55 days after pollination (DAP) with maximum dry matter attained at 40 DAP. Percentage hard seed increased with ripening and also developed during the storage period immediately after harvest (Kowithayakorn and Hill, 1982). Similarly, Lute (1942) reported that mature alfalfa seed had a higher percentage hard seed than immature seed. A very similar seed development pattern was reported in the related species black medic (*Medicago lupulina* L.) (Sidhu and Cavers, 1977).

It has been assumed that temperature during, and immediately after, seed maturation plays a major role in hard seed development (Bass *et al.*,1988). Alfalfa seed produced in the warm climate of southern California usually has a percentage hard seed of less than 20%, while alfalfa grown in the cooler regions of the Pacific northwest has a percentage hard seed between 40-50%. A recent study in Western Canada (Fairey and Lefkovich, 1991)

showed that percentage hard seed in the most northern growing areas in the Peace River region of Alberta and BC ranged from 31 to 51%, while the percentage hard seed elsewhere in Western Canada varied from 22 to 38%.

Fairey and Lefkovich (1991) also reported a genetic response in alfalfa for percentage hard seed. The *falcata* type cultivars had the highest hard seed content (34.1-38.3%) and *media* and *flemish* types were lower (21.6-33.9%). These types or germplasm sources form the genetic basis of most of the currently grown alfalfa cultivars in North America (Barnes *et al.*, 1977). Other researchers have reported a similar genetic response for percentage hard seed in alfalfa (Lute, 1928; Watson, 1948). They also reported that cultivars with a large percentage of *falcata* germplasm in their genetic background generally expressed a high percentage of hard seed.

Hard seededness has ecological advantages in spreading germination over time, but opinions on the potential agricultural benefit are varied. Nelson *et al.* (1968) reported that alfalfa growers in different production areas in U.S. had different tolerance to percentage hard seed. The EEC official germination standards require that alfalfa seed lots have less than 40% hard seed (Pedron, 1978). Hard seededness can be easily overcome by mechanical scarification or other methods (reviewed by Ballard *et al.*, 1976), but under the same storage conditions scarified seed lose viability at a greater rate than non-scarified seed (Bass *et al.*, 1988).

Literature on the genotype and environment influence on seed size is limited. Seed size is considered to be under environmental control to a lesser

extent than hard seed (Bass *et al.*, 1988). Genotypically, non-dormant cultivars were found to produce larger seeds than dormant cultivars (Hesterman *et al.*, 1981). Related research has focused on the relationship of the seed size and subsequent seedling vigour. The results ranged from a strong positive relationship (Beveridge and Wilsie, 1959; Carnahan, 1963) to only a weak relationship (Nel and Burgers, 1968; Smith, 1961) between these two traits. Generally, seed size was positively associated with seedling vigour and early forage growth, but that relationship diminished with plant age (Erickson, 1946; Black, 1959; Pedersen and Barnes, 1973).

The objective of this research was to study the effect of cultivar and environment on the following seed quality characteristics: seed weight/size, seed colour and percentage germination, hard seed and non-viable seed.

4.3. MATERIALS AND METHODS

4.3.1. Experimental design and management

This study consisted of several experiments established during the period 1992-1994 at three locations in Manitoba (Table 4.3.1.1.): Homewood (HW), 56 km southwest of Winnipeg; Glenlea (GL), 15 km south of Winnipeg; and Arborg (AB), 150 km north of Winnipeg.

Table 4.3.1.1. Summary of alfalfa seed quality experiments conducted at 3 locations in southern Manitoba during 1992-1994.

Location\Year	1992	1993	1994
Glenlea (GL)	Y0†§	Y0§/Y1‡§	-
Homewood (HW)	Y0§	Y0§	Y0¶/Y1§
Arborg (AB)	Y0¶	Y0¶/Y1§	Y0¶

† - Y0: Establishment year.
‡ - Y1: First production year, experiment established in the preceding year and survived the winter.
§ - Successful experiments, all or most of the data collected.
¶ - These experiments did not reach reproductive maturity due to low temperatures in 1992, extensive rainfall in 1993 and early spring drought in 1994.

The experimental plots were established on 7 May, 13 May and 22 May, 1992 at Homewood, Glenlea and Arborg, respectively, and at the same sites on 12 May, 5 May and 14 May, 1993, respectively. The soil type at Homewood was a Sperling mixed loam, at Glenlea a Red River clay (lacustrine fine clay) and at Arborg a Tano series clay (Peat meadow). Phosphorus was applied at all locations to soil test recommendations and potassium was at high levels. Monthly precipitation and temperature data were recorded during the experiments (Tables 4.3.1.2 and 4.3.1.3).

Table 4.3.1.2. Mean monthly temperatures (^{0}C) for 3 locations in southern Manitoba during the growing seasons 1992-1994.

	Location/Year					
	Glenlea		Homewood			Arborg
Month	1992	1993	1992†	1993†	1994	1993
May	13.2	11.3	12.5	11.8	15.7	10.3
June	15.1	15.1	15.3	15.4	17.7	15.6
July	15.2	17.5	16.2	17.0	18.4	17.0
August	16.3	17.6	16.0	17.1	17.4	16.5
September	11.4	10.4	10.7	10.0	14.7	8.9
October	4.6	2.9	‡	4.0	6.8	2.6

† - Data from the closest data logger: Graysville, approx. 11 km W from the experiment.
‡ - Data not available.

Table 4.3.1.3. Monthly precipitation (mm) for 3 locations in southern Manitoba during the growing seasons 1992-1994.

	Locations/Years					
	Glenlea		Homewood			Arborg
Month	1992	1993	1992†	1993	1994	1993
May	26	41	8	55†	43	1
June	98	73	87	96†	91	74
July	96	246	62	228	86	66
August	98	160	61	70	70	77
September	70	32	31	16	63	50
October	4	40	‡	40	125	5

† - Data from the closest data logger: Graysville, approx. 11 km W from the experiment.
‡ - Data not available.

Nine alfalfa cultivars (Table 4.3.1.4.) from a range of fall dormancy classes were used in this experiment. The alfalfa seed industry uses a 1-9 rating system to differentiate the fall dormancy of individual cultivars, with "1" designating those cultivars that show the least growth during the fall and "9" those cultivars that show the most fall growth (Barnes *et al.*, 1991). This manuscript will refer to cultivars belonging to dormancy classes 1 and 2 as fall dormant cultivars, 3 and 4 moderately fall dormant, 6 intermediate fall dormancy, and 8 and 9 non-dormant cultivars.

Table 4.3.1.4. Alfalfa cultivars used in seed quality experiments at 3 locations in southern Manitoba during 1992-1994.

Cultivar	Origin	Reference
Florida 77 (9)†	Florida	Horner and Ruelke, 1981.
CUF 101 (9)	California	Lehman *et al.*, 1983.
Nitro (8)	Minnesota	Barnes *et al.*, 1988.
Moapa 69 (8)	Nevada	Hunt, *et al.*, 1979.
Wilson (6)	N. Mexico	Melton *et al.*, 1989.
Cimmaron VR (4)	N. Carolina	Alfalfa Variety Review Board,1989.
Arrow (3)	Iowa	Ag. Canada, 1987.
Algonquin (2)	Ontario	Baenziger, 1975.
Rangelander (1)	Saskatchewan	Heinrichs *et al.*, 1980.

† - Numbers in parenthesis denominate fall dormancy classes.

Two establishment methods were used: 1) direct seeding at a rate of 3.36 kg ha^{-1} and 2) transplanting 9 week old seedlings (11 week old in 1993) . A row spacing of 30 cm was used with both establishment methods in 1992. In

1993, row spacing was increased to 45 cm in an attempt to prevent lodging, and consequently the seeding rate was also reduced to 2.24 kg ha^{-1}. Seed was inoculated with *Rhizobium meliloti* L. Dang. The transplanting treatment was included in the experiment to provide an advantage in development as well as to ensure that all cultivars would attain seed maturation in the year of establishment. The experiments were randomized in a split-plot design with four replications. Establishment treatments were assigned to main plots and cultivar treatments to the sub-plots. Each sub-plot covered an area of 10.8 m^2 (1.8m x 6m).

Weed control was accomplished using a combination of herbicides (Table 4.3.1.5), cultivation and hand weeding. These herbicides were used to control annual grassy and broadleaf weeds with the exception of Roundup which was spot applied in both years to control Canada thistle. Ethalfluralin and trifluralin were applied in the spring and incorporated with a cross directional cultivation one week before planting. Scouting for insects was done frequently to check for any alfalfa pests. Lygus bug, alfalfa plant bug, pea aphid, and other insect control was achieved with specific insecticides (Table 4.3.1.3). Dimethoate was sprayed one week before bee release and Dylox was applied at night when the bees were dormant in their shelters.

Table 4.3.1.3. Pesticides used for weed and insect control in alfalfa seed quality experiments at 3 locations in southern Manitoba during 1992-1994.

Pesticide	Label	Description
Pre-emergence herbicides	Edge 50 DF	Ethalfluralin or [N-ethyl-N-(2-methyl-2-propenyl)-2,6-dinitro-4-(trifluoromethyl) benzenamine]
	Treflan	Trifluralin or {2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl) benzenamine}
Post-emergence herbicides	Pursuit	Imazethapyr or {2-[4,5-dihydro-4-methyl-4-(1-methylenthyl)-5-oxo-1H-imididazol-2-yl]-3-pyridinecarboxylic acid}
	Poast	Sethoxydim or {2-[1-(ethoxyimino)]-5-[2-(ethylthio)propyl]-3-hydroxy-2 cyclohexen-1-one}
Other herbicides	Roundup	Glyphosate or {isopropylamine salt of N-(phosphono-methyl) glycine}
Insecticides	Cygon	Dimethoate or [o,o-dimethyl S-(methylcarbamoylmethyl) phosphorodithioate]
	Dylox	Trichlorfon or [dimethyl (2,2,2-trichloro-1-hydroxyethyl) phosphonate]

Alfalfa leafcutter bees were used for pollination in both years. Six leafcutter bee shelters were placed around the periphery and in the centre of the entire experimental area at each location which included two other experiments. Incubation trays containing recently emerged bees were placed in each shelter when flowering averaged 25% across the experiment. Additional leafcutter bees were placed on the experiments at 100% flowering to provide a bee population of 88,000 ha^{-1} and ensure that bees were not a limiting factor for

seed set. The recommended leafcutter bee stocking rate is 44,000 ha^{-1} to ensure adequate pollination and good bee reproduction.

4.3.2. Measurements of plant development

Plant development was monitored by taking a number of measurements during the growing season. These measurements are summarized in the following table (Table 4.3.2.1.).

Table 4.3.2.1. Measurements of plant development in alfalfa seed quality experiments at three locations in southern Manitoba during 1992-1994.

Measurements	Description
Plant height	Measured in cm on 15 randomly selected stems per sub-plot (ten in 1993) at 7, 9, 11 and 13 weeks after planting†.
Percentage flowering	Visual estimation of percentage stems in flower at approximately two week intervals, starting at first flower development until full flowering.
Total number of racemes per stem	Counting all racemes (flowering and with developing pods) on 15 (ten in 1993) randomly selected stems per sub-plot, at 14, 16 and 19 weeks after planting†.
Number of pollinated racemes per stem	Counting racemes with developing pods on 15 (ten in 1993) randomly selected stems per sub-plot, at 14, 16 and 19 weeks after planting†.

† - Weeks after 1 May in the first production year (Y1) plots.

4.3.3. Seed yield

The desiccant Harvest [(2-amino-4-(hydroxymethylphosphinyl) butanoic acid] was sprayed at all locations/years approximately ten days before harvest.

Harvesting dates were as follows: 28 and 30 September, 1992, Homewood and Glenlea, respectively; 30 September, 4 and 5 October, 1993, Glenlea, Arborg, and Homewood, respectively; and 29 September, 1994, Homewood. Mechanical harvesting was done with a Hege™-plot combine (Hans-Urlich Hege, Waldenburg, Germany). Seed was harvested with the combine set at a low air speed to prevent loss of unthreshed seed still in the pod. A stationery unit (Agriculex, Scarborough, Ont; A.T. Ferrel S.Co. Saginaw, Mich.) was used for subsequent threshing. In 1993, establishment year plots had very low seed yield and they were harvested by hand picking all mature pods. The harvesting, seed threshing and cleaning equipment were adjusted (maximum clearance, low air speed) to minimize casual scarification of the seed.

4.3.4. Seed quality characteristics

Immediately after harvest, seed from all experiments were stored in a drying room for a period of approximately six weeks. After that, seed remained at room temperature until the germination tests. A sample of 150 seeds were taken from each sub-plot and seed weights were determined. The samples were then screened through a computer Digital Image Analysis (DIA) system designed around a DT-2871 (HSI) true colour frame grabber (Data Translation Inc., Marlboro, MA). The measurement software ImageX (Dr. L. Lamari, Dept. Plant Science, Univ. Manitoba) was used to automatically identify and measure seeds for size (area of seed image) and seed colour (hue on 0-255 scale).

Germination tests were performed on the same samples in accordance with the rules of the International Seed Testing Association (1979) and Association of Official Seed Analysts (1981). The germination test conditions included: constant dark, temperature of 20°C and seed rolled in a moist paper towel. The following characteristics were recorded: percentage normal seedlings (germinated by day 7), percentage hard seed and percentage non-viable seed. Seed testing rules also recognize a percentage abnormal seedlings. Abnormal seedlings generally result from mechanical damage to the seed. This class of seed was insignificant due to the precautions undertaken to minimize casual scarification during harvesting and seed processing. Therefore, this manuscript will report percentage normal seed as percentage germination and sum of percentage hard and normal seed as percentage viable seed.

4.3.5. Statistical analysis

Analysis of variance was conducted using the SAS (Statistical Analysis Systems, SAS Institute, 1988) GLM procedure. Differential establishment success and winter survival prevented analysis of the effects of location, year and production year. Therefore, the analysis tested the effect of the environment with the assumption that environment was the combination of year/-location/-production year effects. Using this definition, data was collected and analyzed over 7 environments. The establishment and cultivar treatments

were considered fixed effects and the environment a random effect. Bartlett's Chi-square test (Steel and Torrie, 1980) was used to determine the homogeneity of variance across environments. Flowering percentage data was transformed by arcsin $x^{1/2}$. Treatment means were compared using Fishers's protected least significant difference (LSD) procedure.

4.4. RESULTS AND DISCUSSION

4.4.1. Measurements of plant development

The measurements of plant development were taken at approximately two week intervals. Good winter survival of 1992 experiments resulted in a large number of experiments in 1993, therefore the number of measurements was reduced. A higher priority was given to detailed measurements on the establishment year plots. This resulted in a variation in the number of different measurements that were pooled over the environments.

4.4.1.1. Plant height

The transplanting establishment treatment did not provide an advantage in plant growth and development over direct seeding, but increased within-row spacing resulted in less lodging in comparison to the direct seeding treatment. Therefore, all cultivar means were pooled over the transplanted and seeded establishment treatments. Cultivar effect on plant height diminished as

each season advanced (Table 4.4.1.1.1). At 7 weeks after planting the non-dormant CUF 101 had the highest numerical plant height, with an average of 24.3 cm across all environments. Cultivars from the lowest fall dormancy classes (Rangelander and Algonquin) showed the lowest plant height at 7 weeks after planting (Table 4.4.1.1.2). This same trend was observed 9 and 11 weeks after planting, with Rangelander and Algonquin consistently having the lowest plant height.

This initial difference in plant height was expected since cultivars that are dormant in the fall also tend to have slower growth in the spring than more non-dormant cultivars. Fall dormancy is mostly a function of photoperiod, although low late-summer/fall temperatures also play a significant role in reducing plant growth. Therefore, more fall dormant cultivars are more sensitive to photoperiod and react to short day length by reducing fall growth. Massangale *et al.* (1971) confirmed that the more fall dormant alfalfa cultivars show slower growth in the spring than less fall dormant cultivars.

Table 4.4.1.1.1. Analysis of variance for plant height of nine alfalfa cultivars grown in seed quality experiments under various environments and two establishment practices in southern Manitoba during 1992-1994.

Date	Source	d.f.	MS	F value	
7 weeks after planting	Environment	4†	3431.8	6.5	NS
	Establishment	1	1123.2	2.1	NS
	Cultivar	8	111.9	6.8	***
	Env x Cv	32	16.5	1.4	NS
	Estb x Cv	8	11.6	1.0	NS
9 weeks after planting	Environment	4†	11793.5	8.8	*
	Establishment	1	3512.5	2.6	NS
	Cultivar	8	212.2	4.4	**
	Env x Cv	32	48.1	2.0	*
	Estb x Cv	8	41.0	1.7	NS
11 weeks after planting	Environment	5‡	4334.7	28.6	NS
	Establishment	1	3404.9	8.5	*
	Cultivar	8	172.6	2.4	*
	Env x Cv	40	72.5	1.6	NS
	Estb x Cv	8	32.7	0.7	NS
13 weeks after planting	Environment	4†	3682.7	2.6	NS
	Establishment	1	1012.3	0.8	NS
	Cultivar	8	144.18	1.4	NS
	Env x Cv	32	107.2	4.7	***
	Estb x Cv	8	42.6	1.9	NS

*, **, *** Significance at P≤0.05, 0.01, and 0.001.
† - The following environments: HW-1992-Y0, HW-1993-Y0, GL-1992-Y0, AB-1993-Y1 and AB-1993-Y1.
‡ - The following environments: HW-1992-Y0, HW-1993-Y0, GL-1992-Y0, GL-1993-Y0, AB-1993-Y1 and AB-1993-Y1 (HW-Homewood, GL-Glenlea, AB-Arborg).

Table 4.4.1.1.2. Plant height (cm) of nine alfalfa cultivars grown in seed quality experiments under various environments in southern Manitoba during 1992-1994. Cultivar means averaged across environments and establishment treatments.

Cultivar	Measuring dates (weeks after planting)			
	7 weeks†	9 weeks‡	11 weeks†	13 weeks†§
Florida 77	23.5 *ab*¶	41.4 *a*	54.0 *ab*	69.5
CUF 101	24.3 *a*	42.7 *a*	53.4 *ab*	71.2
Nitro	22.9 *ab*	42.1 *a*	52.7 *ab*	68.1
Moapa 69	23.7 *ab*	40.7 *a*	52.7 *ab*	67.9
Wilson	22.5 *bc*	40.6 *a*	51.4 *bc*	69.1
Cimmaron VR	23.2 *ab*	42.2 *a*	54.8 *a*	72.1
Arrow	22.8 *b*	42.5 *a*	53.6 *ab*	70.0
Algonquin	20.7 *c*	36.5 *b*	49.8 *c*	67.1
Rangelander	18.9 *d*	36.0 *b*	48.8 *c*	65.2

† - Averaged across the following environments: HW-1992-Y0, HW-1993-Y0, GL-1992-Y0, AB-1993-Y1 and AB-1993-Y1 (HW-Homewood, GL-Glenlea, AB-Arborg).
‡ - Averaged across the following environments: HW-1992-Y0, HW-1993-Y0, GL-1992-Y0, GL-1993-Y0, AB-1993-Y1 and AB-1993-Y1.
§ - No significant cultivar differences.
¶ - Cultivar means followed by the same letter are not significantly different according to Fisher's protected LSD at $P \leq 0.05$.

Later in the season (13 weeks after planting) the differences between cultivars diminished, but Rangelander and Algonquin still ranked the lowest. Plant height results indicated that cultivars generally differed in initial spring growth, but the relative growth rates were similar later in the season, therefore the relative differences in plant height diminished as the plants grew taller.

4.4.1.2. Flowering percentage

The 1993 growing season was characterized with extensive rainfall which caused delayed flowering and poor pollinator activity. Most of the establishment year plots did not reach 100% flowering until the end of the 1993 season. Establishment year plots in Arborg started flowering very late in both 1992 and 1993 and did not reach seed set stage in either year. CUF 101 consistently started flowering earliest, while Rangelander was latest (data not shown).

Table 4.4.1.2.1. Flowering percentage of nine alfalfa cultivars grown in seed quality experiments under various environments in southern Manitoba during 1992-1994. Cultivars means averaged across environments and establishment treatments. Mean separation based on arcsin $x^{1/2}$ transformed data.

Cultivar	Scoring dates (weeks after planting)		
	10 weeks†	11 weeks†	12 weeks‡
Florida 77	7.5 *a*§	33.1 *a*	33.5 *ab*
CUF 101	4.7 *a*	17.5 *bc*	27.8 *ab*
Nitro	6.2 *a*	24.4 *ab*	27.2 *ab*
Moapa 69	4.9 *a*	23.9 *ab*	24.9 *ab*
Wilson	2.6 *b*	13.7 *bc*	19.3 *bc*
Cimmaron VR	5.8 *a*	28.0 *ab*	25.8 *a*
Arrow	6.0 *a*	34.4 *a*	27.7 *ab*
Algonquin	2.2 *b*	18.3 *bc*	17.9 *c*
Rangelander	1.6 *b*	8.2 *c*	15.9 *c*

† - Averaged across establishment treatments and following environments: HW-1992-Y0, HW-1993-Y0, GL-1992-Y0 and GL-1993-Y0.
‡ - Averaged across establishment treatments and following environments: HW-1992-Y0, HW-1993-Y0 and GL-1992-Y0 (HW-Homewood, GL-Glenlea).
§ - Cultivar means followed by the same letter are not significantly different according to Fisher's protected LSD at $P \leq 0.05$.

Rangelander also had the lowest flowering percentage at 10, 11 and 12 weeks after planting (Table 4.4.1.2.1), but at 14 weeks after planting all cultivars reached approximately 100% flowering under more favourable environments.

4.4.1.3. Number of racemes per stem

The number of racemes per stem was influenced by both the environment and the cultivar (Table 4.4.1.3.1). Due to unfavourable weather conditions, there was a noticeable decrease in number of racemes per stem from week 16 to week 19 after planting (Table 4.4.1.3.2). Flower and pod abortion are often observed in alfalfa (Carlson, 1928), but extensive lodging in GL-1993-Y1 definitely accounted for the large decrease in number of racemes per stem (both flowering and pollinated) from week 16 to week 19. In addition, poor pollinating conditions in 1993 resulted in an overall low number of racemes per stem.

Four cultivars belonging to the lowest fall dormancy classes (Rangelander, Algonquin, Arrow and Cimmaron VR) and Florida 77 (dormancy of 9) tended to produce more racemes per stem than other cultivars (Table 4.4.1.3.2). This was especially evident later in the growing seasons, at 16 and 19 weeks after planting. All fall dormant/moderately fall dormant (fall dormancy 1-2/3-4) cultivars had higher number of racemes per stem than cultivars belonging to higher fall dormancy classes, with the exception of Florida 77. The number of pollinated racemes per stem showed similar cultivar differences to these observed with total number of racemes per stem. The five cultivars with

highest total number of racemes per stem also had the highest number of pollinated racemes per stem (Table 4.4.1.3.2).

Table 4.4.1.3.1. Analysis of variance for number of racemes per stem for nine alfalfa cultivars grown in seed quality experiments under various environments and two establishment practices in southern Manitoba during 1992-1994.

Date	Source	d.f.	MS	F value	
14 weeks after planting	Environment	3†	42.3	4.1	NS
	Establishment	1	73.8	5.0	NS
	Cultivar	8	28.5	5.6	***
	Env x Cv	24	5.1	1.6	NS
	Estb x Cv	8	4.4	1.4	NS
16 weeks after planting	Environment	4‡	1150.9	7.6	*
	Establishment	1	52.3	0.4	NS
	Cultivar	8	160.7	10.5	***
	Env x Cv	32	15.3	1.6	NS
	Estb x Cv	8	17.0	1.7	NS
19 weeks after planting	Environment	4‡	983.2	46.9	**
	Establishment	1	1.5	0.1	NS
	Cultivar	8	152.3	10.6	***
	Env x Cv	32	14.4	1.6	NS
	Estb x Cv	8	20.8	1.7	NS

*, **, *** Significance at P≤0.05, 0.01, and 0.001.
† - ANOVA over the following environments: HW-1992-Y0, HW-1993-Y0, GL-1992-Y0 and GL-1993-Y0.
‡ - ANOVA over the following environments: HW-1992-Y0, HW-1993-Y0, GL-1992-Y0, GL-1993-Y0 and GL-1993-Y1 (GL-Glenlea, HW-Homewood).

Table 4.4.1.3.2. Number of racemes per stem (total and pollinated) for nine alfalfa cultivars grown in seed quality experiments under various environments in southern Manitoba during 1992-1994. Cultivar means averaged across environments and establishment treatments.

Cultivar	Measuring dates (weeks after planting)					
	14weeks†		16weeks‡		19weeks‡	
	Total	Polli-nated	Total	Polli-nated	Total	Polli-nated
Florida 77	5.9 *ab*§	1.5 *ab*	9.6 *a*	4.4 *ab*	8.0 *abc*	3.7 *ab*
CUF 101	4.4 *c*	0.9 *b*	7.7 *b*	2.6 *b*	5.0 *d*	2.0 *b*
Nitro	4.7 *bc*	1.2 *ab*	7.3 *b*	2.4 *b*	6.2 *bcd*	2.6 *b*
Moapa 69	4.8 *bc*	1.0 *ab*	7.1 *b*	2.5 *b*	5.7 *cd*	2.6 *b*
Wilson	4.3 *c*	1.7 *b*	6.4 *b*	2.0 *b*	5.1 *d*	1.9 *b*
Cimmaron VR	6.7 *a*	1.8 *a*	11.1 *a*	5.2 *a*	10.4 *a*	5.3 *a*
Arrow	6.7 *a*	1.4 *a*	11.3 *a*	5.9 *a*	9.5 *a*	5.4 *a*
Algonquin	5.6 *ab*	1.4 *ab*	11.2 *a*	6.6 *a*	8.1 *ab*	5.6 *a*
Rangelander	4.9 *bc*	1.1 *b*	10.3 *a*	5.2 *a*	9.5 *ab*	5.0 *a*

† - Averaged over the following environments: HW-1992-Y0, HW-1993-Y0, GL-1992-Y0 and GL-1993-Y0.
‡ - Averaged over the following environments: HW-1992-Y0, HW-1993-Y0, GL-1992-Y0, GL-1993-Y0 and GL-1993-Y1 (GL-Glenlea, HW-Homewood).
§ - Cultivar means followed by the same letter are not significantly different according to Fisher's protected LSD at $P \leq 0.05$.

Plant development measurements showed that the most fall dormant cultivars (Rangelander and Algonquin) had slower growth and development (height and flowering) early in the season. The initial hypothesis was that these differences would translate into differences in reproductive development, with the non-dormant cultivars having an advantage over dormant cultivars. However, these differences did not continue throughout the season and fall

dormant cultivars generally produced more racemes per stem than majority of the non-dormant cultivars. There was no fall dormancy related response for early growth and development among cultivars belonging to fall dormancy classes 3-9. Moderately dormant cultivars, Arrow and Cimmaron VR (dormancy 3 and 4, respectively), consistently ranked among the highest for all measurements of plant development.

4.4.2. Seed yield

Unfavourable weather conditions during 1992 and 1993 dramatically reduced alfalfa seed yields for producers across Manitoba and in these experiments. Low temperatures in 1992 reduced seed yields by reducing pollinator activity and delaying seed maturation. Cloudy weather and excessive precipitation in 1993 also affected pollinator activity, and caused localized flooding and lodging. Approximately 38% and 70% of production fields in Manitoba were not even harvested in 1992 and 1993, respectively (Smith *et al.*, 1993, 1994).

The range in seed yields in this study varied from approximately 1 kg ha^{-1} (HW-1993-Y0) to 250 kg ha^{-1} (HW-1994-Y1) (Table 4.4.2.2.). Bartlett's test for homogeneity of variances showed that variances across environments were not homogenous. Therefore, all seed yield data was transformed (log x), which satisfactory homogenized the variances across environments. There were no

changes in the significance between treatment effects due to transformation, therefore the analysis of the original data is presented (Table 4.4.2.1).

Table 4.4.2.1. Analysis of variance for seed yield of nine alfalfa cultivars grown in seed quality experiments under various environments and two establishment practices in southern Manitoba during 1992-1994.

Source	d.f.	MS	F value	
Environment	6	515224.6	11.8	***
Establishment	1	739.6	0.03	NS
Cultivar	8	18615.9	3.3	**
Env x Cv	48	5637.0	2.4	**
Estb x Cv	8	3588.1	1.5	NS

*, **, *** Significance at P≤0.05, 0.01, and 0.001.

The five cultivars (Algonquin, Arrow, Rangelander, Cimmaron VR and Florida 77) which had the highest number of pollinated racemes per stem also had the highest numerical yields under most environments. In 1993, seed was harvested by hand to obtain a sufficient quantity of seed for quality analysis, as well as to document the devastating effect of the extensive rainfall. Surprisingly, even under such extreme conditions the seed yield of these five cultivars tended to be higher than the remaining four cultivars (Table 4.4.2.2). Wilson and CUF 101, generally produced the lowest yields. The one exception to the general trend was observed in GL-1993-Y1, which accounted in large part for the significant cultivar x environment interaction. This experiment had extensive lodging and localized flooding, which may have contributed to the contradictory results.

Table 4.4.2.2. Seed yield (kg ha^{-1}) of nine alfalfa cultivars grown in seed quality experiments under various environments in southern Manitoba during 1992-1994. Cultivar means averaged across establishment treatments.

Cultivar	ENVIRONMENT						
	GL-1992-Y0†	GL-1993-Y0	GL-1993-Y1	HW-1992-YO	HW-1993-Y0	HW-1994-Y1	AB-1993-Y1
Florida 77	20.5 ab‡ 4§	2.2 ab 4	5.5 d 9	56.2 cde 5	1.1 bc 4	274.2 bcd 5	56.8 c 6
CUF 101	14.9 bc 7	0.8 b 9	12.8 bcd 6	25.2 e 8	0.6 d 7	169.8 d 8	62.0 bc 5
Nitro	15.8 bc 5	0.9 b 8	16.8 abc 3	50.4 de 6	0.5 d 9	190.8 cd 7	46.0 c 8
Moapa 69	15.5 bc 6	1.5 ab 7	13.6 abcd 4	40.1 e 7	0.5 d 8	246.6 bcd 6	56.4 c 7
Wilson	8.3 c 9	1.9 ab 6	23.5 a 1	21.3 e 9	0.6 d 6	156.0 d 9	40.3 c 9
Cimmaron VR	21.7 ab 3	2.3 ab 3	6.8 cd 7	88.3 bc 3	1.2 b 2	254.5 bcd 4	107.7 ab 4
Arrow	29.4 a 1	3.3 a 1	13.1 bcd 5	141.5 a 1	1.1 bc 3	308.1 ab 2	133.8 a 2
Algonquin	21.9 ab 2	1.9 ab 5	17.5 ab 2	86.1 bcd 4	1.7 a 1	354.8 a 1	131.2 a 3
Rangelander	14.5 bc 8	2.9 ab 2	5.8 d 8	95.2 b 2	0.7 cd 5	274.4 abc 3	134.7 a 1

† GL-Glenlea, HW-Homewood, AB-Arborg, Y0-establishment year seed production, Y1-seed production in the first year following establishment.

‡ - Cultivar means followed by the same letter are not significantly different according to Fisher's protected LSD at P≤0.05.

§ - Cultivar rank within each environment.

Similar results were observed from an establishment year experiment conducted at the Glenlea research station in 1991 (Smith, 1992). Arrow and Cimmaron VR had the highest yields (583 and 528 kg ha^{-1}, respectively) and cultivars CUF 101 and Wilson had the lowest yields (277 and 296 kg ha^{-1}, respectively).

The advantage in early growth and development of non-dormant cultivars in comparison to the fall dormant cultivars (Rangelander and Algonquin) did not result in higher seed yield. The non-dormant cultivars, with the exception of Florida 77, showed more lodging and summer regrowth, which negatively affected seed yields. In addition, these cultivars started flowering earlier and therefore flowers and developing pods were exposed to variable (mostly unfavourable) weather and pollination conditions, which may also have negatively affected seed yield. The fall dormant cultivars (Rangelander and Algonquin) had a more synchronous or determinate flowering pattern. For example, Rangelander had the fastest transition between first flower and 100% flowering, and had a definite end point to flowering.

A possible explanation of different flowering patterns would assume that fall dormant cultivars were more sensitive to photoperiod not only during the short photoperiod of early spring and fall, but also during the longer photoperiods in the summer. Cultivars that have a greater photoperiod sensitivity may respond to increased photoperiod with more profuse flowering over a shorter period of time. A study on photoperiod response of alfalfa (Major

et al., 1991) showed that photoperiod sensitivity was correlated with winterhardiness, with the most winterhardy cultivar 'Anik' (also most fall dormant) having the highest photoperiod sensitivity. Major *et al.* (1991) reported that there were no cultivar differences in the maximal optimum photoperiod (MOP), in other words the cultivars did not respond to increased photoperiod above approximately 18 h. They only measured photoperiod sensitivity as days to floral initiation, and there are no reports on photoperiod sensitivity related to later reproductive development.

4.4.3. Seed quality

All seed quality characteristics were measured on the seed harvested from seven different environments (Table 4.4.3.1.2). Seed produced under this extreme range of environmental conditions provided a wide range of seed maturities for studying seed quality characteristics. Cultivar means are presented for each environment to allow a comparison of seed quality measurements of particular cultivar/environment seed productions to the seed regulation standards.

4.4.3.1. Seed weight

Seed produced under all environments had lower seed weights than would be considered normal for alfalfa, approximately 0.2 g $(100\ seed)^{-1}$. The environment had a significant effect on seed weight (Table 4.4.3.1.1). Seed

produced in the establishment year (Y0's) tended to have lower seed weights (Table 4.4.3.1.2) than the seed produced in the first production year (Y1's). This was probably due to the fact that the Y1 plots generally set seed earlier and therefore had more time for maturation.

Table 4.4.3.1.1. Analysis of variance for seed weight of nine alfalfa cultivars grown in seed quality experiments under various environments and two establishment practices in southern Manitoba during 1992-1994.

Source	d.f.	MS	F value	
Environment	6	0.00752	11.8	***
Establishment	1	0.00018	0.6	NS
Cultivar	8	0.00078	2.8	*
Env x Cv	48	0.00018	0.7	NS
Estb x Cv	8	0.00054	2.1	NS

*, **, *** Significance at P≤0.05, 0.01, and 0.001.

Cultivar differences for seed weight were significant only for HW-1994-Y1, which provided the most favourable environment for alfalfa seed production (the highest seed yields and seed weights). The poor seed development conditions for the other environments may not have allowed the expression of cultivar differences. Rangelander had the lowest seed weight in HW-1994-Y1 and also consistently ranked among the lowest in other environments (4.4.3.1.2).

Hand harvested seed (HW-1993-Y0, GL-1993-Y0) contained a high percentage immature seed, which resulted in a very high plot to plot variability. Therefore, although cultivar differences for these two environments were numerically large there were no significant differences.

Table 4.4.3.1.2. Seed weight in g (100 seed)$^{-1}$ of nine alfalfa cultivars grown in seed quality experiments under various environments in southern Manitoba during 1992-1994. Cultivars means averaged across establishment treatments.

Cultivar	ENVIRONMENT													
	GL-1992-Y0†		GL-1993-Y0		GL-1993-Y1		HW-1992-YO		HW-1993-Y0		HW-1994-Y1		AB-1993-Y1	
Florida 77	0.150‡	5§	0.156‡	4	0.173‡	6	0.146‡	6	0.165‡	3	0.182 *a*¶	1	0.163‡	5
CUF 101	0.152	2	0.161	2	0.178	1	0.148	5	0.147	8	0.180 *ab*	2	0.156	8
Nitro	0.155	1	0.153	5	0.174	4	0.151	3	0.170	1	0.177 *ab*	5	0.163	4
Moapa 69	0.149	6	0.146	7	0.169	7	0.143	9	0.144	9	0.177 *ab*	6	0.158	6
Wilson	0.149	7	0.145	9	0.168	8	0.151	2	0.152	5	0.178 *ab*	3	0.157	7
Cimmaron VR	0.151	3	0.159	3	0.176	3	0.144	8	0.168	2	0.177 *ab*	4	0.167	2
Arrow	0.150	4	0.164	1	0.176	2	0.152	1	0.163	4	0.170 *bc*	8	0.167	3
Algonquin	0.146	8	0.151	6	0.173	5	0.149	4	0.150	6	0.171 *bc*	7	0.167	1
Rangelander	0.141	9	0.145	8	0.164	9	0.145	7	0.149	7	0.163 *c*	9	0.153	9

† GL-Glenlea, HW-Homewood, AB-Arborg, Y0-establishment year seed production, Y1-seed production in the first year following establishment.
‡ - Cultivar differences were not significant.
§ - Cultivar rank within each environment.
¶ - Cultivar means followed by the same letter are not significantly different according to Fisher's protected LSD at P≤0.05.

4.4.3.2. Percentage hard seed

The percentages hard seed recorded in this study were higher than that surveyed for alfalfa production in Western Canada between 1984-1988 (Fairey and Lefkovich, 1991). Mechanical harvesting is known to reduce percentage hard seed, which may partly account for this difference. Hand-harvested seed lots may contain 100% hard seed, but mechanically harvested lots generally have less than 60% (Lute, 1927). The lower hard seed percentages for HW-1992-Y0 and GL-1992-Y0 were as a result of high percentages of non-viable seed (Table 4.4.3.2.2). The results of this research confirmed a strong influence of the environment on percentage hard seed (Table 4.4.3.2.1). There was also a significant cultivar effect on percentage hard seed.

Table 4.4.3.2.1. Analysis of variance for percentage hard seed of nine alfalfa cultivars grown in seed quality experiments under various environments and two establishment practices in southern Manitoba during 1992-1994.

Source	d.f.	MS	F value	
Environment	6	22979.3	69.0	***
Establishment	1	8.5	0.1	NS
Cultivar	8	551.9	2.9	**
Env x Cv	48	194.2	1.7	*
Estb x Cv	8	133.8	1.2	NS

*, **, *** Significance at P≤0.05, 0.01, and 0.001.

This study showed that the more fall dormant cultivars generally had higher percentages of hard seed. Rangelander, Algonquin and Arrow (fall dormancy 1, 2 and 3, respectively) had the highest numerical hard seed percentage in the three environments where significant cultivar differences occurred (Table 4.4.3.2.2). Rangelander, which had the highest percentage of *falcata* germplasm consistently ranked highest for percentage hard seed. Research results dating back to the 1920's have suggested that cultivars with *M. falcata* in their genetic background express higher percentage hard seed (Lute, 1928; Watson, 1948; Fairey and Lefkovich, 1991).

The mechanism that triggers hard seed development in alfalfa is not known, therefore it is difficult to explain why certain genotypes and environments produce more hard seed than others. Hard seed development may depend on the amount of stress (photoperiod, temperature, moisture) imposed on a plant and the stress responsiveness of that plant (assuming fall dormant cultivars are more stress responsive). Such scenario would assume that increased endogenous ABA concentrations act as a stress signal, which would result in hard seed development. However, ABA is usually associated with embryo-imposed germination inhibition and its role in coat-imposed germination inhibition (hard seededness) is not clear.

Table 4.4.3.2.2. Percentage hard seed of nine alfalfa cultivars grown in seed quality experiments under various environments in southern Manitoba during 1992-1994. Cultivar means averaged across establishment treatments.

Cultivar	ENVIRONMENT						
	GL-1992-Y0†	GL-1993-Y0	GL-1993-Y1	HW-1992-YO	HW-1993-Y0	HW-1994-Y1	AB-1993-Y1
Florida 77	11.0 c‡ 9§	59.3 abcd 5	52.0 abcd 4	20.9¶ 8	75.6¶ 1	67.7¶ 7	57.0¶ 5
CUF 101	20.9 b 4	46.4 de 8	47.2 cde 6	23.2 6	63.2 7	70.7 2	59.8 2
Nitro	15.2 bc 8	43.7 e 9	42.2 e 9	26.3 2	62.2 8	68.6 4	52.9 8
Moapa 69	15.5 bc 7	52.2 bcde 6	46.6 de 7	21.7 7	59.6 9	68.3 5	56.3 6
Wilson	16.1 bc 6	51.3 cde 7	47.8 bcde 5	24.8 4	65.1 6	68.9 3	57.8 4
Cimmaron VR	18.5 bc 5	61.2 abc 4	44.6 de 8	29.6 1	71.5 3	68.0 6	52.1 9
Arrow	21.0 b 3	66.0 ab 3	55.1 ab 2	17.9 9	74.0 2	65.3 9	55.6 7
Algonquin	30.6 a 2	68.4 a 2	54.5 abc 3	25.7 3	67.5 4	65.9 8	61.8 1
Rangelander	36.9 a 1	72.6 a 1	56.1 a 1	23.8 5	66.4 5	72.7 1	58.0 3

† GL-Glenlea, HW-Homewood, AB-Arborg, Y0-establishment year seed production, Y1-seed production in the first year following establishment.
‡ - Cultivar means followed by the same letter are not significantly different according to Fisher's protected LSD at P≤0.05.
§ - Cultivar rank within each environment.
¶- Cultivar differences were not significant.

4.4.3.3. Percentage non-viable seed

An alfalfa seed lot should contain a minimum of 85% viable seed to qualify for Canada Foundation No.1, the highest grade of production according to the Canada Seeds Act and Seeds Regulation (1989). Since normal and hard seed comprise the viable seed, in this experiment the percentage viability = 100% - % non-viable seed.

Although there was no cultivar effect on percentage non-viable seed, the environment did influence this seed quality parameter (Table 4.4.3.3.1). Despite the poor seed development conditions all seed produced from experiments harvested during the first production year (Y1's) had a low percentage non-viable seed (4.3-14.4%), thereby qualifying these seed lots for Canada Foundation No.1 (Table 4.4.3.3.2). Seed produced from experiments harvested during the establishment year (Y0's) had a higher percentage non-viable seed (8.5-48.7%).

Table 4.4.3.3.1. Analysis of variance for percentage non-viable seed for nine alfalfa cultivars grown in seed quality experiments under various environments and two establishment practices in southern Manitoba during 1992-1994.

Source	d.f.	MS	F value	
Environment	6	8715.2	99.8	*
Establishment	1	262.1	2.1	NS
Cultivar	8	51.7	0.6	NS
Env x Cv	48	83.9	1.0	NS
Estb x Cv	8	94.3	1.1	NS

*, **, *** Significance at P≤0.05, 0.01, and 0.001.

Table 4.4.3.3.2. Percentage non-viable seed of nine alfalfa cultivars grown in seed quality experiments under various environments in southern Manitoba during 1992-1994. Cultivar means averaged across establishment treatments.

Cultivar	ENVIRONMENT													
	GL-1992-Y0†		GL-1993-Y0		GL-1993-Y1		HW-1992-YO		HW-1993-Y0		HW-1994-Y1		AB-1993-Y1	
Florida 77	48.7‡	1§	15.9‡	5	5.1‡	7	29.8‡	4	8.5‡	9	6.9‡	1	10.3‡	5
CUF 101	36.9	7	13.4	6	7.4	2	30.9	3	16.9	5	4.8	8	8.7	9
Nitro	38.3	5	10.0	9	8.1	1	28.7	5	14.2	7	5.2	7	12.4	3
Moapa 69	40.9	3	20.0	1	6.9	4	26.7	9	24.2	1	5.5	5	10.1	6
Wilson	43.8	2	16.2	3	5.6	5	27.9	7	19.9	2	5.7	4	11.3	4
Cimmaron VR	37.9	6	16.2	4	7.1	3	26.9	8	10.8	8	5.8	3	14.4	1
Arrow	39.1	4	12.1	8	5.1	8	35.0	1	15.1	6	6.5	2	13.3	2
Algonquin	33.2	8	18.1	2	5.3	6	28.4	6	18.9	3	5.4	6	9.1	8
Rangelander	31.3	9	12.8	7	4.3	9	32.3	2	18.8	4	4.1	9	9.5	7

† GL-Glenlea, HW-Homewood, AB-Arborg, Y0-establishment year seed production, Y1-seed production in the first year following establishment.
‡ - Cultivar differences were not significant.
§ - Cultivar rank within each environment.

These results indicated that seed yield may not be the only problem in an establishment year alfalfa seed production system. In 1992, seed yields obtained from establishment year plots in this experiment were in the range of the production seed yields in the region. In spite of that, the percentage of non-viable seed was extremely high (26.7-48.75).

4.4.3.4. Percentage germination

Percentage germination (normal seedlings) was influenced by the environment, cultivar and their interaction in the same manner as percentage hard seed. Since percentage germination was negatively associated to the percentage hard seed ($r = -0.742^{***}$, N = 456, sub-plot seed samples as units of observation), the cultivar differences for percentage germination were very similar to those for hard seed, but the order was reversed (data not shown).

4.4.4. Seed characteristics measured through digital image analysis

Seed screening through digital image analysis (DIA) provides precise measurements of individual seed image characteristics, such as: size, colour and shape. When hue is measured through the DIA the value for yellow seed is placed between the values for green and brown seed. Alfalfa seed lots always contain a percentage brown and green seed, therefore the average hue does not graphically describe seed colour, but rather is a measure of the tendency of a seed lot toward green (immaturity) or brown (weathering) seed

colour. Therefore, the variability in seed colour (hue) of a seed lot may be a more appropriate measurement of seed quality and market value of the seed. Sub-plot seed samples (150 seeds) from this experiment were screened through the DIA for individual seed size and colour, and average values and standard deviations were calculated. Average seed colour (hue) was influenced by the environment (Table 4.4.4.1), but cultivar did not influence average seed hue.

Table 4.4.4.1. Analysis of variance for seed colour, measured as hue (0-255 scale) through Digital Image Analysis, of nine alfalfa cultivars grown in seed quality experiments under various environments and two establishment practices in southern Manitoba during 1992-1994.

Source	d.f.	MS	F value	
Environment	6	275.7	47.0	***
Establishment	1	1.9	0.4	NS
Cultivar	8	0.9	0.3	NS
Env x Cv	48	3.4	1.4	NS
Estb x Cv	8	1.7	0.7	NS

*, **, *** Significance at P≤0.05, 0.01, and 0.001.

The highest value for hue (high values lean toward green colour) was observed in HW-1993-Y0 and GL-1993-Y0. These two environments also had extremely low yields.

A correlation analysis was conducted to establish a relationship between conventional seed quality characteristics (seed weight, percentage germination,

hard and non-viable seed) and seed characteristics measured through DIA. Pearson correlation coefficients were calculated with sub-plots as units of observation (Table 4.4.4.2).

Table 4.4.4.2. Correlation coefficients between seed quality characteristics and seed characteristics measured through DIA

Variable		Average Size	$\sigma^{\dagger}$ size	Average hue	$\sigma^{\dagger}$ hue
seed weight	r‡	0.211**	0.000 NS	-0.042 NS	-0.271***
	N§	449	449	449	448
% non-viable	r	-0.112*	0.119*	-0.154***	0.467***
	N	452	452	452	451
% germinable	r	-0.050 NS	0.261***	-0.308***	0.557***
	N	452	452	452	451
% hard	r	0.304***	-0.250***	0.304***	-0.677***
	N	452	452	452	451

*, **, *** Significance at P≤0.05, 0.01, and 0.001.
† - Standard deviation.
‡ - Pearson correlation coefficient.
§ - Sub-plot seed samples were used as units of observation.

Seed size, measured as the area of seed image, was positively correlated to seed weight (r = 0.211**, N = 449). Although significant, this correlation coefficient was much smaller than correlation coefficients that have been observed on seed produced under controlled environments (r > 0.9, unpublished data). A correlation between seed weight and seed size (based on sieve diameter) of r = 0.991 (d.f. 7) was reported by Pedersen and Barnes (1973). Furthermore, correlation coefficients between seed size and seed

weight were considerably higher for more favourable environments (AB-1993-Y1 and HW-1994-Y1). This indicated that less favourable environments produced more flat or less dense seed, which resulted in greater discrepancy between seed weight and seed size.

The very high number of observation increased the chances for a significant correlation without actual biological significance. The correlation coefficients between variability (σ) of seed hue and most conventional seed quality parameters were both significant and numerically high. The variability of seed hue was negatively correlated to the percentage hard seed and positively correlated to the percentage germination and non-viable seed. These results agreed with previous reports indicating that both green and brown seed contained lower percentage hard seed than yellow seed (Staker, 1925). Hard seed develop at later stages of seed maturation, after maximum dry matter accumulation is attained, which means that a portion of immature seed may develop into viable seed without hard seededness. Weathering, sunlight and frost injury cause brown coloration of seed and are known to reduce percentage hard seed. In both cases the extremes will result in non-viable seed, either from immaturity or extensive injury. These results, combined with the fact that fall dormant cultivars had a more synchronous flowering pattern and higher percentage hard seed, suggested a possible inter-relationship between flowering pattern, variability in seed colour (maturity) and percentage hard seed.

In conclusion, over seven location-years provided a wide range of seed yields with the highest yields consistently recorded for 'Arrow', 'Algonquin', 'Cimmaron VR', 'Rangelander' and 'Florida 77', respectively. All seed quality characteristics were under a strong influence of the environment. Seed produced in the establishment year contained higher percentage non-viable seed, indicating that both seed yield and seed quality may be affected in an establishment year alfalfa seed production system. Cultivars had significant effect on seed weight and percentage hard seed. The most fall dormant cultivar, Rangelander, consistently developed the highest percentage hard seed and the lowest seed weight. The characteristics of alfalfa seed image measured through DIA were related to conventional seed quality parameters, therefore indicating that DIA has potential for developing alternative or supplementary measurements of alfalfa seed quality.

V. INHERITANCE OF ALFALFA SEED SIZE: Quantitative Analysis and Response to Selection

5.1. ABSTRACT

A model of the genetic control of alfalfa seed size is needed to design a breeding program that specifically target seed size. The main objective of this research was to estimate the components of genetic variance involved in the inheritance of alfalfa seed size. An additional objective was to determine the most efficient selection method to increase alfalfa seed size. Three selection methods, differing in parental control and selection pressure, were used to determine response to selection. The results of this research indicated that alfalfa seed size was influenced by the seed parent genotype to a greater extent than by the genotype of the embryo comprising the seed. Seed size was controlled by both additive and non-additive components of genetic variance. Parental control appeared to be essential for successful selection for seed size and could not be compensated by increased selection pressure. Alfalfa seed size was found to be a highly heritable trait for the reference population according to both estimated and realized heritability. These results suggest that there is considerable potential for improvement for seed size in alfalfa.

5.2. INTRODUCTION

The relationships between alfalfa seed size, seedling vigour, and subsequent forage yield, have stimulated considerable research interest over the past 50 years. Previous research results indicated that there was no consistent relationship between these traits. The results ranged from a strong correlation among these traits to no correlation at all, but the apparent contradictions may have largely resulted from seedling vigour being scored at different maturity stages. Black (1959) summarized data for most agricultural plants and concluded that early growth and development was related to seed size, but harvestable yield was often not related to seed size. As expected, species with larger seed were able to emerge from greater depths than small seeded species.

In alfalfa, a high correlation between seed size and seedling vigour was demonstrated by Beveridge and Wilsie (1959), and the advantage of larger seed increased with seeding depth (Erickson, 1946). Other researchers have reported only a weak relationship between seed size and seedling vigour (Nel and Burgers, 1968), and that relationship diminished with plant age (Smith, 1961). Carnahan (1963) reported a positive correlation between alfalfa seed weight and unifoliate leaf area, and unifoliate leaf area and seedling height at 4 weeks, but there was no direct correlation between seed size and seedling height. In spite of the interest in alfalfa seed size, there has been limited

research on the genetic control of this trait, and no reports on the selection response for seed size. Several studies, mostly using diallel crossing designs, have investigated the genetic basis of seed set (Singh, 1978; Rice and Gray, 1969, Michaud and Busbice, 1978), but few dealt with the genetics of seed size.

Pedersen and Barnes (1973) postulated that since alfalfa seed has essentially no endosperm, seed size should reflect only embryo size and therefore could serve as an indication of seed hybridity. They reported a positive relationship between seed size and seed hybridity. In their results, hybrid seed (F_1) were approximately 5% larger than sibbed seed, and sibbed seed were approximately 5% larger than selfed (S_1) seed. Dunbier and Bingham (1975) suggested that heterozygosity was important in conditioning the maximum performance for seed weight. They found the highest seed weight in populations with the highest proportion of tri- and tetra-allelic loci. In contrast to these results, Bowley (1980) found that seed weight was greater in S_1 than in F_1 seed. He suggested that reduced fertilization caused by self-incompatibility may have reduced the number of S_1 seed and resulted in less within-pod competition.

More recently, a model of genetic control of seed weight was established by Peterson and Barnes (1982). Their results suggested that seed weight was primarily controlled by additive gene action, and seedling vigour was primarily controlled by non-additive gene action. They also reported a significant

maternal (seed parent) effect on seed weight. Subsequent research (Katepa-Mupondwa *et al.*, 1995) also showed that the influence of the pollen parent on seed weight was less consistent than the influence of the seed parent. Expected response to selection for seed weight was assumed to be higher when selection was performed among seed parent families than among pollen parent families.

There have been reports on the selection response for seed size in other herbage legumes. In three cycles of selection for large seed in birdsfoot trefoil (*Lotus corniculatus L.*) the average gain per cycle ranged between 6.25-20% (Draper and Wilsie, 1965). Previous genetic studies on alfalfa seed size relied on measurements taken on seed developed on the seed parent involved in the crossing design, which may have biased the results. The very strong seed parent effect and inconsistent influence of the pollen parent suggests that genetic expression for seed size may need to be measured on the seed developed on the progeny plants.

The objective of this research was to estimate the components of genetic variance involved in the inheritance of alfalfa seed size. In addition, three selection methods were conducted to determine the most efficient selection method to increase alfalfa seed size.

5.3. MATERIAL AND METHODS

5.3.1. Quantitative analysis

A controlled crossing study was designed to estimate the components of genetic variance for alfalfa seed size. The reference population for this research was 'BIC-7WH' (Barnes *et al.*, 1977), a broad based germplasm developed for experimental purposes at the USDA-ARS Research Station, Beltsville, Maryland. Twenty-four plants, 3 sets x (4 seed parents + 4 pollen parents) were randomly selected from an initial population of BIC-7WH and arranged in a crossing design (N.C. Design II; Hallauer, A.R. and J.B. Miranda Fo, 1981) under growthroom conditions (Table 5.3.2.1). Three racemes were cross-pollinated, with vacuum emasculation, for each cross at five crossing dates. Crossing dates were used as replications. All seed produced by cross-pollination was harvested and individually screened through computer digital image analysis (DIA). Seed size was measured as the area of seed image.

Five full-sib progeny per cross (3 x 4 x 4 = 48 crosses), totalling 240 plants were randomized in five replications, with one full-sib from each cross per block. All plants were crossed with emasculation to a single pollen parent plant and also self-pollinated. Seed from individual full-sibs were harvested and screened through DIA for genetic analysis for seed size.

5.3.2. Response to selection

Three selection methods, differing in parental control and selection pressure, were applied on an alfalfa population to determine the most efficient selection method to increase seed size.

The first selection method allowed control over both the selected seed and pollen parent (full control) and utilized a 10% selection pressure for both large and small seed. One hundred and fifty randomly selected plants from the germplasm BIC-7WH were seeded under growthroom conditions (Table 5.3.2.1). One hundred and twenty plants produced a sufficient number of seed (≥150) for subsequent screening through the DIA for seed size. Twelve plants were identified as large seeded (LS) and 12 plants as small seeded (SS), based on average seed size. These plants were placed back in the growthroom and then randomly intercrossed (within each selected population) without emasculation and seed was harvested.

The second selection method allowed control over only the selected seed parents (half control) with a 10% selection pressure for both large and small seed. The mature seed collected from 12 LS and 12 SS plants were used for this selection method. For both LS and SS populations, families containing 8 half-sib progeny from each selected plant (12 x 8 = 96 plants) were randomly intercrossed in a greenhouse (Table 5.3.2.1), without emasculation and seed was harvested.

The third selection method was a form of mass selection with no control over the selected parents and a selection pressure of less than 1% for both large and small seed. A representative seed lot from BIC-7WH was mechanically separated using a series of dockage screens of varying sizes (Seedburo Equipment Co., Chicago, IL). The largest seed fraction (screen size 1.8 mm) and smallest seed fraction (1.0 mm) were seeded in the growthroom (Table 5.3.2.1) (25 plants each from the LS and SS population). Plants were randomly intercrossed without emasculation and seed was harvested.

The three selection methods produced the following six experimental populations: large seed full control (LSFC), small seed full control (SSFC), large seed half control (LSHC), small seed half control (SSHC), large seed no control (LSNC) and small seed no control (SSNC). A greenhouse experiment was established using the 6 selected populations and the initial population BIC-7WH in order to determine the success of different selection schemes. The experimental design was a completely randomized block design (RCBD), with 9 replications and 4 plants per plot. The plants were randomly intercrossed without emasculation within each respective population. Seed was harvested and samples of approximately 300 seeds per plot were screened through DIA for seed size. Seed weight was also determined on a seed sample of 150 seeds per plot.

The following methods, growing conditions and practices (Table 5.3.2.1) apply to all stages of the above experiments.

Table 5.3.2.1. Materials, growing conditions and methods used for research on quantitative analysis and selection response for seed size in alfalfa.

Materials and methods	Description
Growthroom conditions	18 h light (approx. 400 $\mu E\ m^{-2}\ s^{-1}$) - 6 h dark; 25°C-18°C; 15-35% relative humidity (light-dark, respectively).
Greenhouse conditions	Constant 22±2°C; additional light to 17 h when needed.
Growing medium	Soil:sand:pith:metromix (2:1:1:1 by volume) in 0.5 L milk-cartons.
Fertilizers	Initially 4 g NPK (10-48-0) per 1 L of growing medium, additional soluble NPK (10:20:0 + micronutrients) 5 mL L^{-1} water every 3 weeks while watering.
Watering	2-3 times a week until saturated.
Pest control	Decis or Trumpet once a week to control thrips and spider mites.
Planting	Seeding three seeds per container, then thinning to one at 1st trifoliate leaf.
Management	Cutting at 50% bud stage an crossing on the second regrowth to ensure better flowering synchronization. The average generation cycle including cutting was 19 weeks.
Controlled crossing	With vacuum emasculation, vacuum tube sterilized between two crossings with 96% ethanol, pollen collected with folded cardboard, standard petal partly removed.
Open pollination	Without emasculation using flat toothpick.
Self-pollination	Gently rolling racemes between fingers.
Seed harvest	Manual.
Seed threshing	Manual.
Digital image analysis	Image processing system designed around a DT-2871 (HSI) true colour frame grabber (Data Translation Inc., Marlboro, MA). Measurement software ImageX (Dr. L. Lamari; Dept. Plant Science, Univ. Manitoba)

The analysis of variance was calculated with mean squares pooled over sets of parents as proposed for N.C. Design II (Hallauer and Miranda Fo, 1981). The components of genetic variance were calculated using the model for a diploid species (Table 5.3.2.2). The diploid model is appropriate to determine the short term heritability for a tetraploid species, but is limited because it slightly underestimates the dominant genetic variance (Levings and Dudley, 1963).

Table 5.3.2.2. Half-sib and full-sib covariances expressed as functions of the components of genetic variance, for diploid and autotetraploid inheritance (from Levings and Dudley, 1963).

Genetic covariance	Ploidy	Coefficients of components of genetic variance						
		σ^2_A †	σ^2_D	σ^2_T	σ^2_F	σ^2_{AA}	σ^2_{AD}	σ^2_{DD}
Half-sib	2n	1/4	0	-	-	1/16	0	0
	4n	1/4	1/36	0	0	1/16	1/144	1/1296
Full-sib	2n	1/2	1/4	-	-	1/4	1/8	1/16
	4n	1/2	2/9	1/12	1/36	1/4	1/9	4/81

† σ^2_A - additive/monoallelic variance (V), σ^2_D - dominant/diallelic V, σ^2_T - triallelic V, σ^2_F - tetraallelic V, σ^2_{AA} - additive*additive interaction V, σ^2_{AD} - additive*dominant interaction V,
σ^2_{DD} - dominant*dominant V.

Realised heritability was calculated as suggested by Hill (1971). Statistical analysis was performed using the Statistical Analysis System (SAS, 1988) GLM procedure. Mean separation analysis used Fisher's protected least significant difference test.

5.4. RESULTS AND DISCUSSION

5.4.1. Quantitative analysis

Size of the seed developed on the seed parents (females) from the mating design experiment (N.C. Design II) was not correlated with the size of the seed developed on the progeny crossed to a common pollen parent (r=0.256 NS; N=44). There was a significant seed parent effect on seed size, but the pollen parent (male) did not influence seed size (Table 5.4.1.1). There was also an influence of crossing dates on seed size, which were used as replication in this experiment.

Table 5.4.1.1. ANOVA table for seed size, N.C. Design II random model, BIC-7-Wh used as reference population.

Source	d.f.	MS	d.f.	MS
	Parental	plants	Progeny	plants
Set	2	5.571 NS	2	0.273 NS
Rep(Set)	12	0.385 ***	12	0.026 NS
Female(Set)	9	1.416 ***	9	0.476 ***
Male(Set)	9	0.101 NS	9	0.223 *
Female*Male(Set)	27	0.065 NS	23	0.075 **
Error	161	0.058	116	0.034

*, **, *** - Significance at P≤0.05, 0.01 and 0.001.

When size was measured for seed produced on the progeny plants (from mating design crosses) there was a seed parent and a pollen parent effect on seed size and an interaction between the two. Replications in this case were

represented with five full sibs and did not have significant effect. Self-pollination on the progeny plants was followed by very low seed set, which caused reduced number of observations and very high experimental error.

Design II analysis provides two independent estimates of additive variance and h^2, since two sets of half-sibs are produced, through both female and male parents. Higher estimates for h^2 are expected through female parent half-sibs due to the effect of the seed parent. The h^2 estimates calculated from this research confirmed a positive seed parent effect with $h^2_{(f)}$=98.0% vs. $h^2_{(m)}$=62.4% (Table 5.4.1.3). The discrepancy between h^2 estimates through the female and male half sibs was smaller when seed was measured on the progeny ($h^2_{(f)}$=89.9% vs.d $h^2_{(m)}$=76.3%).

Table 5.4.1.3. Estimates of components of genetic variance and heritability for alfalfa seed size using N.C. Design II and BIC-7WH as a reference population.

Component of genetic variance	Parental plants	σ^2 of estimate	Progeny plants	σ^2 of estimate
$\sigma^2_{A(f)}$ †	0.300	0.028	0.130	0.022
$\sigma^2_{A(m)}$ ‡	0.008	0.003	0.049	0.011
σ^2_D §	0.006	0.010	0.046	0.021
$h^2_{(f)}$ ¶	98.0%		89.9%	
$h^2_{(m)}$ ††	62.4%		76.3%	

† $\sigma^2_{A(f)}$ - additive genetic variance estimated through female parent.
‡ $\sigma^2_{A(m)}$ - additive genetic variance estimated through male parent.
§ σ^2_D - dominant genetic variance.
¶ $h^2_{(f)}$ - narrow sense heritability estimated through female parent.
†† $h^2_{(m)}$ - narrow sense heritability estimated through male parent.

Alfalfa seed size appeared to be a highly heritable trait ($h^2_{(f)}$=89.9%; $h^2_{(m)}$=76.3). Components of genetic variance, when estimated on parental plants, showed that only $\sigma^2_{A(f)}$ had a significant role in determining seed size, which would be expected with a maternally inherited trait. When seed size was measured on the progeny plants, both additive and non additive components of genetic variance were found to be involved in the inheritance of the seed size.

Reports from previous controlled environment research indicate that the seed parent effect had the greatest influence in determining alfalfa seed size, but these genetic studies were based on seed developed on parental plants involved in crossing designs. The results of this research suggested that it is not applicable to measure the genetic expression for seed size on the parental plants involved in the crossing design, but seed size should be measured on the progeny plants. In other words, seed size is controlled by maternal plant genotype to greater extend then by the genotype of the embryo comprising that seed.

When studying the genetics of seed size in alfalfa it is important to consider the relationship between self-pollination and seed size. Pedersen and Barnes (1973) indicated that selfing resulted in smaller seed. They suggested that it may be possible to increase the portion of outcrossed (F_1) seed by removing the smallest fraction of seed. This type of technique would provide an economic method to increase the percentage of F_1 seed in a hybrid seed lot.

In this experiment, self-pollination did not have a consistent effect on the size of resulting S_1 seed. Measurements of seed size after cross- and self-pollination on 48 individual plants showed that selfed (S_1) seed was significantly larger in certain crosses, but also smaller than F_1 seed in other crosses. There was a significant correlation ($r=0.727^{***}$, $N=46$) between S_1 and F_1 seed developed on individual plants, further confirming the importance of the seed parent in determining seed size.

5.4.2. Response to selection

In order to develop seed sampling techniques, seed from 18 plants was harvested and the following information was recorded for each individual seed: (1) seed parent, (2) number and position of pod on raceme, and (3) number and position of seed in the pod. The analysis showed that the seed parent had the greatest influence on seed variability, while other factors had inconsistent effects (data not shown). It was assumed that sampling a large number of individual seeds per plant or all seed produced on a plant would effectively compensate for any sampling error. Therefore, the sample size was set at 150 to 350 seeds. This range provided a sufficiently precise estimate of the mean value for seed size on an individual plant.

Based on individual plant means, the initial base population (120 plants, BIC 7 WH) had an average seed size of 2.633 mm^2 and standard deviation of 0.236, with a slight skew toward the higher values. The selected population,

large and small seeded, had an average of 3.058 mm^2 and 2.296 mm^2, or selection differential of 0.425 mm^2 and 0.337 mm^2, respectively.

After one cycle of selection, the three selection methods had attained different selection gains (Table 5.4.2.1.). Selection methods FC-10% and NC-1% provided a significant separation between the large and small seeded selected population.

Table 5.4.2.1. Average seed size (mm^2) and seed weight (g/100 seeds) for selected populations and unselected BIC-7WH (RCBD, 9 reps).

	Seed size mm^2				Seed weight	
Population	Mean		Gain	Realized h^2	Mean	
LSFC†	3.029	*a*‡	0.244	57.3%	0.291	*a*
LSNC	2.897	*ab*	0.094	N/A	0.281	*ab*
LSHC	2.822	*bc*	0.019	8.6%	0.268	*bc*
BIC-7WH	2.785	*bc*			0.261	*bc*
SSFC	2.710	*c*	-0.095	22.4%	0.259	*c*
SSHC	2.698	*cd*	-0.106	26.0%	0.250	*cd*
SSNC	2.526	*d*	-0.277	N/A	0.237	*d*

† - LSFC (large seed full control), SSFC (small seed full control, LSHC (large seed half control), SSHC (small seed half control), LSNC (large seed no control), SSNC (small seed no control, and BIC-7WH (unselected population)
‡ - Population means followed by the same letter are not significantly different according to Fisher's protected LSD test at $P \leq 0.05$.

There was a significant shift for seed size between the original population (BIC-7WH) and FC-10% for large seed (LSFC), and NC-1% for small seed (SSNC). The maximum realized heritability for increased seed size was 57.3% (LSFC), confirming that alfalfa seed size is a highly heritable trait. The

differences for realized gain from different selection methods suggest that parental control was very important for the success of selection. Theoretically, HC-10% should have shown approximately half as much gain for seed size as FC-10%, since these populations only differed in parental control (full control=1, half control=½) (Fehr, 1987). The intense selection pressure of <1% applied in NC-1% did not compensate for lack of parental control for large seed. However, NC-1% did show considerable gain for small seed size.

Seed weight was strongly associated with seed size, and therefore the selected population showed the same differences for seed weight as did for seed size (Table 5.4.2.1). The correlation coefficient based on the selected population means was r=0.978*** (N=7). A similar strong association (r=991***, d.f. 7) between seed weight and seed size, given as sieve diameter, was previously reported by Pedersen and Barnes (1973).

The apparent differences in selection gain for large and small seed within the same selection method could be attributed to several factors, including: non-normal distribution of the initial population, and/or possibly different inheritance mechanisms associated with the large and small seed trait. Heterozygosity, or the presence of tri- and tetra-allelic loci, has been reported to maximize seed size (Dunbier and Bingham, 1975). Therefore, it would be very unlikely that heterozygosity, usually associated with vigour, could act toward reducing seed size. A plausible hypothesis is that only additive genetic variance determines small seed and that both additive and non-additive genetic

variance (heterozygosity) determines large seed, and may explain the seed size skewness of the initial population toward high values.

In conclusion, this research indicated that it was more appropriate to measure genetic expression for seed size on the progeny plants than on the mating parental plants. Alfalfa seed size was shown to be a highly heritable trait and selection for increased seed size was effective.

VI. IDENTIFICATION OF CROSS-POLLINATED AND SELF-POLLINATED PROGENY IN ALFALFA THROUGH *RAPD* NULLIPLEX LOCI ANALYSIS

6.1. ABSTRACT

An efficient method to estimate outcrossing rates is needed to facilitate development of hybrid alfalfa cultivars. A method termed RAPD nulliplex loci analysis distinguishes between F_1 and S_1 progeny. Marker selection is based on polymorphism between a seed parent and its bulk open-pollination progeny. Polymorphic markers automatically identify the seed parent as nulliplex (aaaa) for that particular loci, which allows the individual open-pollinated progeny from that seed parent to be classified as F_1 or S_1 progeny based on the presence/absence of these markers. The objective of this research was to demonstrate the applicability of RAPD nulliplex analysis to estimate outcrossing/selfing rates in autotetraploid alfalfa. Two alfalfa genotypes served as seed parents in crossing studies which differed in pollination control and number of pollen parents. The RAPD nulliplex analysis was conducted on these two seed parents and their progeny. Controlled crossing and selfing provided independent control over S_1s and F_1s. Five polymorphic markers were identified for both seed parents and these markers were sufficient to identify S_1 and F_1 progeny. In conclusion, RAPD nulliplex loci analysis can provide accurate identification of F_1 and S_1 progeny and estimation of outcrossing rates in autotetraploid alfalfa.

6.2. INTRODUCTION

Alfalfa *(Medicago sativa* L.) is currently cultivated on more than 32 million hectares worldwide (Michaud *et al.*, 1988) and is considered the most important forage crop in the world. Surprisingly, improvement in the genetic yield potential of alfalfa has been relatively small in comparison to the majority of cultivated crop species. Hill *et al.* (1988) reported that there was only a 3% increase in the genetic yield potential of alfalfa between 1956 and 1974. All current alfalfa cultivars are synthetic populations created by intercrossing selected parents and advancing their offspring through a limited number of generations of random mating (usually three). The first generation of a synthetic cultivar, developed from unrelated parents, usually shows a dramatic improvement in yield potential. This initial yield improvement is attributed to heterosis, but the heterotic effect declines during each successive generation of seed increase (Bowley and McKersie, 1992). The inability to maintain this initial heterotic effect in traditional synthetic cultivars has encouraged breeders to consider the development of hybrid alfalfa cultivars.

The development of a successful hybrid alfalfa cultivar requires an efficient pollination control system. A number of methods have been proposed for controlling pollination in hybrid alfalfa seed production including: (1) self-incompatibility (Tysdal and Kiesselbach, 1944), (2) cytoplasmic male sterility (CMS) (Barnes *et al.*, 1972), and (3) male- or self-sterile seed parents and

female-sterile pollen parents (Brown and Bingham, 1984). Since environmental conditions are extremely important for the stability of the CMS system (Viands *et al.* 1988) and the self-incompatibility system (Barnes *et al.*, 1972), there are major concerns as to whether either system will provide complete control over pollination in alfalfa. In a more recent study, Campbell and Bauchan (1990) reported that it was possible to select alfalfa genotypes with consistently high levels of self-incompatibility and concluded that the self-incompatibility system had potential for hybrid alfalfa seed production.

Since none of the proposed pollination systems provides 100% pollination control, an efficient method to estimate the percentage of outcrossing/selfing is needed to facilitate the development of hybrid alfalfa cultivars. Such a method could be used as follows: (1) to select stable self-incompatible genotypes; (2) to control the stability of the CMS system; or (3) to accurately determine outcrossing/selfing rates when the level of pollination control is not complete or is questionable.

Simply inherited phenotypic traits, (Barnes and Hanson, 1967) provide one method to distinguish outcrossing from selfed progeny. Flower colour has been used in most studies that estimated outcrossing rates in alfalfa (reviewed by Steiner *et al.*, 1992), despite the fact that pollinators preferentially visit certain colour of flowers and therefore bias selfing rates. The practical use of phenotypic markers is limited by a number of factors. They must be introduced into the breeding material and may not contribute to the merit of hybrid cultivar.

Progeny populations must be grown to the stage of maturity that will allow the marker to be scored. Finally, since S_1 progeny are less viable, growing the progeny to maturity may bias any estimates in favour of the F_1 progeny.

Another method to determine outcrossing/selfing rates was proposed by Pedersen and Barnes (1973). They demonstrated a strong relationship between seed size and outcrossing rate in alfalfa and proposed that the percentage of hybrid (F_1) seed could be estimated through seed size. Subsequently, Charlesworth (1988) also described a method for estimating outcrossing rates in natural plant populations based on traits such as seed size and seed viability. He found that these traits were useful because they could be scored relatively soon after pollination. Conversely, other researchers have reported an inconsistent relationship between seed size and the presence of S_1 seed (Bowley, 1980; Gjuric and Smith, unpublished), therefore raising questions about the validity of estimating outcrossing rates based on seed size.

In recent years, molecular markers have proven to be a useful method for determining outcrossing rates in plants. Knapp and Teuber (1993) used allozyme markers and 'TETRAT' software (Ritland, 1990) to estimate outcrossing rates in populations selected for an "easy-to-trip" trait. Newer molecular markers including RFLPs (restriction fragment length polymorphism) and RAPDs (random amplified polymorphic DNA) have been used to map the alfalfa genome (Brummer *et al.*, 1994), but mapping was done on diploid populations and therefore was not directly transferable to cultivated tetraploid

alfalfa. Neither RFLPs or RAPDs have been used to estimate outcrossing rates in alfalfa.

RFLP markers require the use of Southern blots which are often laborious, time-consuming, and expensive. Therefore, it would be difficult to justify screening large numbers of plants for RFLPs to estimate outcrossing rates. RAPD markers (Williams *et al.*, 1990) are obtained by PCR amplification of random DNA segments from single arbitrary primers. Since RAPDs require minimum target DNA quantity and tolerate crude extraction their use is technically very simple (Waugh and Powell, 1992; Yu *et al.*, 1993). The limitation to the use of RAPDs is that they are dominant markers. Only two phenotypes can be distinguished, dominant (+) and null (-), and allelic variants cannot be detected. In an autotetraploid crop species like alfalfa, four out of five possible genotypes (A...) on a single tetrasomic locus have the same RAPD phenotype (+), and the nulliplex genotype (aaaa) is the only one that can be scored directly from the (-) phenotype. Dominant Mendelian inheritance of RAPDs was reported in diploid alfalfa (Echt *et al.*, 1991), and dominant tetrasomic inheritance was reported in autotetraploid potato (Quiros *et al.*, 1993). Yu and Pauls (1993) analyzed the segregation of RAPDs in tetraploid alfalfa. Their results indicated that random chromosome segregation was likely the predominant, but not exclusive mode of inheritance in alfalfa. The level of double reduction, typical for chromatid segregation, was very low (1 out of 121). They also reported the first molecular linkage groups in tetraploid alfalfa.

The objective of this research was to determine if RAPD markers can be used to estimate outcrossing/selfing rates in autotetraploid alfalfa through the proposed RAPD nulliplex loci analysis.

6.3. MATERIALS AND METHODS

6.3.1. RAPD nulliplex loci analysis

'RAPD nulliplex loci analysis' has been proposed as an accurate analysis method to identify individual F_1 progeny. The remaining progeny is then considered S_1 based on a probability which depends on the number of markers used. When this analysis method is used to estimate outcrossing rates, it gives the minimum outcrossing rate, but total outcrossing rate can be estimated with a very high degree of probability.

Marker selection for RAPD nulliplex loci analysis is based on the identification of RAPD polymorphism between the seed parent (SP) and its bulked open pollination progeny (OPP). Polymorphic markers are identified that are present (+) in the bulk OPP and absent (-) in the SP, therefore automatically identifying the SP as nulliplex for that particular loci. The other type of polymorphism, SP (+) and bulk OPP (-), would be impossible because if the marker is present in the SP at least 50% of the individuals in the OPP will also carry that marker.

Individual OPP progeny are screened for the presence/absence of selected markers. Self-pollination of a nulliplex SP will always give nulliplex (-) progeny for all markers, while outcrossing may give (+) or (-) for individual markers depending on the pollen parent genotype. An individual OPP is classified as an F_1 progeny, if it has (+) phenotype for any of the selected markers. Therefore, for any number of loci, identified F_1 progeny represent the minimum portion of total F_1 progeny. When the number of markers is increased the proportion of F_1 progeny that can be accurately identified subsequently increases. The following model is used to calculate the number of nulliplex loci needed to identify an individual as S_1 progeny with a certain probability.

The genotypic array of the cross-pollinated progeny can be derived from the seed parent genotype and the genotypes of the pollen parents with their genotypic frequencies (Table 6.3.1.1).

Table 6.3.1.1. Seed parent and pollen parent genotypes and genotypic frequencies. Seed parent nulliplex, pollen parents represent the whole random mating population.

Seed parent	Pollen parent			
	Genotype	Frequencies	Gametic array	Segregation
aaaa	AAAA	p^4	AA	all A...
	AAAa	$4p^3q$	AA+Aa	all A...
	AAaa	$6p^2q^2$	AA+4Aa+aa	5:1 A...:aaaa
	Aaaa	$4q^3p$	Aa+aa	1:1 A...:aaaa
	aaaa	q^4	aa	all aaaa

Assuming a nulliplex seed parent and pollen parents represented with the whole random mating population, it is possible to calculate the proportion of nulliplex F_1 progenies. In other words, it is possible to calculate the maximum probability that an individual is nulliplex at that particular locus, but still F_1 progeny.

$P = q^4 \times 1 + 4pq^3 \times 1/2 + 6p^2q^2 \times 1/6$; (if p=0.5; then P=0.25).

Assuming no linkage, the above probability can be extended to any seed parent nulliplex locus. By combining the probabilities for independent loci, it is possible to calculate the maximum probability that an individual is nulliplex at 2, 3, 4, etc.. loci, but still F_1 progeny. That probability will depend on the allele frequencies and will asymptotically approach zero as the number of loci increases. Under common assumptions (no linkage, allele frequencies p=0.5 for all loci, random mating), if an individual OPP is nulliplex at five SP's nulliplex loci, the probability that this individual is still a F_1 progeny is less than 0.001.

6.3.2. Plant material

RAPD nulliplex loci analysis was demonstrated on two alfalfa seed parents and their progenies, randomly selected from different crossing studies. The first seed parent was the genotype BIC-7WH-S65 which was crossed with 4 pollen parents with vacuum emasculation. An additional number of S_1 progeny were produced by self-pollination, therefore allowing control over both

F_1 and S_1 progeny. The seed parent was reassembled from a bulked DNA sample which included 11 individual S_1 progenies. An advantage of RAPD analysis is that a bulked DNA sample has a RAPD pattern which is superposition of the individual samples included in the bulk (Yu *et al.*, 1993).

The second seed parent was a genotype randomly selected out of a winterhardy population of 'Alfagraze' and designated Alfagraze 36. Vegetative clones from this genotype were randomized in a polycross with clones from 30 other parents from the same population. In addition to random intercrossing (without emasculation), the clones of Alfagraze 36 were selfed, providing control over S_1 progeny. Open pollinated (OP) progeny contained a mixture of F_1 and S_1 progeny. DNA sampling for SP was done on the original Alfgraze 36 plant. With both seed parents, 21 individual DNA samples (F_1s with BIC-7WH-S65 or OPs with Alfagraze 36) were used to form the bulked OPP DNA sample.

6.3.3. DNA extraction and PCR conditions

A crude-DNA extraction protocol (Table 6.3.3.1) and specific PCR reaction conditions (Table 6.3.3.2) were used to screen arbitrary primers for polymorphic markers as well as to screen individual progeny with identified markers. Bulk DNA samples were made by combining equal volumes of DNA extract from individual samples.

Table 6.3.3.1. DNA extraction protocol (Edwards *et al.*, 1991; Yu *et al.*, 1993)

Step	Description
1	Add 400 µL extraction buffer into a 1.5 mL Eppendorf tube.
2	Drop a fresh leaflet or imbibed seed into the tube.
3	Homogenize tissue with a pestel that fits tightly in the tube.
4	Centrifuge for 1 min, and transfer 300 µL of the supernatant to a fresh tube.
5	Add one volume 300 µL isopropanol, allow precipitation of the DNA for 5 min, then centrifuge for 5 min.
6	Discard the supernatant and air dry the pellet completely.
7	Add 100 µL TE buffer (pH 7.5) and allow the DNA to dissolve for 10 min or longer.
8	Centrifuge the sample for 1 min, and collect the supernatant.

Extraction buffer	
Materials	Concentration
1 *M* Tris-HCl buffer Ph 7.5	200 m*M*
5 *M* NaCl	250 m*M*
0.5 EDTA	25 m*M*
10 % SDS	0.5 %

Table 6.3.3.2. PCR Reaction mixture 25 µL per reaction (Yu *et al.*, 1993).

Materials	Concentration
10X PCR Buffer†	
MgCl†	2 m*M*
dATP, dTTP, dCTP, dGTP‡	0.1 m*M* each
Primer§	0.4 µ*M*
DNA extract	2 µL/reaction
Taq polymerase†	2 units/reaction

† GibcoBRL, Life Technologies, Inc., Gaithersburg, MD.
‡ Promega, Madison, WI.
§ University of British Columbia.

PCR reactions were carried out in a 60 well model PTC-100™ programable thermal controller (MJ Research, Inc., Watertown, MA) for 45 cycles. Each cycle consisted of 1 min at 94°C; 1 min at 36°C; and 2 min at 72°C using the fastest available transition phase between each temperature. The PCR products plus electrophoretic mix (Table 6.3.3.3) were separated by electrophoresis using 1.2% agarose in 1 X TAE buffer. The bands were then detected with ethidium bromide staining [5µL(10mg/mL)/100mL] and their size determined using 'λ/Hind III' and 'pUC 19/Hin f1' size markers.

The DNA extraction protocol and PCR reaction conditions used in this research produced consistent RAPD patterns over multiple DNA sampling from the same plant material (leaf tissue). Each specific combination of DNA sample and arbitrary primer was repeated at least twice.

Table 6.3.3.3. Electrophoretic mix used for PCR products separation.

5X Electrophoretic Mix	
2 mL	10X Stop solution
4 mL	0.5% BPB†, 0.5% XC‡
2 mL	Glycerol
8 mL	
10X Stop Solution	
3.75 mL	filter sterilized ddH_2O
0.25 mL	10% SDS ([final])=0.5% SDS)
1.00 mL	250 mM EDTA ([final]=50mM EDTA)
5 mL	

† - Bromophenol blue.
‡ - Xylene cyonile.

6.4. RESULTS AND DISCUSSION

Eighty-five 10-mer arbitrary primers from the University of British Columbia (UBC) set no.4 (301-400) were screened with the BIC-7WH-S65 and its bulk OPP. There were only 4 primers which produced no bands. Three primers were identified that produced 5 polymorphic bands with one of these primers producing three polymorphic bands (Table 6.4.1). Only 30 primers from the same set were screened with Alfagraze 36 and its bulk OPP combination. Five primers were identified, each producing one polymorphic band (Table 6.4.2).

Fourteen cross-/open-pollinated progeny and 15 self-pollination progeny, not involved in bulks, were screened with the identified markers (Tables 6.4.1, 6.4.2). Only one individual (#4) from the cross-pollinated progeny of the of BIC-7WH-S65 was identified as an S_1 progeny. Considering that vacuum emasculation often does not provide 100% pollination control (Viands *et al.*, 1988), this individual was considered a genuine S_1 progeny. Almost all F_1 individuals (12/13) were identified with 3 markers amplified with a single primer (UBC 389). Among the 14 open pollinated progeny of Alfagraze 36, 10 were identified as F_1 (71.4%) and 4 as S_1 progeny. Crossing without emasculation usually results in lower outcrossing rates (approximately 85%) in comparison to crossing with emasculation (Barnes, 1980). Two markers, 0.822 kb (UBC 309) and 0.476 kb (UBC 400), were sufficient to identify all ten F_1 progeny. The

progeny produced by self-pollination from both seed parents were nulliplex for all selected polymorphic markers, therefore confirming that they were actually S_1 progeny.

According to our model, five RAPD markers will allow estimates of outcrossing/ selfing rates with very high degree of confidence within a random mating population. However, the number of markers could be increased or decreased, depending on the number of pollen parents and their genotypes. For example, pollen parents that were quadruplex or triplex (AAA.) for a phenotypic trait in combination with a nulliplex (aaaa) seed parent were used to estimate outcrossing rates in autotetraploid alfalfa (Campbell and Bauchan, 1990). This same technique could be applied for RAPD nulliplex analysis, consequently only a single marker would be sufficient for complete analysis. If the number of pollen parents was increased, especially with unknown genotypes, more markers would be needed for the analysis. The results of this research showed that for BIC-7WH-S65 only one F_1 progeny (#12) was identified by the presence of a single marker, compared to half of the F_1 progeny for Alfagraze 36 (Tables 6.4.1 and 6.4.2). This difference can be easily credited to the different number of potential pollen parents that fathered these two F_1 progenies (4 and 30, for BIC-7WH-S65 and Alfagraze 36, respectively) .

Identifying a sufficient number of polymorphic RAPD markers in alfalfa should not be a problem, as alfalfa is known as a polymorphic species for many

traits (Brummer *et al.*, 1994). Extensive RFLP polymorphism has been reported among alfalfa plants of the same population (Brummer *et al.*, 1991). In this research abundant RAPD marker variability was observed among individual alfalfa plants, suggesting that null (-) RAPD phenotypes frequently occur in alfalfa. This could be partly due to the inability of RAPDs to detect multiple allelism. Multiple allelism does not directly affect the analysis. In the case of multiple allelism the allele frequencies would be lower, which could be compensated with an increased number of markers. If the parental plants were closely related it would be very likely that more primers would need to be screened to identify a sufficient number of polymorphic markers. However, once polymorphic markers have been identified, analysis would not be affected by the degree of relatedness of the parents.

In conclusion, use of RAPD markers to estimate outcrossing rates in autotetraploid alfalfa has several advantages over the traditional approach using phenotypic markers. RAPD markers can be identified in almost any plant material and RAPD polymorphism is abundant among individual alfalfa plants. In addition, RAPD markers allow the use of simple and quick procedures for DNA extraction. Proposed RAPD nulliplex analysis provides accurate identification of F_1 and S_1 progeny and estimation of outcrossing rates in alfalfa crosses regardless the number of parents.

Table 6.4.1. Identification of alfalfa F_1 and S_1 progeny through RAPD nulliplex loci analysis on individual progeny plants of BIC-7WH-S65 following controlled cross-pollination and self-pollination.

Primer	Marker			Individual progeny plants of BIC-7WH-S65†																										
		O P	S	Cross-pollinated‡													Self-pollinated														
UBC	size kb	P§	P¶	1	2	3	4	5	6	7	8	9	10	11	12	13	14	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
309	1.968	+	-	+	+	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
389	1.188	+	-	+	-	-	-	-	-	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
389	0.939	+	-	-	+	-	-	+	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
389	0.736	+	-	-	+	+	-	-	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
400	0.941	+	-	-	-	+	-	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

† - BIC-7WH-S65 used as seed parent and crossed to 4 pollen parents.

‡ - Crossing with vacuum emasculation.

§ - OPP, bulk open pollinated progeny.

¶ - SP, seed parent reassembled with bulk self pollinated progeny.

Table 6.4.2. Identification of alfalfa F_1 and S_1 progeny through RAPD nulliplex loci analysis on individual progeny plants of Alfagraze 36 following open-pollination and controlled self-pollination.

Primer	Marker			Individual progeny plants of Alfagraze 36†																										
		O P	S	Open-pollinated‡														Self-pollinated												
UBC	size kb	P§	P¶	1	2	3	4	5	6	7	8	9	10	11	12	13	14	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
309	0.822	+	-	-	-	+	-	+	-	+	-	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
316	1.624	+	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
352	1.098	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
362	0.503	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
400	0.476	+	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

† - Alfagraze 36 used as seed parent and crossed to 30 pollen parents.

‡ - Crossing without emasculation.

§ - OPP, bulk open pollinated progeny.

¶ - SP, seed parent, DNA extracted from the vegetative tissue .

VII. GENERAL DISCUSSION

This research was comprised of three major studies, each designed to provide important information related to alfalfa seed quality. In addition, a number of small experiments were conducted to demonstrate the applicability of Digital Image Analysis (DIA) for assessment of alfalfa seed quality.

The study on the effect of cultivar and environment on alfalfa seed quality characteristics included seven alfalfa seed yield experiments in southern Manitoba, using alfalfa cultivars covering the whole range of fall-dormancy. These experiments included establishment year seed production and the traditional practice of harvesting seed in the second year of the stand. The results of these experiments were negatively affected by the poor environmental conditions for seed production in 1992 and 1993. Seed yields ranged from 1 to 250 kg ha^{-1}. The extremely low yields for these experiments were not surprising since during the years of this research many producers fields were not even harvested in Manitoba. Surprisingly, cultivar ranking for seed yield was consistent over all experiments, which may have implications for future cultivar testing for seed yield. In other words, it may be possible to conduct preliminary evaluation trials to determine the highest seed yielding alfalfa cultivars regardless of the environmental conditions.

Alfalfa seed quality was also strongly influenced by the environment. All cultivars produced very high percentage hard seed (up to 80% in certain experiments). As expected, fall-dormant cultivars produced higher percentage

hard seed than non-dormant cultivars, but all seed lots would have required scarification to reduce percentage hard seed to acceptable levels. If agronomic management practices are developed for non-dormant seed production in Western Canada, the high percentage hard seed may have negative marketing implications.

Establishment year seed production, which allows non-dormant alfalfa seed production, showed a high percentage of non-viable seed, or lower seed quality. These results should be considered within the specific environmental conditions during 1992 and 1993. An initial experiment conducted in 1991 showed that there is a potential of producing high yields (300-600 kg ha^{-1}) of high quality seed (percentage non-viable seed only 2-3%) in the establishment year. Establishment year alfalfa seed production may be associated with a higher risk related to decreasing both seed yield and seed quality. The future of such a production system will depend primarily on economic factors which include the benefits of non-dormant alfalfa in a short term crop rotations, price of the seed and price of the leafcutter bees.

The study on inheritance of alfalfa seed size was conducted under controlled environment using a specific mating design (N.C. Design II) and different selection procedures. This research showed that seed parent had a major role in determining seed size, therefore any genetic study of alfalfa seed size should rely on seed measurements on the progeny plants. These results will have a significant impact on future genetic studies and breeding procedures

for seed size, and consequently may represent the single most important finding provided by this research.

A new model of genetic control of alfalfa seed size was established, showing that both additive and non-additive components of genetic variance control alfalfa seed size. Response to selection for seed size has not been previously reported in alfalfa. In this research seed size was significantly improved after only one cycle of selection. First cycle of selection usually show the strongest response to selection, therefore improvement in subsequent cycles may be slower. This research suggests that alfalfa cultivars can be developed with lager seed size through a breeding program that specifically selects for seed size with full parental control and selection based on the progeny plants.

The last study developed a procedure termed 'RAPD nulliplex loci analysis' to provide an accurate method to estimate outcrossing rates in alfalfa. This method is based on the newer generation of DNA markers (Random Amplified Polymorphic DNA) and has several advantages over the existing methods. Existing phenotypic marker methods require presence and expression of a visible plant characteristics and isozyme methods require sampling at a specific growth stage. RAPD nulliplex loci analysis does not have any restrictions on the plant material and can even be conducted on seed. This method may have an application in hybrid alfalfa development and subsequent regulation of alfalfa hybrid seed production.

VIII. SUMMARY

A series of field experiments were conducted to determine the genotype and environment effect on alfalfa seed quality. Over seven location-years, with seed yields ranging from approximately 1 to 250 kg ha^{-1}, the highest yields were consistently recorded for five cultivars (Arrow, Algonquin, Cimmaron VR, Rangelander and Florida 77).

Previous reports on the effect of environment on percentage hard seed in alfalfa were based on survey type research, which did not account for the different management practices, harvesting equipment and seed cleaning equipment used in different regions. This research showed that a high percentage hard seed was produced under a specific set of environments in southern Manitoba during 1992-1994, and these were the first reports on the percentage hard seed in non-dormant alfalfa cultivars produced in Western Canada. Interestingly, the environments that were more favourable for seed production tended to produce higher percentage hard seed. This research also showed that higher percentage hard seed was found in seed lots with more uniform colour (maturity), in agreement with previous reports. These results question whether the high percentage hard seed produced under some environments should be considered a detrimental seed quality factor.

Controlled environment studies were conducted to study the inheritance of alfalfa seed size and response to selection for seed size. The results of this

research suggested that it was not applicable to measure the genetic expression for seed size on the parental plants involved in the mating design. This information may have a big impact on future genetic studies and breeding procedures for alfalfa seed size. When seed size was measured on seed developed on progeny plants both additive and non-additive genetic variance appeared to be involved in the inheritance of alfalfa seed size. This research suggests that alfalfa seed size is a highly heritable trait and could have a considerable potential for improvement. A significant deviation for seed size was obtained after one cycle of bidirectional selection.

This research demonstrated the potential application of measuring alfalfa seed characteristics through computer Digital Image Analysis (DIA). The DIA has the ability to precisely characterise images of various objects (in this case seeds) for size, shape and colour, and alfalfa seed size and colour are related to the conventional seed quality parameters. The DIA also provided rapid and accurate measurements of individual seed size, which allowed it to serve as useful research tool for the genetic and selection method research.

An important part of this research was devoted to development of a new method to estimate outcrossing/selfing rates in alfalfa. A method of random amplified polymorphic DNA (RAPD) nulliplex loci analysis was proposed and demonstrated. This method uses RAPD polymorphism between a given seed parent and its bulk open-pollinated progeny to identify a number of RAPD nulliplex loci on the seed parent. Open-pollinated progeny from the seed parent

is classified as self- (S_1) or cross-pollinated (F_1) progeny based on their genotype for these loci. This method is technically very simple, but provides accurate identification of F_1 and S_1 progeny and estimation of outcrossing/selfing rates in autotetraploid alfalfa.

In conclusion, these results indicate that there is a genetic effect for most of the alfalfa seed quality characteristics, but these effects are often masked by a combination of the environmental conditions and seed parent effect.

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APPENDICES

Appendix 1. Seed yield and seed quality measurements of eight alfalfa cultivars grown in Glenlea experimental station in 1991. Cultivar means averaged over two establishment treatments. (Source: Smith, 1992).

Cultivar	Measurements				
	Seed yield kg ha^{-1}	seed weight g/100 seed	hard seed % ‡	dead seed % ‡	germination % ‡
Florida 77	595.3 *bc*†	0.212 *abc*	73.7	2.0	24.3
CUF 101	464.4 *d*	0.221 *a*	75.4	2.9	21.4
Nitro	572.1 *bcd*	0.215 *ab*	73.1	2.1	24.8
Wilson	489.5 *cd*	0.199 *d*	77.5	2.5	20.0
Grande	607.7 *b*	0.210 *abcd*	73.6	2.5	23.9
Cimmaron VR	748.1 *a*	0.212 *ab*	77.3	2.7	20.0
Arrow	725.4 *a*	0.202 *abc*	73.6	1.8	24.6
Algonquin	539.9 *bcd*	0.205 *bcd*	74.0	2.6	23.4

† - Cultivar means followed by the same letter are not significantly different at 0.05.

‡ - Cultivar means were not significant.

Appendix 2. Plant height (cm) at 7 weeks after planting of nine alfalfa cultivars grown in seed quality experiments under different environments in southern Manitoba during 1992-1994. Cultivar means averaged over establishment treatments.

Cultivar	ENVIRONMENT						
	GL-1992-Y0	GL-1993-Y0	HW-1992-Y0	HW-1993-YO	AB-1993-Y0	AB-1993-Y1	§
Florida 77	21.4 *abc*† 5‡	30.9 *a* 6	30.8 *ab* 2	17.1 *abc* 4	17.5 *ab* 3	17.7 *a* 2	**3**
CUF 101	23.7 *a* 1	30.3 *a* 7	33.3 *a*	18.2 *a* 1	15.9 *abc* 5	15.6 *ab* 6	**1**
Nitro	23.7 *a* 2	31.6 *a* 3	29.8 *b*	15.1 *cd* 7	14.5 *bc* 8	16.2 *ab* 5	**5**
Moapa 69	22.6 *ab* 3	31.5 *a* 4	29.3 *b*	17.6 *ab* 2	17.6 *ab* 2	15.6 *ab* 7	**8**
Wilson	20.3 *bc* 7	31.6 *a* 2	29.2 *b*	15.6 *bcd* 6	15.6 *abc* 6	12.9 *b* 9	**7**
Cimmaron VR	21.3 *abc* 6	31.1 *a* 5	28.4 *bc*	17.4 *ab* 3	17.9 *a* 1	19.1 *a* 1	**4**
Arrow	21.9 *abc* 4	32.3 *a* 1	28.8 *bc*	16.9 *abc* 5	13.9 *c* 9	13.1 *b* 8	**6**
Algonquin	19.2 *cd* 8	28.9 *ab* 8	25.1 *d*	13.8 *d* 8	16.6 *abc* 4	16.4 *ab* 4	**8**
Rangelander	17.0 *d* 9	25.7 *b* 9	25.6 *cd*	11.0 *e* 9	15.4 *abc* 7	17.6 *a* 3	**9**

GL-Glenlea, HW-Homewood, AB-Arborg, Y0-establishment year seed production, Y1-seed production in the first year following establishment.

† - Cultivar means followed by the same letter are not significantly different at 0.05.

‡ - Cultivar rank within each environment.

§ - Cultivar rank over all environments.

Appendix 3. Plant height (cm) at nine weeks after planting of nine alfalfa cultivars grown in seed quality experiments under different environments in southern Manitoba during 1992-1994. Cultivar means averaged over establishment treatments.

Cultivar	ENVIRONMENT				
	GL-1992-Y0	GL-1993-Y0	HW-1992-Y0	HW-1993-YO	§
Florida 77	49.0 *b*† 7‡	47.7 *abc* 5	51.5 *ab* 2	35.5 *ab* 4	**3**
CUF 101	51.4 *ab* 2	46.2 *bcd* 7	54.7 *a* 1	37.7 *a* 1	**1**
Nitro	53.7 *b* 1	47.4 *abc* 4	51.4 *ab* 3	35.3 *ab* 7	**5**
Moapa 69	49.2 *b* 5	47.1 *bc* 6	47.6 *bcd* 6	37.3 *a* 2	**8**
Wilson	50.2 *ab* 6	50.2 *ab* 2	46.7 *bcd* 7	37.3 *a* 6	**7**
Cimmaron VR	51.1 *ab* 4	48.3 *abc* 3	50.1 *abc* 4	37.7 *a* 3	**4**
Arrow	41.4 *c* 3	51.9 *a* 1	50.0 *abc* 5	38.9 *a* 5	**6**
Algonquin	40.7 *c* 8	45.1 *cd* 8	42.9 *d* 9	31.8 *bc* 8	**8**
Rangelander	17.0 *d* 9	42.3 *d* 9	45.4 *cd* 8	29.5 *c* 9	**9**

GL-Glenlea, HW-Homewood, AB-Arborg, Y0-establishment year seed production, Y1-seed production in the first year following establishment.

† - Cultivar means followed by the same letter are not significantly different at 0.05.

‡ - Cultivar rank within each environment.

§ - Cultivar rank over all environments.

Appendix 4. Plant height (cm) at 11 weeks after planting of nine alfalfa cultivars grown in seed quality experiments under different environments in southern Manitoba during 1992-1994. Cultivar means averaged over establishment treatments.

Cultivar	ENVIRONMENT												
	GL-1992-Y0			GL-1993-Y0			HW-1992-Y0			HW-1993-YO			AB-1993-Y0			AB-1993-Y1			§
Florida 77	65.8	ab†	5‡	54.1	ab	2	58.4	ab	5	52.5	bc	5	44.4	ab	2	47.3	a	2	3
CUF 101	64.3	a	1	53.2	ab	3	62.2	a	1	52.3	bc	7	43.3	ab	5	43.5	ab	5	4
Nitro	63.5	a	3	55.5	a	4	59.9	a	3	50.3	c	8	39.9	ab	8	45.2	ab	8	6
Moapa 69	63.2	ab	4	53.5	ab	6	58.5	ab	4	54.1	bc	4	43.3	ab	4	41.0	bc	4	5
Wilson	63.4	ab	7	54.7	ab	5	55.0	ab	7	56.6	ab	3	39.4	b	9	35.1	d	9	7
Cimmaron VR	62.9	ab	6	55.6	a	7	57.4	ab	6	59.6	a	1	45.9	a	1	45.1	ab	1	1
Arrow	66.9	a	2	52.5	ab	1	59.9	a	2	59.3	a	2	41.8	ab	7	37.1	cd	7	2
Algonquin	60.2	b	9	49.9	bc	8	48.2	b	9	52.4	bc	6	42.5	ab	6	44.3	ab	6	8
Rangelander	56.1	ab	8	46.0	c	9	51.7	ab	8	49.9	c	9	43.6	ab	3	44.2	ab	3	9

GL-Glenlea, HW-Homewood, AB-Arborg, Y0-establishment year seed production, Y1-seed production in the first year following establishment.

† - Cultivar means followed by the same letter are not significantly different at 0.05.

‡ - Cultivar rank within each environment.

§ - Cultivar rank over all environments.

Appendix 5. Plant height (cm) at 13 weeks after planting of nine alfalfa cultivars grown in alfalfa seed quality studies under different environments in southern Manitoba during 1992-1994. Cultivar means averaged over establishment treatments.

Cultivar	ENVIRONMENT					
	GL-1992-Y0	HW-1992-Y0	HW-1993-YO	AB-1992-Y0	AB-1993-Y1	§
Florida 77	80.7 abc† 4‡	67.9 ab 4	70.2 b	64.6 a 1	62.5 abc 6	4
CUF 101	85.8 ab 3	72.2 a 1	73.1 b	60.6 a 7	61.9 abc 7	2
Nitro	80.5 abc 5	67.0 ab 6	6938 b	59.1 a 9	62.9 abc 4	6
Moapa 69	77.0 c 7	65.7 bc 7	70.6 b	62.3 a 5	62.6 abc 5	7
Wilson	86.1 a 2	67.3 ab 5	70.8 b	62.5 a 3	55.7 c 9	5
Cimmaron VR	79.6 bc 6	70.2 ab 3	79.9 a	64.1 a 2	65.1 ab 2	1
Arrow	86.2 a 1	72.1 a 2	79.4 a	59.5 a 8	57.6 bc 8	3
Algonquin	74.7 cd 8	61.1 c 9	72.0 b	60.9 a 6	66.8 a 1	8
Rangelander	70.3 ab 9	64.4 bc 8	64.2 c	62.4 a 4	64.6 ab 3	9

GL-Glenlea, HW-Homewood, AB-Arborg, Y0-establishment year seed production, Y1-seed production in the first year following establishment.

† - Cultivar means followed by the same letter are not significantly different at 0.05.

‡ - Cultivar rank within each environment.

§ - Cultivar rank over all environments.

Appendix 6. Total number of racemes per stem (flowering and pollinated) at 14 weeks after planting of nine alfalfa cultivars grown in seed quality experiments under different environments in southern Manitoba. Cultivar means averaged over establishment treatments.

Cultivar	ENVIRONMENT								
	GL-1992-Y0			GL-1993-Y0			HW-1992-YO			HW-1993-Y0			§
Florida 77	5.3	ab†	5‡	4.9	abc	4	6.8	ab	2	6.5	ab	4	3
CUF 101	4.9	b	6	2.4	d	9	6.7	ab	3	3.5	c	9	8
Nitro	5.6	ab	3	4.2	bc	5	4.7	bc	8	4.5	bc	7	7
Moapa 69	4.8	b	7	3.6	bcd	7	6.2	abc	6	4.8	bc	6	6
Wilson	4.2	b	9	3.4	cd	8	4.2	c	9	5.2	abc	5	9
Cimmaron VR	6.9	a	1	5.2	ab	2	7.5	a	1	7.5	a	2	1
Arrow	6.8	a	2	5.9	a	1	6.3	abc	5	7.8	a	1	2
Algonquin	4.3	b	8	5.1	ab	3	6.5	abc	4	6.6	ab	3	4
Rangelander	5.5	ab	4	3.8	bcd	6	6.0	abc	7	4.3	bc	8	5

GL-Glenlea, HW-Homewood, AB-Arborg, Y0-establishment year seed production, Y1-seed production in the first year following establishment.

† - Cultivar means followed by the same letter are not significantly different at 0.05.

‡ - Cultivar rank within each environment.

§ - Cultivar rank over all environments.

Appendix 7. Total number of racemes per stem (flowering and pollinated) at 16 weeks after planting of nine alfalfa cultivars grown in seed quality studies under different environments in southern Manitoba during 1992-1994. Cultivar means averaged over establishment treatments.

Cultivar	ENVIRONMENT												
	GL-1992-Y0			GL-1993-Y0			GL-1993-Y1			HW-1992-YO			HW-1993-Y0			§
Florida 77	10.1	a†	1‡	6.0	cd	5	9.9	bc	5	17.2	a	4	4.9	bc	6	5
CUF 101	5.9	c	9	4.1	e	7	9.0	bcd	6	13.6	bc	7	3.9	c	9	7
Nitro	6.6	c	7	3.9	e	8	7.6	cd	7	14.2	bc	6	4.2	c	8	6
Moapa 69	7.2	bc	6	4.3	de	6	5.8	d	8	13.2	bc	8	5.0	bc	5	8
Wilson	6.2	c	8	3.7	e	9	5.4	d	9	12.4	c	9	4.4	c	7	9
Cimmaron VR	9.5	ab	2	8.1	ab	3	12.1	ab	4	17.6	a	2	8.2	a	3	3
Arrow	9.2	ab	3	8.4	a	1	12.4	ab	3	18.0	a	1	8.2	a	2	1
Algonquin	7.5	bc	4	8.3	a	2	15.8	a	1	15.8	ab	5	8.5	a	1	2
Rangelander	7.2	bc	5	6.6	bc	4	14.2	ab	2	17.5	a	3	6.2	b	4	4

GL-Glenlea, HW-Homewood, AB-Arborg, Y0-establishment year seed production, Y1-seed production in the first year following establishment.

† - Cultivar means followed by the same letter are not significantly different at 0.05.

‡ - Cultivar rank within each environment.

§ - Cultivar rank over all environments.

Appendix 8. Total number of racemes per stem (flowering and pollinated) at 19 weeks after planting of nine alfalfa cultivars grown in seed quality experiments under different environments in southern Manitoba during 1992-1994. Cultivar means averaged over establishment treatments.

Cultivar	ENVIRONMENT												
	GL-1992-Y0			GL-1993-Y0			GL-1993-Y1			HW-1992-YO			HW-1993-Y0			§
Florida 77	8.7	bc†	5‡	5.5	b	4	5.2	abcd	4	15.9	abc	3	4.8	bc	5	5
CUF 101	3.1	cd	8	3.0	d	9	2.9	cd	7	10.0	de	7	2.6	d	9	9
Nitro	7.8	bcd	7	3.3	d	8	3.2	bcd	6	12.7	cde	5	3.5	cd	8	6
Moapa 69	7.9	bcd	6	4.2	cd	7	2.2	d	9	9.7	de	8	4.1	bc	6	7
Wilson	5.7	d	9	4.9	bc	6	2.9	cd	8	8.6	e	9	3.6	cd	7	8
Cimmaron VR	11.5	a	2	7.0	a	2	6.7	abc	3	18.8	a	1	7.8	a	1	1
Arrow	12.0	a	1	7.2	a	1	4.3	abcd	5	17.4	ab	2	7.0	a	2	2
Algonquin	10.2	ab	3	6.2	ab	3	7.0	ab	2	11.9	cde	6	5.3	b	3	4
Rangelander	9.7	ab	4	5.0	bc	5	7.3	a	1	13.8	bcd	4	5.0	bc	4	3

GL-Glenlea, HW-Homewood, AB-Arborg, Y0-establishment year seed production, Y1-seed production in the first year following establishment.

† - Cultivar means followed by the same letter are not significantly different at 0.05.

‡ - Cultivar rank within an environment.

§ - Cultivar rank over all environments.

Appendix 9. Number of pollinated racemes per stem at 14 weeks after planting of nine alfalfa cultivars grown in seed quality experiments under different environments in southern Manitoba during 1992-1994. Cultivar means averaged over establishment treatments.

Cultivar	ENVIRONMENT								
	GL-1992-Y0			GL-1993-Y0		HW-1992-YO		HW-1993-Y0		§
Florida 77	1.4	*b*†	5‡	0.9	*abc*	1.6	*a* 3	1.9	*ab* 4	**3**
CUF 101	1.2	*b*	7	0.4	*c*	1.2	*a* 8	0.9	*c* 9	**9**
Nitro	1.7	*ab*	3	0.6	*bc*	1.3	*a* 6	1.4	*bc* 5	**5**
Moapa 69	1.2	*b*	6	0.9	*abc*	1.4	*a* 4	1.3	*bc* 6	**6**
Wilson	1.1	*b*	8	0.4	*c*	1.1	*a* 9	1.3	*bc* 7	**8**
Cimmaron VR	1.7	*ab*	2	1.2	*ab*	1.7	*a* 1	2.5	*a* 2	**2**
Arrow	2.2	*a*	1	1.2	*a*	1.4	*a* 5	2.6	*a* 1	**1**
Algonquin	1.0	*b*	9	1.1	*ab*	1.7	*a* 2	2.0	*ab* 3	**4**
Rangelander	1.4	*b*	4	0.7	*bc*	1.3	*a* 7	0.9	*c* 8	**7**

GL-Glenlea, HW-Homewood, AB-Arborg, Y0-establishment year seed production, Y1-seed production in the first year following establishment.

† - Cultivar means followed by the same letter are not significantly different at 0.05.

‡ - Cultivar rank within each environment.

§ - Cultivar rank over all environments.

Appendix 10. Number of pollinated racemes per stem at 16 weeks after planting of nine alfalfa cultivars grown in seed quality experiments under different environments in southern Manitoba during 1992-1994. Cultivar means averaged over establishment treatments.

Cultivar	ENVIRONMENT												
	GL-1992-Y0			GL-1993-Y0			GL-1993-Y1			HW-1992-YO			HW-1993-Y0			§
Florida 77	4.3	a†	1‡	2.2	cde	5	5.3	bcde	5	8.5	a	4	1.6	c	6	**5**
CUF 101	1.9	d	8	1.0	ef	8	4.8	cde	6	4.9	b	6	0.6	c	9	**6**
Nitro	2.2	cd	7	1.1	ef	7	3.3	cde	7	4.6	b	8	0.9	c	8	**8**
Moapa 69	2.9	abcd	4	1.5	edf	6	2.6	de	8	3.9	b	9	1.7	c	5	**7**
Wilson	1.7	d	9	0.7	f	9	1.7	e	9	4.9	b	7	1.2	c	7	**9**
Cimmaron VR	3.5	abc	3	3.5	bc	3	6.6	bc	3	8.9	a	2	3.7	ab	3	**3**
Arrow	3.9	ab	2	4.0	ab	2	6.3	bcd	4	10.9	a	1	4.3	ab	2	**2**
Algonquin	2.5	bcd	5	5.4	a	1	11.8	a	1	8.4	a	5	4.8	a	1	**1**
Rangelander	2.4	cd	6	2.7	bcd	4	9.0	ab	2	8.6	a	3	3.2	b	4	**4**

GL-Glenlea, HW-Homewood, AB-Arborg, Y0-establishment year seed production, Y1-seed production in the first year following establishment.

† - Cultivar means followed by the same letter are not significantly different at 0.05.

‡ - Cultivar rank within each environment.

§ - Cultivar rank over all environments.

Appendix 11. Number of pollinated racemes per stem at 19 weeks after planting of nine alfalfa cultivars grown in seed quality experiments under different environments in southern Manitoba during 1992-1994. Cultivar means averaged over establishment treatments.

Cultivar	ENVIRONMENT												
	GL-1992-Y0			GL-1993-Y0			GL-1993-Y1			HW-1992-YO			HW-1993-Y0			§
Florida 77	4.2	bc†	6‡	1.6	cd	5	1.9	b	5	9.3	ab	3	1.2	b	6	**5**
CUF 101	3.1	cd	8	0.7	d	8	0.6	b	8	5.2	cd	7	0.5	b	9	**8**
Nitro	3.6	cd	7	0.7	d	9	1.0	b	6	6.5	bcd	6	1.0	b	7	**7**
Moapa 69	4.7	bc	4	1.2	d	7	0.6	b	9	4.6	d	8	1.4	b	5	**6**
Wilson	1.9	d	9	1.6	cd	6	0.6	b	7	4.4	d	9	0.9	b	8	**9**
Cimmaron VR	5.7	ab	3	3.4	ab	3	2.4	ab	3	11.7	a	2	3.3	a	4	**3**
Arrow	6.6	a	2	3.4	ab	2	1.9	b	4	11.8	a	1	3.5	a	2	**2**
Algonquin	6.7	a	1	4.2	a	1	5.5	a	2	7.7	bcd	5	3.8	a	1	**1**
Rangelander	4.5	bc	5	2.8	bc	4	5.6	a	1	9.0	abc	4	3.4	a	3	**4**

GL-Glenlea, HW-Homewood, AB-Arborg, Y0-establishment year seed production, Y1-seed production in the first year following establishment.

† - Cultivar means followed by the same letter are not significantly different at 0.05.

‡ - Cultivar rank within each environment.

§ - Cultivar rank over all environments.