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Biochemical and physiological adaptations of alfalfa to germination stresses imposed by NaCl

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BIOCHEMICAL AND PHYSIOLOGICAL ADAPTATIONS OF ALFALFA TO GERMINATION STRESSES IMPOSED BY NaCl

by

David Charles Poteet

A Thesis Submitted to the Faculty of the

DEPARTMENT OF PLANT SCIENCES

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE
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In the Graduate College

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1989

STATEMENT BY AUTHOR

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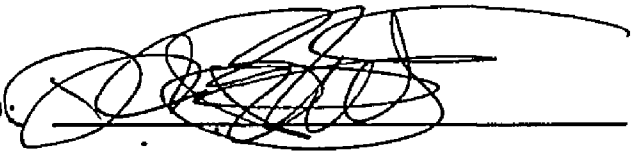
SIGNED: 

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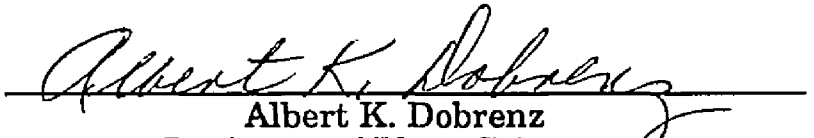

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5/8/89
Date

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ABSTRACT

Nine cycles of recurrent selection for germination salt tolerance in alfalfa (*Medicago sativa* L.) were compared with their parental cultivar, 'Mesa-Sirsa'. Test seeds were produced in the same season and locale. Cycle 9 and Mesa-Sirsa showed 90% and 2.5% germination, respectively, in a -1.7 MPa NaCl medium. Cycle 8 germinated more vigorously compared to Mesa-Sirsa in stressed and non-stressed environments. Selection also enhanced germination speed and radicle length. Fresh seed and one year old seed showed similar percent germination. Scarification decreased germination in a saline solution. Mesa-Sirsa and Cycle 8 displayed the same pattern of water uptake in a salt solution. Salinity decreased water uptake in Cycle 8 and Mesa-Sirsa compared to the control. Cycle 8 and Mesa-Sirsa contained 7% galactomannan and 3.2% stachyose. Galactomannan was not an important factor in seed salt tolerance. Seed protein content was stable throughout the cycles of selection. Selection for germination salt tolerance in alfalfa significantly affected the percentage of seed amino acids.

INTRODUCTION

Alfalfa contributes greatly to the economy of the Southwest. Alfalfa is the most important forage crop grown under irrigation in the state of Arizona. The Food Security Act of 1985, which discourages cotton production, may work to increase alfalfa acreage by 10 percent in Arizona to a total of 165,000 acres over the next few years (Blank and Ayer, 1986). Water availability and salinity, however, threaten alfalfa production in the future in many parts of Arizona. Salinity and other water-related issues excited public concern to the point of promoting Cadillac Desert on to the best-seller list. In Cadillac Desert, Marc Reisner pointed to increasing salt levels in river water and drew conclusions about the decline of irrigated agriculture in the Southwest (Reisner, 1986). Following the Groundwater Management Act of 1980, it is certain that in the near future water availability in Arizona will determine which industries flourish and to what extent. A group of real estate lobbyists before the Arizona legislature recently claimed that artificial lakes for housing developments "would not waste water but instead would be an appropriate method of water conservation as they evaporate less water than is used when the same amount of land is planted with cotton or alfalfa" (Farrell, 1987). Arizona farmers will need to find innovative responses to water issues and pressures, such as the use of otherwise unsuitable saline irrigation water and recycled tail-water. These measures, combined with existing problems of salinity in Arizona agriculture, call for innovative development of plant

materials, including the improvement of salinity tolerance in alfalfa at all growth stages.

The germination stage is an important focus in any plant improvement program for salt tolerance. Selection for germination salt tolerance has been one of the objectives of the alfalfa breeding program of Dr. A. K. Dobrenz at the University of Arizona. Nine cycles of recurrent mass selection have been completed, culminating in an alfalfa population demonstrating improved germination in various concentrations of salt (NaCl) compared to 'Mesa-Sirsa', the parental cultivar. The availability of the selected germplasm and its closely related parent present a unique opportunity to study the physiological and biochemical mechanisms of germination salt tolerance.

The objectives of this research are to 1) characterize the germination response of Mesa-Sirsa and the selected cycles to a wide range of saline media; 2) evaluate water absorption of seed in stressed and non-stressed environments and 3) evaluate the relationships between seed biochemical constituents and germination salt tolerance.

LITERATURE REVIEW

The quintessential condition of plant life is a variable environment (Cheeseman, 1988). In this variable environment germination is the single most vulnerable period of a plant's existence. The immediate environment of the seed may be the most challenging that the plant will ever face. Microorganisms, animal and insect pests and environmental factors simultaneously stress the seed. Plant distribution during seedling establishment is strongly influenced by water stress (Osmond et al., 1987). Osmond et al. (1987) noted that seedling mortality during drought is higher than adult mortality due to rapid changes in water availability at the soil surface. Salinity is a particularly pervasive stress in the environment of the seed, especially in irrigated fields where the greatest salt concentrations often occur in the first few centimeters of soil. Most plants suffer their greatest salt damage during germination and seedling establishment (Khatib and Massengale, 1966). Approximately half of the world's arid areas have very saline soils and are often bountiful agriculturally (Epstein, 1976). In other cases, the progressive deterioration of previously arable lands has had enormous economic and social repercussions, especially in Third World countries (Wyn Jones and Gorham, 1986). Epstein (1976) recommended emphasizing plant breeding and manipulation of adaptable plant materials genetically to improve salt tolerance rather than engineering to fit field conditions to crops. Noble (1983) also pointed out the heritability and variation for salt tolerance in many plants and stated that it

should be possible to increase tolerance without major changes to existing plant stress mechanisms.

Hayward and Wadleigh (1949) noted that observations concerning the osmotic inhibition of plant growth had been made as early as 1896 by Buffum. They added that Stewart had remarked in 1898 that various crops had different responses to salinity and that severe osmotic stress suppressed germination entirely. Mulwani and Pollard (1939) also determined that seeds of different crops displayed a wide range of sensitivity to salt and ranked red clover (*Trifolium pratense* L.), alfalfa and rice (*Oryza sativa* L.) as among the most sensitive to NaCl at germination. Maize (*Zea mays* L.), barley (*Hordeum vulgare* L.) and oats (*Avena sativa* L.) were among the crop seeds ranked as the least sensitive to salt stress during germination. Small concentrations of NaCl stimulated germination of certain crop seeds (Mulwani and Pollard, 1939). Hayward and Wadleigh (1949) observed that alfalfa displayed sensitivity to salt at germination although it was generally regarded as a salt tolerant crop. Alfalfa planted on salinized plots had a higher salt tolerance index compared to lettuce (*Lactuca sativa* L.), birdsfoot trefoil (*Lotus corniculatus* var. *tennuifolius*) and six other leguminous forages (Brown and Hayward, 1955). Large differences in response to NaCl were noted among three varieties of alfalfa. Furthermore, germination among the three varieties decreased nonuniformly in response to decreasing osmotic potentials. A -1.4 MPa NaCl solution suppressed germination of two alfalfa varieties entirely (Redmann, 1974). A -1.5 MPa NaCl solution inhibited alfalfa germination

for ten days after which the alfalfa demonstrated only 2% germination (Uhvits, 1946). Barley germinated at 80% in a -2.0 MPa NaCl solution whereas alfalfa and sugar beet showed 80% and 50% germination in -0.7 and -0.6 MPa NaCl solutions, respectively (Hayward and Wadleigh, 1949). Both percent germination and rate of germination of alfalfa were affected by increased salt levels (Khatib and Massengale, 1966). Although alfalfa germination in a variety of salt solutions was less than for some vegetable crop seeds, the alfalfa seed germinated faster, reducing the time for salt buildup in seed tissues (Assadian and Miyamoto, 1987).

NaCl has both an osmotic and toxic ion effect on alfalfa germination (Uhvits, 1949). Alfalfa germination decreased as concentrations of NaCl and mannitol increased. At equal osmotic pressures the inhibitory effect of NaCl on germination was greater than that of mannitol (Uhvits, 1949). More recently Allen (1986) observed osmotic and toxic ion inhibition in germination of Mesa-Sirsa compared to five alfalfa populations selected for NaCl tolerance at germination. He showed that selection for tolerance to NaCl during germination also resulted in increased tolerance to other salts as well as to mannitol and polyethylene glycol and that germination in mannitol was higher than in any of the salt solutions tested (Allen, 1984). Six varieties of alfalfa showed significantly different responses to osmotic pressures indicating that the ability to germinate under osmotic stress is heritable (Dotzenko and Dean, 1959). Carlson et al. (1983) successfully used plates containing NaCl imbued agar to screen fifteen alfalfa cultivars for their ability to germinate under salt stress. 'Ladak 65' demonstrated a 3.75

fold increase in its tolerance to salt at germination after only one cycle of selection in agar containing 1.75% NaCl (Carlson et al., 1983).

Recurrent phenotypic selection has proved successful in producing alfalfa tolerant of extreme salinities at germination (Robinson et al., 1986). The fifth cycle of recurrent selection in the alfalfa breeding program for seed salt tolerance showed 96% germination in a -1.35 MPa NaCl solution and was released as a registered germplasm in 1983 (Dobrenz et al., 1983). Heritability of salt tolerance during germination was estimated at 49.9% (Allen et al., 1985). Selection after seven cycles increased salt tolerance by one MPa over that of the parental cultivar, Mesa-Sirsa (Robinson, 1986). Seed of the first synthetic generation of the ninth cycle of mass recurrent selection exhibited a 20% increase in salt tolerance over that of the fifth cycle of selection (Dobrenz et al., 1989).

When it is not fractured or scarified the testa of alfalfa seed is largely impermeable due to thickened palisade cells below the seed cuticle (Teuber and Brick, 1988). Alfalfa seed that was hand-harvested and cleaned had a germination percentage of 41%, indicating a propensity to hard-seededness (Watson, 1948). Imbibitional water uptake is through an opening which is located in the lens of the alfalfa seed, a weak point in the seed palisade layer (Gunn, 1972). Time course studies revealed a hydration gradient from the lens towards the radicle hook (Teuber and Brick, 1988). Alfalfa is classified as an endospermic legume and contains a galactomannan polysaccharide located entirely in the endosperm which is stored in the endosperm cell

walls (Halmer, 1985; Reid, 1985). The breakdown of galactomannan, the major seed storage carbohydrate in alfalfa, is largely undertaken by the aleurone layer, the closest layer of the endosperm to the seed coat (McCleary and Matheson, 1974; Reid and Meier, 1972). The aleurone is prevented from commencing hydrolysis by an inhibitor whose action is relieved by a signal from the embryo axis, indicating a point of control in the hydrolysis process (Spyropoulos and Reid, 1985). Aged alfalfa seed responds differently to salt stress than does fresh seed (Smith and Dobrenz, 1987). Comparisons of alfalfa seed lots of different years of production gave inaccurate representations of germination salt tolerance. Smith and Dobrenz (1987) recommended evaluating seed salt tolerance using seed produced in the same season and environment. The exact combination of factors contributing to salt tolerance in alfalfa, as well as other species, remains largely undefined.

Carbohydrate-Water Interactions

Carbohydrate-water interactions are an important topic in studies of plant stress physiology. Certain plant polysaccharides have a noted tendency to hold water and moderate effects of water stress in plant tissues. Plant sugars in particular have been studied. The hydrogen bonding of water directly to specific sites on the solute molecule determined the physical and chemical polyfunctionality of sugars (Franks, 1975). He observed that the interaction between water and sugar is orientation specific and depended greatly on the stereochemistry of the sugar molecule.

Franks (1975) added that different sugars had different hydration interactions with water. Pyranose sugars with equatorial -OH substituents had the most favorable interactions with water (Franks, 1975).

Going beyond solutions of monosaccharides, it is possible that conformations taken up in aqueous solutions, gels and *in vivo* by oligo- and polysaccharides are determined by specific solvent effects (Franks, 1975). These conformations, or hydration structures, may give the plant the ability to withstand certain environmental stresses (on a structural basis). Suggett (1975) commented on the capability of polysaccharide gels to hold water and also observed that the networks formed by sugars are more important than the effects of individual molecules. Suggett (1975) added that it has been suggested that biological systems are protected against cold or drought damage by sugars and sugar-like molecules. Suggett (1975) noted that this kind of biological protection is based on the hydrophilic hydration properties of sugars and the compatibility between sugar stereochemistry and water structure.

Franks (1975) reported that gelling polysaccharides have a more complex relationship with water than monosaccharides. Gelation mechanisms in foods which were heated and cooled formed junction zones holding polymer chains together in a three-dimensional network (Suggett, 1975). The water relations of these networks varied according to which of the three structures the particular gels formed. The solid quality of these gels occurred as a result of polymer to polymer interaction and not because

of large-scale ordering of water. In this sense, water acted as a catalyst in polysaccharide gel formation (Suggett, 1975). Polysaccharides in plants may form similar structures and sequester water, protecting the plant against dessication or other damage.

The formation and water relations of gels are, in fact, different from the mucilage formation and polysaccharide-water interactions which take place in plant tissues. Gels *per se* do not form easily, if at all, in plants. Galactomannan, a major seed polysaccharide in certain species, was described as a mucilage by Aspinall (1973). Meier and Reid (1982) defined galactomannan solutions as mucilaginous and added that galactomannans are referred to as "gums" or mucilages. The behavior of gels, however, may throw some light on carbohydrate-water interactions in plants. The role of polysaccharide mucilages in the plant function of interest (NaCl tolerance) may have little to do with the ordering of water so much as with the hydrophilic properties of the polysaccharides or of their dissolved products (Mercier, 1985). A review of polysaccharide conformation in solution and as gels was offered by Rees (1973) although he had little to say directly about polysaccharide conformation and hydration in plant tissues.

Observations concerning the water relations role of galactomannan and plant mucilages in seeds have been made (Halmer, 1985; Mercier, 1985; Reid and Meier, 1972). Certain carbohydrates have a role in plant-water relations at other stages in plant development also. Distelbarth and Ulrich

(1972) commented on the water relations role of a leaf mucilage composed of complex water-soluble polysaccharides of high molecular weight in the needles of *Taxus baccata* L. They suggested that the mucilage stabilized water relations leading to improved frost resistance. Carbohydrates in mature alfalfa plants were implicated in both drought and cold tolerance (Jung and Larson, 1972).

Seed Polysaccharides and Free Sugars

Hydrophilic polysaccharides are commonly found in plant tissues at all stages of growth. Meier and Reid (1982) speculated that species which have seeds with hydrophilic cell wall storage polysaccharides have been underestimated and that their numbers may equal those of the more well known starch-bearing seeds. Some polysaccharides are in such quantity in their tissues of origin that they are extracted for commercial purposes as varied as additives in oil well drilling slurries, sizing for paper products and the sealing of dynamite (Dickey and Roth, 1981).

Galactomannan is the polysaccharide which dominates carbohydrate and water interactions in alfalfa seed. The seeds of many of the *Leguminosae*, *Palmaceae* and the *Rubiaceae* also store galactomannan polysaccharides in their endosperm (Stepanenko, 1960). In fenugreek (*Trigonella foenum-graecum* L.), galactomannan deposition continued during seed development until it occupied nearly the entire volume of the endosperm (Reid, 1985). McCleary and Matheson (1975)

determined the amounts of galactomannan and free galactose and mannose in non-imbibed and imbibed alfalfa. Non-imbibed alfalfa contained 8.8 milligrams of galactomannan per one hundred milligrams of seed. At all measured points during germination, alfalfa contained very low or trace amounts of free galactose and mannose (McCleary and Matheson, 1975). Dea and Morrison (1975) reported 5.5 % "yield of gum", or galactomannan in alfalfa. Gonzalez-Murua et al. (1985) measured the amount of galactomannan in 'Tierra de Campos', a European alfalfa which demonstrated substantial drought and salt tolerance as a mature plant compared to 'Aragon', another European alfalfa. 'Tierra de Campos' and 'Aragon' contained 81% and 84% carbohydrate in the endosperm, which represented 14.0% and 14.5% of seed weight, respectively. Galactose and mannose together represented 85% and 79% of the endosperm polysaccharide in 'Tierra de Campos' and 'Aragon' or 11.9% and 12.3% of seed weight, respectively. Endospermic legumes other than alfalfa contain various amounts of galactomannan ranging from 30%, 21%, 5% and 2% of the seed for fenugreek, guar (*Cyamopsis tetragonoloba* L. Taub.), subclover (*Trifolium brachycalycinum*) and soybean (*Glycine max* L. Merr.) (Reid, 1985; McCleary and Matheson, 1975; Gonzalez-Murua et al., 1985 and Dea and Morrison,1975).

Alfalfa also contains several other important sugars as mono- and oligosaccharides. McCleary and Matheson (1974) reported that the raffinose series oligosaccharides were the first storage carbohydrates depleted upon germination in alfalfa, guar, carob (*Ceratonia siliqua* L.) and soybean.

Stachyose was the major reserve oligomer in alfalfa. Raffinose, sucrose, glucose and fructose were also present in lesser amounts in the leguminous species which McCleary and Matheson (1974) examined. Arabinose and xylose were among the neutral sugars released by acid hydrolysis of alfalfa endosperms (Gonzalez-Murua et al., 1985) . Escalada (1970) assayed the carbohydrates in alfalfa seed produced in four locations and during different years in the Western United States but identified only three carbohydrates by name; sucrose, maltose and raffinose. On the basis of seed size, Escalada (1970) determined that there was a lower percentage of free sugars, total sugars, combined sugars (by 80% ethanol extract and H_2SO_4 hydrolysis) and total hydrolyzable carbohydrates in larger seed than in smaller seed. Escalada (1970) also demonstrated that alfalfa seed produced in cool, stressed conditions had a higher percentage of free sugars than did alfalfa seed grown out in warmer (non-temperature stressed) climates.

Galactomannan belongs to the mannan group of cell wall storage polysaccharides in endospermic seeds. Meier and Reid (1982) showed that cell wall storage polysaccharides with a mannan foundation are based on a linear (1→4)-β linked chain or "backbone". The mannan group reserves are subclassified into the mannans, the glucomannans and the galactomannans. The mannan backbone in galactomannans carried (1→6)-α linked D-galactosyl substituents (Meier and Reid, 1982). Mannose:galactose ratios varied per species from a 1:1 to a 4:1 ratio (Reid, 1985) with alfalfa galactomannan containing about 45% galactose

(McCleary and Matheson, 1975; Reid, 1985). Galactomannans were soluble in water or alkaline solutions (Stepanenko, 1960). Seed galactomannans were hydrophilic and became mucilaginous in contact with water (Reid,1985). Meier and Reid (1982) observed that galactomannans have a linear structure which is highly branched making them very different from unbranched water-insoluble mannans and glucomannans. Reid (1985) referred to galactomannan as a "multipurpose macromolecule" and noted that the multifunctionality of galactomannan was remarked upon as early as 1877 by DeVries.

In a study of seed hydration in relation to polysaccharides, Reid and Bewley (1979) claimed that the spatial location (in the endosperm) and the hydrophilic properties of galactomannan constituted the molecular basis for drought tolerance in fenugreek. Galactomannan, Reid and Bewley (1979) observed, allowed the seed to imbibe large quantities of water which buffered it against dessication. Seed galactomannan may have two biological functions which it fulfills one after the other. Galactomannan interacted with water during germination and nourished the embryo following radicle protrusion (Reid and Bewley,1979). Meier and Reid (1982) proposed that the endosperm surrounds the embryo and acts like a water-buffer because it can lose significant amounts of water without injurious changes in its water potential. On the basis of water loss studies performed by Reid and Bewley (1979), Meier and Reid (1982) maintained that endospermic seed may face a dessicating environment and lose water to external gradients, but that galactomannan allowed the germinating

embryo to be challenged only by the endosperm's water potential. Salt stress is similar to water stress because salts lower the effective concentration of water (Osmond et al.,1986). Considering this, it may be that galactomannan offers the same benefit to salt-stressed seed that it extends to water-stressed seed.

Seed Germination and Water Relations

Bewley and Black (1985) described water uptake in seeds during germination and seedling emergence as triphasic. The imbibition phase is a consequence of the seed matric potential, whereas the two other phases, the static (or "lag") phase and the radicle elongation phase have more to do with the living processes of the germinating seed. The static phase of water uptake is highly active in terms of various synthetic activities, including enzyme synthesis, and in this regard is referred to as the activation phase (Hegarty, 1978). Allen's (1984) speculations on water relations in alfalfa seed suggested that the osmotic benefit of galactomannan may be expressed after the matric potential of the seed is played out, during the lag phase of water uptake. Allen (1984) remarked that osmotic water uptake in viable seed becomes more crucial after the initial imbibitional water absorption. He felt that his study measured primarily imbibitional water absorption and might not have reflected important physiological differences between seed germplasm - such as rate of ion absorption and cell solute concentration - which influenced osmotic water uptake (Allen, 1984). Osmotic uptake, presumably, is a less dramatic event than matric uptake

but the small incremental increases in water uptake may have more meaning to the plant during its activation phase.

Bliss et al. (1986) confirmed the importance of water relations in the seed beyond matric water uptake. They explored the possibility that NaCl affects barley differently during the imbibition phase as compared to the germination (or activation) phase. Seed hydration had to reach a critical threshold value for germination to occur. Barley seed were prevented from attaining the hydration threshold due primarily to the inhibitory osmotic effects of NaCl (Bliss et al., 1986). Allen (1986) determined that selection for NaCl tolerance resulted in greater tolerance to osmotic stress than NaCl stress. Bliss et al. (1986) concluded that if insufficient hydration prevented germination during imbibition, any means which boosted hydration over a "critical threshold" would promote germination. Even a momentary increase in the seed's internal water potential, they stated, might be sufficient to initiate germination.

Plant growth has been shown to be immediately responsive to changes in water supply. Acevedo et al. (1971) examined elongation of intact young maize leaves where changes were made in the water available in the root environment. They found that maize leaf growth was highly sensitive to very small reductions in soil water potential and also that growth (measured as elongation) resumed in less than a few seconds in a mildly stressed plant after rewatering. Matsuda and Riazi (1981) measured the growing regions of stressed barley leaves and found an immediate

cessation of leaf elongation upon sudden exposure to osmotic solutions ranging from -3 to -11 bars. They also found that leaf growth resumed after a delay which increased with proportional stress, and that the resumed growth rate was reduced proportionally to stress.

Robinson's (1986) data supported the hypothesis of Bliss et al. (1986) concerning the hydration threshold. Robinson (1986) compared the percent germination (expressed in natural logarithms) between Mesa-Sirsa and Cycle 7 Syn 1 of a selection program for germination salt tolerance in alfalfa. Across a gradient of osmotic potentials, Mesa-Sirsa showed percent germination similar to Cycle 7 Syn 1 until a very specific point and then declined abruptly. Cycle 7 Syn 1 germinated in media of a lower osmotic potential than Mesa-Sirsa did, but also began to decline at a very specific point and in a parallel manner to the Mesa-Sirsa decline. The data suggests a critical level of hydration and that the value for this point may have simply been readjusted in Cycle 7 Syn 1 as compared to Mesa-Sirsa.

The above reports do not describe conclusively how galactomannan confers the ability to withstand water stress to the seed. There is a need for a more rigorous and exact definition of the biochemical/biophysical means that have been proposed to explain drought, salt and other stress tolerance as a polysaccharide function, especially in seeds. Stout et al. (1980) indicated that osmotic solutions had little effect on the total amount of water taken up by sorghum (*Sorghum bicolor* L. Moench) seeds. Allen (1984) reported that the differences in germination salt tolerance between salt

tolerant seed and its non-salt tolerant parent were not due to differences in ability to absorb water in saline media. This may indicate, contrary to Reid and Bewley (1979) that in the case of fenugreek and other endospermic legumes there was little difference in the gross imbibitional ability that may be conferred by galactomannan. Reid and Bewley (1979) proposed that galactomannan moderated water stress in seeds but they did not suggest in more than general terms how galactomannan contributed to the ability of seeds to tolerate drought or salt stress. Reid and Bewley (1979) did not describe the drought tolerance of the fenugreek variety they studied or refer to the dessication tolerance of their test fenugreek in relation to other varieties of fenugreek. The development of nine cycles of increasingly germination salt tolerant alfalfa from the same parent (Dobrenz et al., 1989) provides the ideal base for comparative studies of galactomannan and other cooperative factors which lead to seed salt tolerance.

Water Stress and Enzyme Inhibition

Three enzymes are responsible for galactomannan hydrolysis in alfalfa (McCleary and Matheson, 1976; Spyropoulos and Reid, 1985). Gonzalez-Murua et al. (1985) reported that the presence of polyethylene glycol 6,000 and NaCl in a germination media separately diminished the activity of α-galactosidase, the most important enzyme in galactomannan hydrolysis. Hadas (1976) observed that diminished germination of chickpea (*Cicer arietinum* L.) and vetch (*Vicia faba* L.) seed in a polyethylene glycol solution was due to the effect of a low water potential on enzymatic activity

and not to lessened water uptake. Reid (1972) indicated that before galactomannan is broken down in germinating fenugreek seeds, polyribosomes form and endoplasmic reticulum proliferated in the seed aleurone cells which suggested intensive protein synthesis. Hesterman et al. (1981) reported that environmental factors, including abiotic stresses, have an effect on the protein composition of germinating seeds and seedlings.

Reid (1972) observed in fenugreek a high degree of indentation and distortion of the aleurone plasmalemmae which suggested a secretion of protein materials just before the beginning of galactomannan breakdown. Armstrong and Jones (1973) reported that water stress induced by 0.6 M solutions of polyethylene glycol in barley reduced the binding of ribosomes to the endoplasmic reticulum. Reid did not provide any direct evidence of the behavior of fenugreek polyribosomes under water stress. It can only be assumed that the response to water stress in fenugreek is similar to that of barley in the manner suggested by Armstrong and Jones (1973). In alfalfa, where two of the major hydrolytic enzymes which break down galactomannan are synthesized *de novo* in the aleurone layer, the lack of formation of the organelles responsible for polysaccharase synthesis would inhibit and delay germination. Cheeseman (1988) recommended that mRNA and protein synthesis patterns in mature plants under a variety of growth conditions be scrutinized in order to understand response to salt stress.

The role of galactomannan described by Reid and Bewley (1979) is not exclusive of the possible prevention of enzyme inhibition effects. Gonzalez-Murua et al. (1985) accepted Reid and Bewley's (1979) hypothesis and also noted that water stress may inhibit hydrolytic enzymes in alfalfa. Gonzalez-Murua et al. (1985) also implied that some of the solutes used for osmotic adjustment in seeds may come from hydrolyzed endosperm polysaccharides or other seed sugars thereby contributing to osmotic adjustment. Reid (1972) indicated, however, that galactomannan breakdown was very quick once it started and that there was a rapid uptake of the products of enzymatic catabolism. As stated previously, there is little free mannose or galactose present in endospermic legumes at any point during germination. Reid (1971) showed that galactomannan only begins its complete hydrolysis as much as twenty four hours after radicle protrusion. If there is a solute effect it may be very important in terms of the overall seed water balance, but it may also be very transitory and useful only to the germinating seedling.

MATERIALS AND METHODS

Selection for germination salt tolerance has been one of the objectives of the alfalfa breeding program of Dr. A. K. Dobrenz and colleagues at the University of Arizona. The techniques of the breeding program have been described by Allen (1984), Robinson (1986) and McKimmie (1986). The alfalfa seed used in this study represented the original population, Mesa-Sirsa and nine cycles of recurrent selection for germination salt tolerance (Dobrenz et al., 1989). Many of the experiments which form this research shared methods and materials in common. Conditions specific to a particular experiment are noted in tables below. Sodium chloride was the osmotic agent in all experiments. In almost every experiment, seed of the same age was used. Seed representing several synthetic (Syn) generations of increase was used, but most experiments used seed of the Syn 2 generation.

All experiments involving germination performance or imbibition were performed at 26° ±1° C in a dark growth chamber. Prior to planting, the seed was bulk scarified in a scarifier (Forsberg Inc., Thief River Falls, MN). Any minimal scarification which the seed had already undergone in the field or during cleaning was effectively complemented in the scarified treatment by a 2 to 3 second cycle in the scarifier accompanied by a single, upward twist of the cannister containing the seed. Seeds were planted in 4.5 to 5 ml of germination solution inside 10 x 2 cm plastic petri dishes lined with one sheet of Whatman #5 filter paper. Captan (0.2 % W/V) was added to most germination solutions prior to planting to prevent fungal

contamination of the samples. Osmotic solutions were verified for osmotic potential with a Wescor Model 1500C vapor pressure osmometer. After planting, petri dishes were placed in Ziplock bags and sealed to prevent evaporation of the test solutions. Petri dishes were randomly placed in basins and covered with a large plastic bag to further prevent environmental moisture loss. Experiments measuring germination performance ran for ten consecutive days, with one reading per day at standard intervals. Completed germination was defined and counted as the protrusion of the radicle to 2 mm beyond the seedcoat.

All analysis of variance and other statistical operations were performed using SAS (SAS Institute, Cary, NC). Percent data and germination speed data were arcsine or square root transformed (Gomez and Gomez, 1984) prior to analysis depending on the range of the test values. Significant differences were established by the LSD method at the .05 level of probability.

Experiment 1

Table 1: Techniques used to compare Mesa-Sirsa and Cycles 1 through 7.

No. of populations tested:	8
Seed source:	Mesa-Sirsa and Cycles 1-7 Syn 2 (1986)
No. of osmotic treatments:	3
Osmotic potentials (MPa):	0, -1.1 and -1.7
No. of replications:	4
No. of seed per replication:	50
Measurements taken:	Percent germination, germination speed and seed weight

Seven cycles of selection which had been developed for germination salt tolerance were compared with Mesa-Sirsa. A germination speed index was calculated based on a formula proposed by Guneyli et al. (1969) and modified by Robinson (1986). Germination speed describes the onset and rate of germination and constitutes a weighted measure of the vigor of seed in saline solutions. Robinson's (1986) formula for germination speed was:

$$\text{Germination Speed} = (N_1/D_1 + N_2/D_2 + \ldots + N_{10}/D_{10}) / P$$

N equals the seed germinating each day, D is the respective day and P is the total number of seed which germinate by the last day of the experiment. Seed which doesn't germinate scores a germination speed of 0 whereas seed which germinates entirely on the first day of the experiment scores a 1 (Robinson, 1986). For the purposes of graphing, all scores were multiplied by one hundred following statistical analysis. Seed in this experiment was not scarified and Captan was not added to the germination solutions. Hard seed, defined as non-imbibed seed, was counted at the end of the test period and excluded from analysis.

Seed weight was obtained to determine the effect of selection for germination salt tolerance on seed size and to evaluate whether seed weight has an effect on germination performance. Seed dry weights were measured using a modified air-oven-130° C technique (Roberts, 1972) and a Mettler PE-160 electronic balance. Percent germination, germination speed and dry seed weight were analyzed as a CRD design.

Experiment 2

Table 2: Techniques used to compare Mesa-Sirsa and Cycles 1 through 9.

No. of populations tested:	10
Seed source:	Mesa-Sirsa, Cycles 1-8 Syn 2 and Cycle 9 Syn 1 (1987)
No. of osmotic treatments:	2
Osmotic potentials (MPa):	0 and -1.7
No. of replications:	2
No. of seed per replication:	40
Measurements taken:	Percent germination and germination speed

Due to the small amount of seed remaining in the 1986 seed lot and also because of the relatively larger amount of seed produced in 1987, this research switched from the use of the 1986 Syn 2 seed to the use of the 1987 Syn 2 seed for this and all following experiments. Additionally, seed from all nine cycles of selection were available for testing and research in 1987. Cycles 1-9 were compared with Mesa-Sirsa for percent germination and germination speed. Captan was added to the saline treatment and not to the control. All seed in this experiment was scarified. Percent germination was analyzed as a CRD design.

Experiment 3

Table 3: Techniques used to compare Cycles 1 through 7 produced in 1986 and Mesa-Sirsa and Cycles 1 through 8 produced in 1987.

No. of populations tested:	16
Seed source:	Cycles 1-7 Syn 2 (1986) and Mesa-Sirsa and Cycles 1-8 Syn 2 (1987)
No. of osmotic treatments:	3
Osmotic potentials (MPa):	0, -1.1 and -1.7
No. of replications:	4
No. of seed per replication:	50
Measurements taken:	Percent germination and germination speed

All of the cycles of selection produced in 1986 and 1987 were compared with Mesa-Sirsa to determine the genetic stability of germination salt tolerance in seed produced in different environments (i.e., year of production). The seed lots were tested together under the same experimental conditions and were analyzed as groups in an orthogonal contrast, a type of comparison in which the aggregate mean of one group is compared to that of any other specified group (Gomez and Gomez, 1984). All seed in this experiment was scarified and Captan was added to all germination solutions.

Experiment 4

Table 4: Techniques used to determine genetic shift in the salt tolerance of Mesa-Sirsa and Cycle 8.

No. of populations tested:	4
Seed source:	Mesa-Sirsa and Cycle 8 Syn 1-3 (1987)
No. of osmotic treatments:	4
Osmotic potentials (MPa):	0, -0.6, -1.2 and -1.7
No. of replications:	4
No. of seed per replication:	40
Measurements taken:	Percent germination, germination speed and radicle length

Synthetic generations 1 through 3 of Cycle 8 were compared with Mesa-Sirsa to determine whether several generations of seed produced in a non-saline environment maintains its tolerance to salinity. The three generations of Cycle 8 and Mesa-Sirsa were produced in adjacent bee cages on experimental sites with negligible salinity. All seed was scarified in this experiment and Captan was added to all germination solutions. Replications were run successively and were considered blocks in the experiment. Radicle length at 72 and 120 hours after planting was evaluated using a scoring technique developed by Smith and Dobrenz (1987). Seedlings with radicles greater than 2.5 m after 72 hours were counted and scored 5. Any remaining seedlings were counted at 120 hours. Seedlings with radicles greater than 2.5 cm were scored 4. Seedlings with radicles measuring between 1.5 to 2.5, 1.0 to 1.5 or 0.2 to 1 cm were scored 3, 2 or 1, respectively. The experiment was analyzed as a RCB design.

Experiment 5

Table 5: Techniques used to evaluate the effect of seedcoat scarification on Mesa-Sirsa and Cycle 8.

No. of populations tested:	4
Seed source:	Mesa-Sirsa and Cycle 8 Syn 1-3 (1987)
No. of osmotic treatments:	2
Osmotic potentials (MPa):	0 and -2.0
No. of replications:	4
No. of seed per replication:	50
Measurements taken:	Percent germination, germination speed and radicle length

Three generations of Cycle 8 and Mesa-Sirsa were evaluated in a saline medium to determine whether scarification affects the germination of alfalfa seed in control and saline media. Prior to planting, half the seed was bulk scarified. Percent germination and germination speed were scored in the scarified treatment as the percent of non-hard seed. Hard seed, defined as non-imbibed seed, was counted at the end of the test period. The experiment was replicated four times and analyzed in a split split-plot design. Post hoc testing (Tukey HSD) was used to determine the significant differences between the means in the salt x treatment interaction.

Experiment 6

Table 6: Techniques used to evaluate relative fresh weight and percent germination of Mesa-Sirsa and Cycle 8.

No. of populations tested:	2
Seed source:	Mesa-Sirsa and Cycle 8 Syn 2 (1987)
No. of osmotic treatments:	2
Osmotic potentials (MPa):	0 and -1.1
No. of replications:	1-3
No. of seed per replication:	20
Duration of test:	18.75 hours
Times of observations:	1 to 3.75 hours
Measurements taken:	Seed fresh weight and percent germination

Seed fresh weights and percent germination were simultaneously measured to determine if water uptake and growth of Cycle 8 varied according to the level of salinity stress compared to Mesa-Sirsa. Seed was weighed prior to planting to obtain non-imbibed fresh weight. This experiment was performed twice. For the first run of this experiment, the same seed samples were imbibed, removed from a petri dish and blotted dry, weighed and replaced in the petri dish, and the whole process repeated at hourly intervals. These manipulations may have caused internal damage to the seed and evaporation of the germination solutions resulting in erroneous readings. The second run of the experiment was designed so that seed samples were imbibed at the same time, weighed and counted at intervals of 1 to 3.75 hours and discarded, removing the confounding effect of prolonged seed handling. There were three replications of the three seed populations at 0, 3.75, 7.5, 11.25, 15 and 18.75 hours after planting. Single replications of Cycle 8 and Mesa-Sirsa were measured at 2, 5, 9, 13 and 17 hours after planting.

Water uptake was expressed as a relative value in order to take into account the differences in seed weight between the populations. Relative fresh weight was calculated by dividing the measured fresh weight of the imbibed seed by the initial fresh weight of the non-imbibed seed. Standard deviation was calculated for both relative fresh weight and percent germination.

Experiment 7

Table 7: Techniques used to determine the effect of selection on seed polysaccharide content.

No. of populations tested:	3
Seed source:	Mesa-Sirsa, Cycle 5 and 8 Syn 2 (1987)
No. of replications:	3
No. of seed per replication:	50
Measurements taken:	milligrams per 50 seed and percent of seed

Cycles 5 and 8 were compared to Mesa-Sirsa to evaluate whether the amounts of certain polysaccharides and cell wall sugars, especially galactomannan, changed as a consequence of selection for germination salt tolerance. Samples of fifty intact and unscarified seed (embryo not removed) were ground in a mortar and pestle and placed in centrifuge tubes. One hundred µl of one mg mannitol/ml H_2O was added to the the sample in each centrifuge tube as an internal standard. Extraction of the soluble free sugars in boiling ethanol followed a modified technique of Reid (1971). Five ml of 95% ethanol were added to each centrifuge tube. The centrifuge tubes were placed in a boiling water bath for fifteen minutes and vortexed twice during this period. Each sample was then centrifuged for fifteen minutes. The solubilized free sugars were pipetted and collected from each centrifuge tube. This extraction was repeated three times for each seed sample. The residues insoluble in ethanol were dried at room temperature.

Hydrolysis of the insoluble residues followed a combination of modified procedures of Albersheim (1967) and Grimes (1976). The bulk of the residues were transferred dry from the centrifuge tubes to round bottom test tubes. One hundred µl of one mg mannitol/ml H_2O was then added to

each centrifuge tube, followed by the addition of one ml of 2N HCl. Remaining residues were thoroughly scraped from the sides of the centrifuge tubes and transferred to the test tubes. The centrifuge tubes were washed twice more with one ml of 2N HCl for a total of three ml of 2N HCl/tube. Once they were emptied the same steps were followed with any residues remaining in the tubes used to collect the soluble sugars. The test tubes containing the insoluble samples were quickly transferred to a special enclosure designed to seal them under nitrogen during hydrolysis. Inside the enclosure, the test tubes were individually flushed with nitrogen. The samples were boiled at 100° C for 2.5 hours and stirred with a glass rod twice during that time. The samples were adjusted to pH = 7 with approximately three ml of 4M NaOH.

Due to the concentration of salts in the samples following hydrolysis, the samples were purified five times using ion exchange columns. In order to separate anions and cations from the neutral sugars, samples were initially passed through four columns containing a layered 1:1 resin combination of Amberlite IRA-45 and Dowex 50-W. Finally, the samples were passed through a column containing Dowex MR-3, a 1:1 resin mixture of Dowex HCR-5 and Dowex SBR. The samples were dried *in vacuo* following each pass through a column.

The samples were filtered separately. One µl of each sample was injected into a High Performance Liquid Chromatograph (HPLC). A HPX-87C carbohydrate analysis column (Bio-Rad Laboratories, Richmond, CA)

with a mobile phase of H_2O measured the refractive index of the samples. Column temperature was set at 85° C, column flow rate at 0.6 ml/min and column pressure at approximately sixty bars atmospheric with a run time of twenty minutes. Monosaccharide standards were also injected and analyzed separately by HPLC and compared with the sample results. The ion exchange column resins and the monosaccharide standards were obtained from Sigma Chemical Company, St. Louis, Missouri. Results were analyzed as a CRD design. Galactose and mannose coeluted. Where the sugars did not coelute, the amount of galactomannan in a sample was arrived at by adding together the two separate peak area retention times.

Experiment 8

Table 8: Techniques used to determine the effect of selection on seed free sugar content.

No. of populations tested:	3
Seed source:	Mesa-Sirsa, Cycle 5 and 8 Syn 2 (1987)
No. of replications:	3
No. of seed per replication:	50
Measurements taken:	milligrams per 50 seed and percent of seed

Seed from Cycles 5 and 8 and Mesa-Sirsa were analyzed to determine if there were changes in their content of free sugars as a consequence of selection for germination salt tolerance. The seed free sugars were obtained from the boiling ethanol extraction in Experiment 7. Following extraction, the samples were centrifuged a fourth time to remove any traces of insoluble residues. The samples were separately passed through ion exchange columns containing a layered 1:1 resin combination of Amberlite IRA-45 and Dowex 50-W. After this step the free sugars were treated and analyzed by HPLC in the same way as the polysaccharides in Experiment 7. Results were analyzed as a CRD design.

Experiment 9

Table 9: Techniques used to determine the effect of selection on seed protein content.

No. of populations tested:	10
Seed source:	Mesa-Sirsa, Cycles 1 through 8 Syn 2 and Cycle 9 Syn 1 (1987)
No. of replications:	3
No. of seed per replication:	51 seed (approximately 108 mg)
Measurements taken:	Percent total nitrogen and total percent protein

The seed from all the cycles of selection and Mesa-Sirsa were compared to evaluate any potential differences in seed nitrogen and percent protein as a result of selection for salt tolerance. Unscarified seed was dried at 60° C for four hours prior to weighing and grinding. The seed was analyzed by the Soil, Water and Plant Testing Laboratory at the University of Arizona. Results were expressed as percent total nitrogen. Percent protein was calculated using a conversion factor of 6.25 x N (Hamilton and Vanderstoep, 1979). Results were analyzed as a CRD design.

Experiment 10

Table 10: Techniques used to determine the effect of selection on total seed amino acid content.

No. of populations tested:	3
Seed source:	Mesa-Sirsa, Cycles 4 and 7 Syn 2 (1987)
No. of replications:	3
No. of seed per replication:	98 seed (approximately 210 mg)
Measurements taken:	μmoles of amino acid/g seed tissue and amino acids as percent of total protein

Seed amino acid content was measured in dry seed from Cycles 4 and 7 and compared to the amino acid content of Mesa-Sirsa. Amino acids were analyzed by the Animal Sciences Laboratory at the University of Arizona. Unscarified seed was dried at 60° C for four hours, weighed and ground. A 0.1 gram sample of each population was hydrolyzed with 6N HCl, autoclaved for sixteen to eighteen hours at 121° C (15 psi) and then dried. Samples were filtered and reacted with o-phthaldehyde in order to form a fluorescent derivative. 0.1 μmole/ml of α-amino butyric acid was added to each sample as an internal standard. Amino acids were analyzed at room temperature on a Beckman HPLC with a Rainin C^{18} reverse phase column (methanol mobile phase) and a Beckman fluorescence detector over a run time of thirty minutes. Amino acid standards were analyzed in concentrations of 0.1 μmole/ml for each amino acid sampled. Proline and trytophan were not analyzed due to equipment constraints. Results were analyzed as a CRD design.

RESULTS AND DISCUSSION

EXPERIMENT 1: Comparison of Mesa-Sirsa and Cycles 1 through 7.

Percent Germination

Seeds must show high vigor under stressful conditions for successful emergence and establishment. Although the average difference in percent germination between the eight populations in the control was less than one percent, Cycles 2 through 7 germinated significantly better than Cycle 1 and Mesa-Sirsa (Figure 1A). Breeding for the ability to sprout under salt stress did not detract from germination under normal conditions and may have even enhanced it.

In a -1.7 MPa solution, a steady progressive increase in percent germination occurred through the cycles of selection with an average increase of 11% between all populations (Figure 1B). Cycle 6 showed only a 3% increase in percent germination in the -1.7 MPa solution compared to Cycle 5. Cycle 7 germinated nearly 17% better than Cycles 5 and 6 which indicated that the limit of the ability to productively exploit genetic variability for this trait had not been reached. Percent germination is a useful technique with which to evaluate seed tolerance to salt at high concentrations. Salt tolerance is a heritable characteristic and can be improved through recurrent selection and maintained over a generation of seed production.

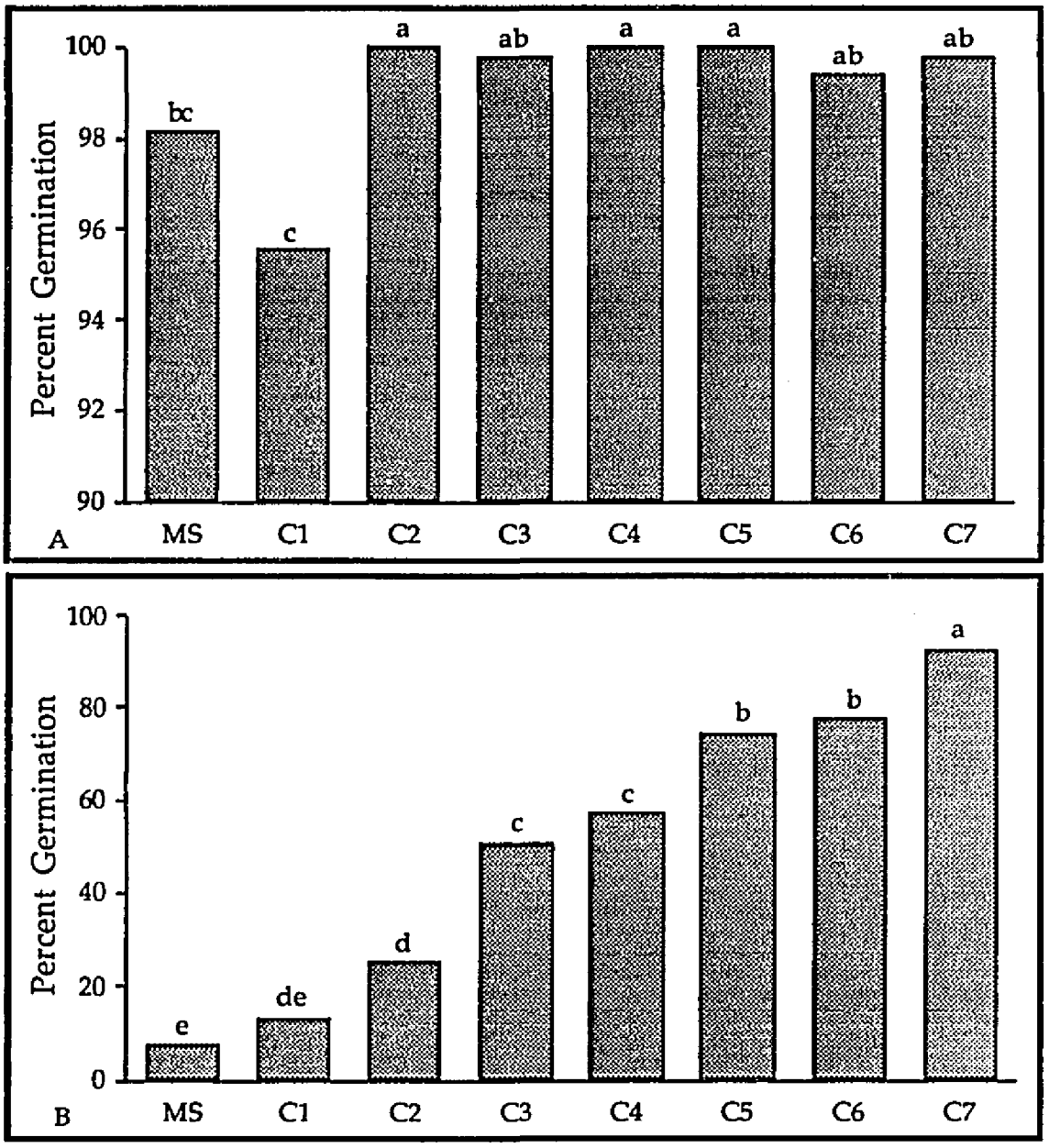

Figure 1: Percent germination of Mesa-Sirsa and Cycles 1 through 7 in a control (A) and a -1.7 MPa NaCl solution (B).

Germination Speed

No clear trend was observed in the differences between seed populations in the control as measured by germination speed (Figure 2). In contrast to percent germination, germination speed showed more differences in the intermediate -1.1 MPa solution compared to those at -1.7 MPa or in the control (Figure 3). In the -1.7 MPa solution there was an average difference in germination speed of only 2.87 between populations. Although the differences between populations as measured by germination speed were small, seed vigor as reflected by germination percent may be an important expression of seed salt tolerance. The speed of germination appeared to be a more sensitive index of germination salt tolerance and may be especially useful in screening seed response in moderately saline environments. On the other hand, percent germination is a much simpler technique and is suitable for measuring germination salt tolerance in very concentrated saline solutions.

Seed Weight

Robinson (1986) concluded that seed weight had no affect on salt tolerance of alfalfa populations and also that differences in seed weight were due to environmental or processing phenomena. Although there were significant differences in seed weight between the populations (Figure 4), there is no suggestion that these differences were related to seed salt tolerance. The test seed was produced in the same place and season under similar conditions implying that seed weight is highly sensitive to

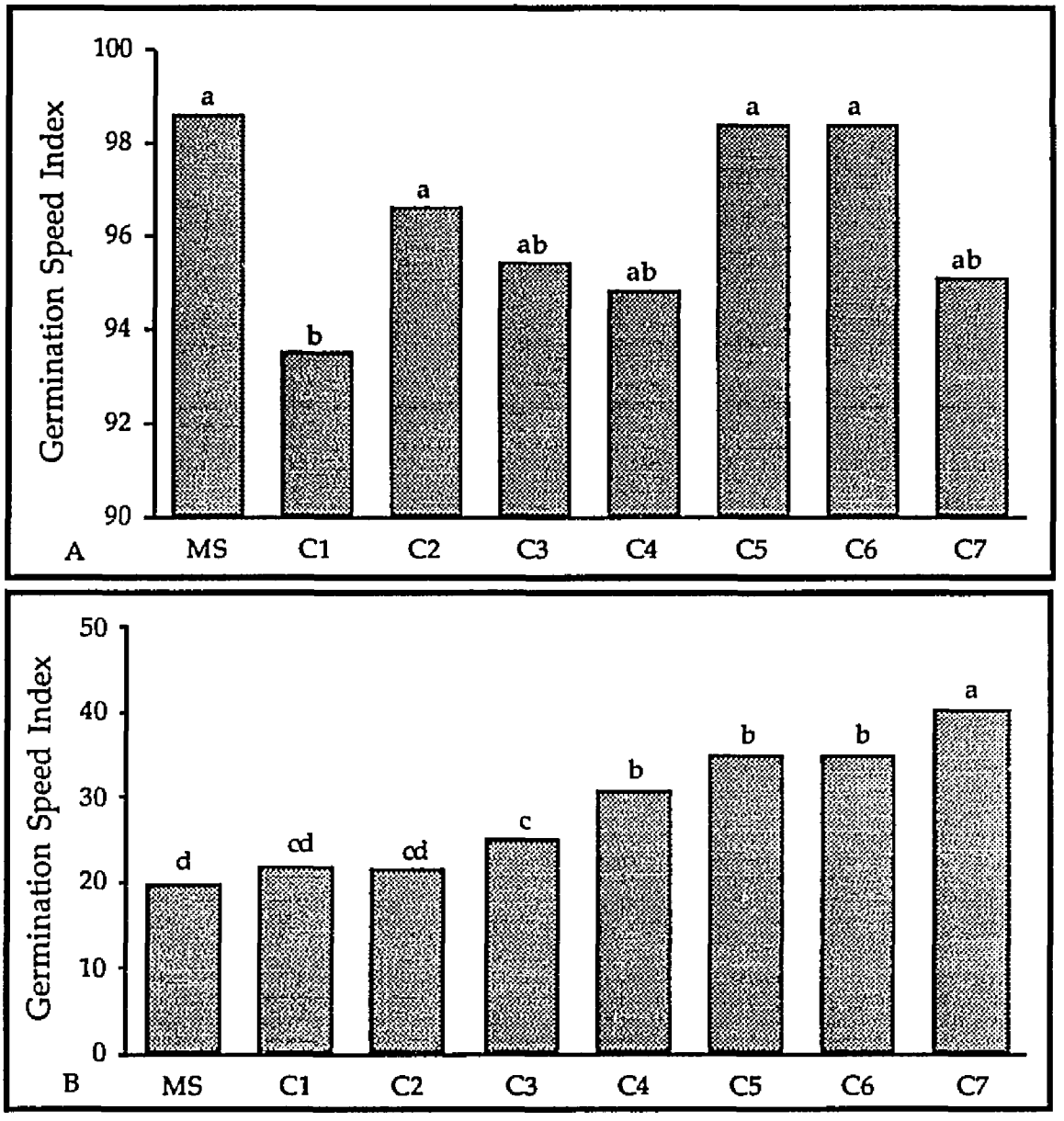

Figure 2: Germination speed of Mesa-Sirsa and Cycles 1 through 7 and in a control (A) and a -1.7 MPa NaCl solution (B).

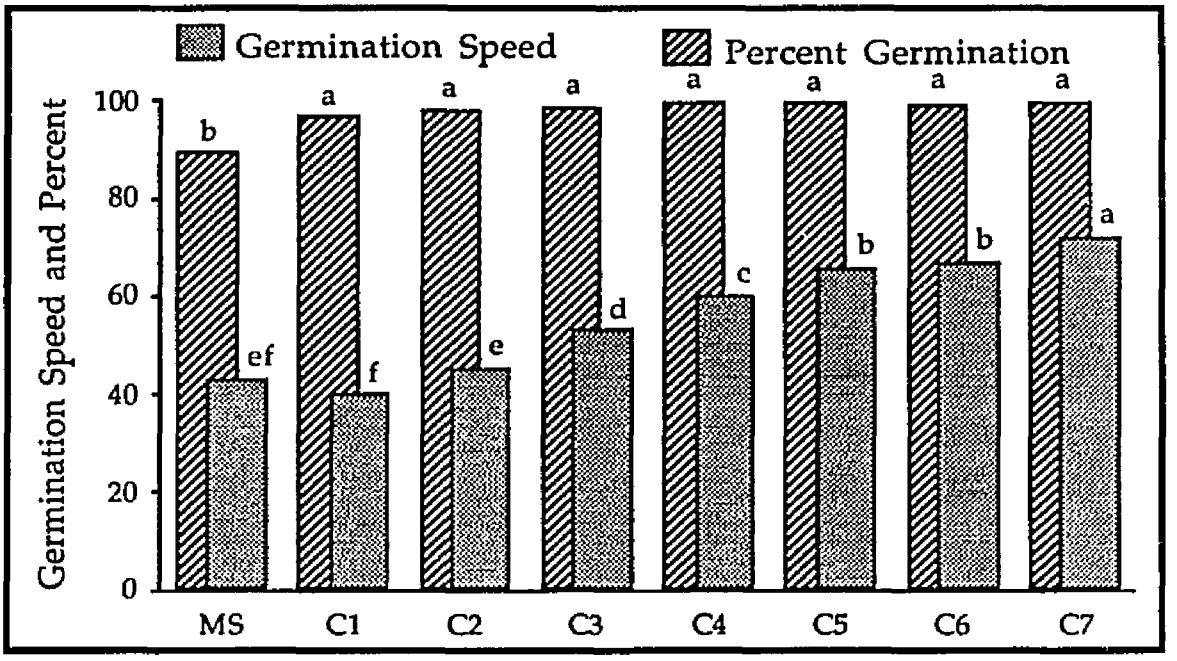

Figure 3: Germination speed and percent germination of Mesa-Sirsa and Cycles 1 through 7 in a -1.1 MPa NaCl solution.

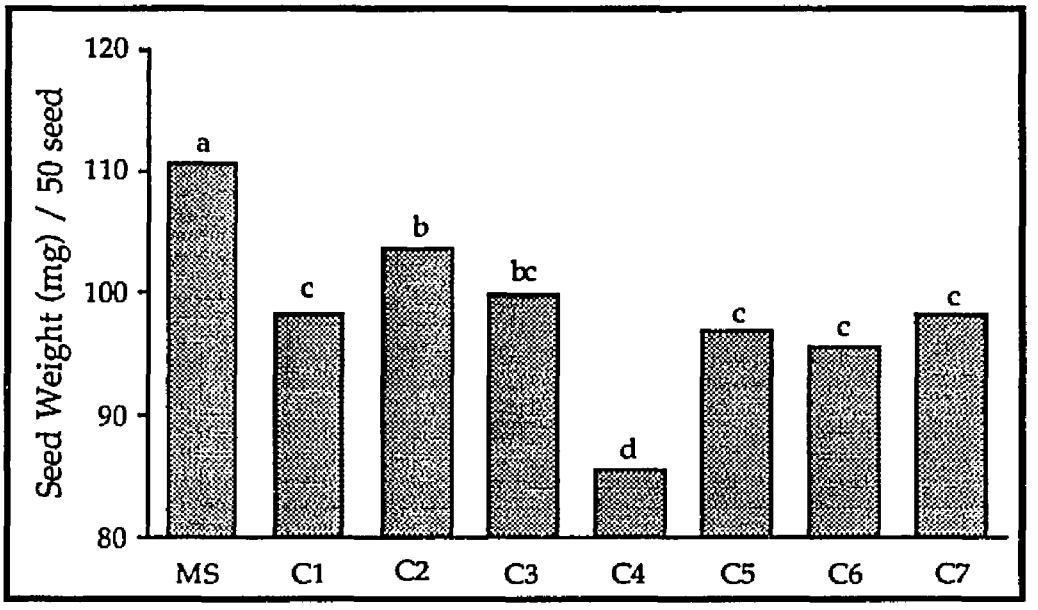

Figure 4: Seed weight of Mesa-Sirsa and Cycles 1 through 7.

undetermined environmental variation. Mesa-Sirsa weighed significantly more than any of the salt tolerant germplasm. In Experiment 4, however, two synthetic generations of Cycle 8 produced in 1987 weighed significantly more than Mesa-Sirsa. Selection for salt tolerance has not decreased seed weight.

EXPERIMENT 2: Comparison of Mesa-Sirsa and Cycles 1 through 9.

Percent Germination

The 1987 seed presented similar patterns of percent germination as the 1986 seed, with an average difference of 10.5% between cycles increased in 1987 (Figure 5). Between Cycles 7, 8 and 9, however, there was only a 1% difference in percent germination. Thus the final three cycles of recurrent selection appeared to be ineffective in enhancing germination salt tolerance.

Germination Speed

In contrast to experiment 1 (Figure 2), the germination speeds of the later cycles of selection at -1.7 MPa were not significantly different from each other (Figure 6). Cycle 9 showed a slight decline in germination speed. Figure 6 demonstrates one of the difficulties in using germination speed alone to measure seed salt tolerance. Mesa-Sirsa had a better speed of germination in a saline solution than selected cycles 1 through 6. Figure 5 shows, however, that Mesa-Sirsa only germinated 2.5% under salt stress.

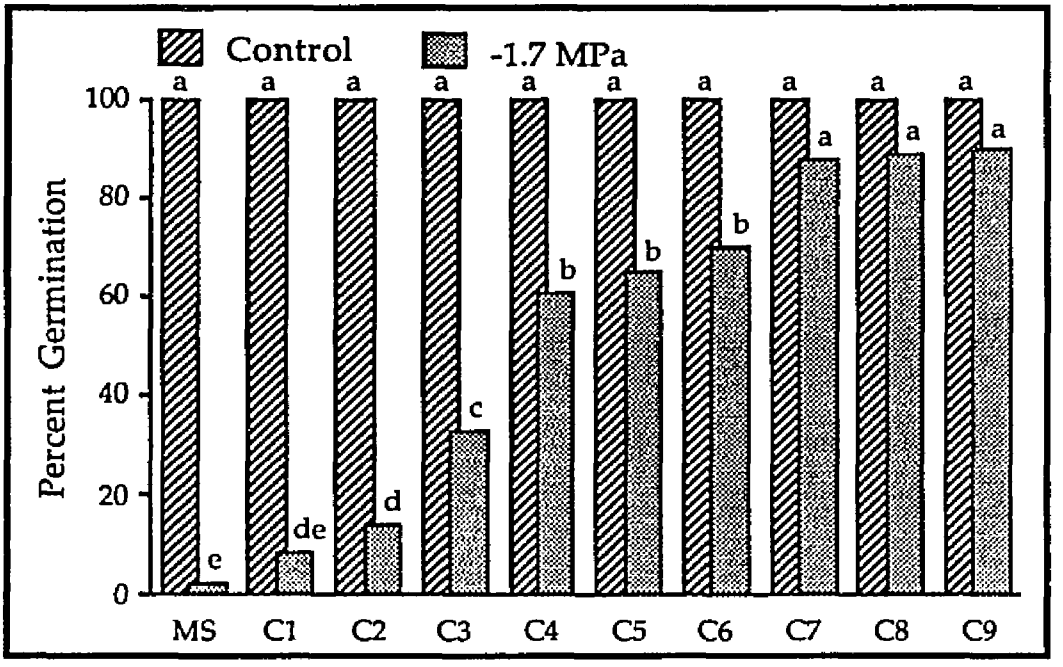

Figure 5: Percent germination of Mesa-Sirsa and Cycles 1 through 9 in a control and a -1.7 MPa NaCl solution.

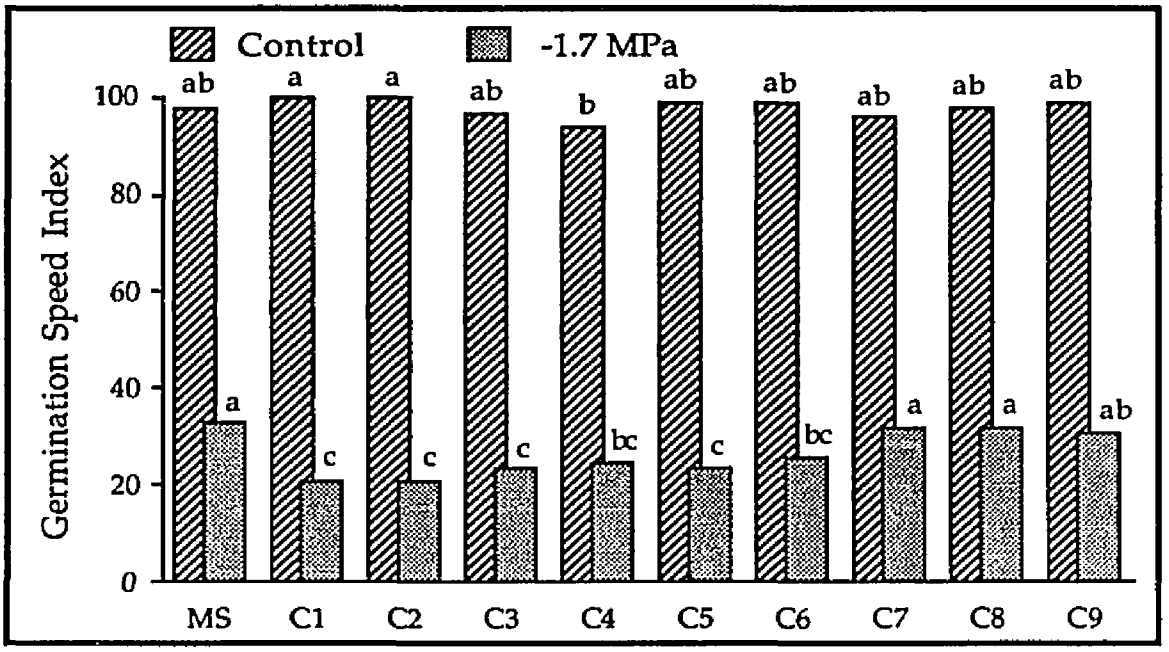

Figure 6: Germination speed of Mesa-Sirsa and Cycles 1 through 9 in a control and a -1.7 MPa NaCl solution.

Although only one Mesa-Sirsa seed out of forty managed to germinate (on the third day in both cases), it did so early enough to score well in terms of germination speed. It is recommended that germination speed not be measured without also determining percent germination. Alternatively, an index can be used which considers both germination speed and percent germination by eliminating the denominator from the germination speed index equation.

EXPERIMENT 3: Comparison of Cycles 1 through 7 produced in 1986 and Mesa-Sirsa and Cycles 1 through 8 produced in 1987.

Figures 7A and 7B show the percent germination and germination speed of the 1986 seed lot compared to the 1987 seed lot. The 1986 and 1987 seed of Cycle 1 in this case germinated at 0 and 0.3% at -1.7 MPa. The p-value (or significance level) for the test of percent germination of the 1986 seed compared to the 1987 seed in a -1.7 MPa solution was 0.25 indicating no significant differences between the two groups of seed. The p-value for the test of germination speed of the two seed lots compared to each other was 0.1 indicating that the two seed lots had comparable speeds of germination. This experiment demonstrated the genetic stability of germination salt tolerant alfalfa seed produced in different environments. Smith and Dobrenz (1987) determined that seed age influenced the expression of germination salt tolerance negatively. This experiment indicated that the seed age effect did not take place within one year.

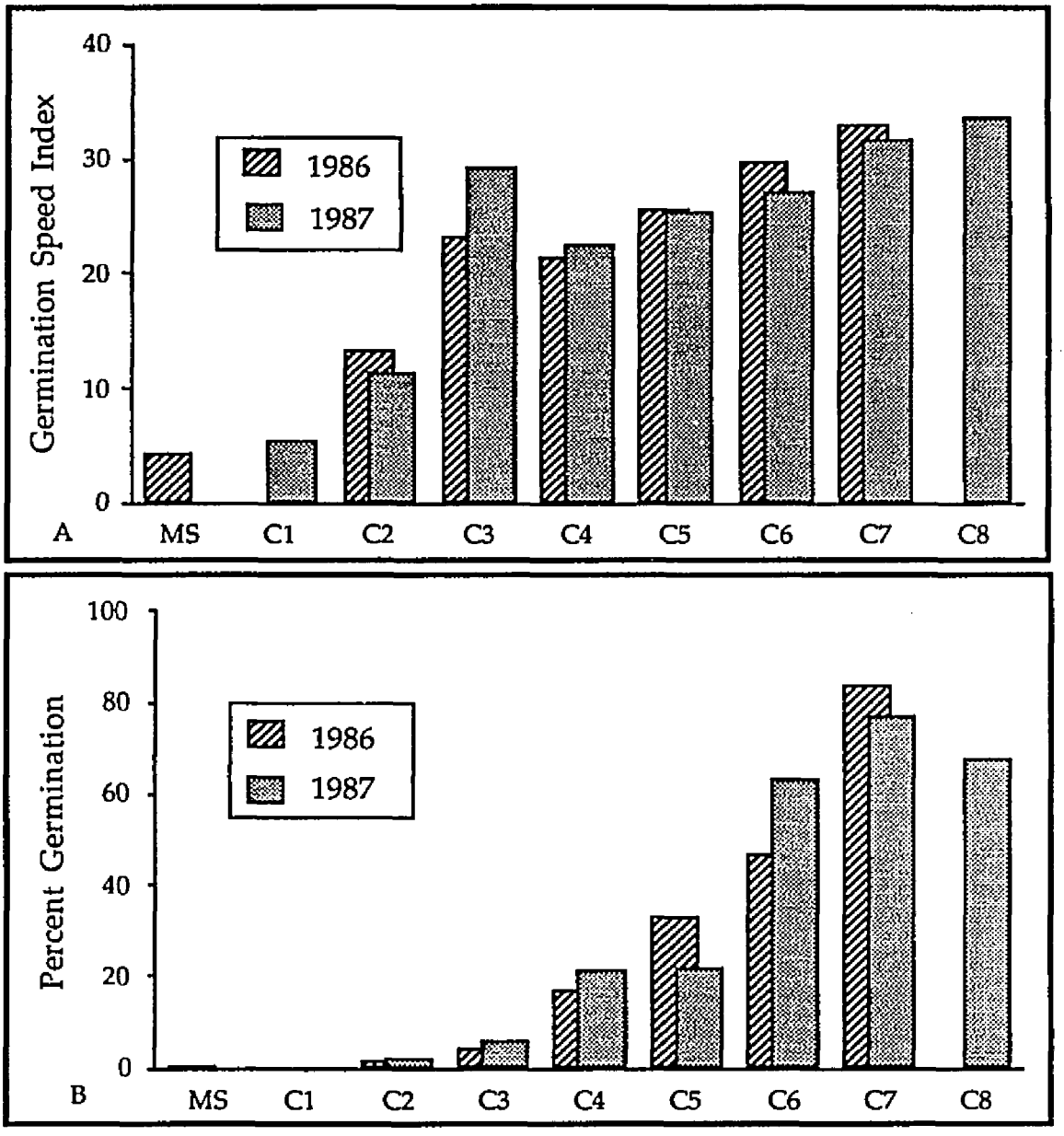

Figure 7: Germination speed (A) and percent germination (B) of Cycles 1 through 7 produced in 1986 and Mesa-Sirsa and Cycles 1 through 8 produced in 1987 in a -1.7 MPa NaCl solution.

EXPERIMENT 4: Determination of genetic shift in the salt tolerance of Mesa-Sirsa and Cycle 8.

Mesa-Sirsa and the three synthetic generations showed the most vigorous percent germination, germination speed and radicle length in the absence of NaCl. Percent germination of Mesa-Sirsa steadily decreased at each succeeding salt concentration (Table 11). The Syn 1-3 generations of Cycle 8 displayed greater than 90% germination until -2.0 MPa, where average germination dropped to 55% overall. There was no evidence of a consistent decline in percent germination between the three synthetic generations. Cycle 8 Syn 3 showed superior germination at all salt levels compared to Cycle 8 Syn 1 and 2 which may have been due to inadvertent selection for seedling vigor during seed multiplication. Germination speed showed a significant decline for all three generations at -1.2 MPa and -2.0 MPa (Table 12). Cycle 8 Syn 3 also showed greater germination speed compared to Syn 1 and 2 at all salt levels. Radicle length was the most sensitive measure of response to stress. Radicle length declined an average of 91% between 0 and -2.0 MPa overall compared to a decrease of 43% and 70% for percent germination and germination speed, respectively. (Tables 11-13). As with germination speed there were no reductions in tolerance as measured by radicle length between the three Cycle 8 populations at any of the levels of salinity tested (Table 13). Three generations of seed multiplication did not lead to any adverse changes (genetic shift) in germination salt tolerance. This suggests that seed production of germination salt tolerant alfalfa may be conducted without loss of tolerance at non-saline sites.

Table 11: Mean separations for dry seed weight and percent germination of Mesa-Sirsa and three synthetic generations of Cycle 8 under four NaCl regimes.

			Percent Germination			
Population	Generation	Dry weight. 50 seed (mg)	NaCl concentration (-MPa) 0	0.6	1.2	2.0
Cycle 8	Syn 1	107	97.0	97.8	93.9	49.0
Cycle 8	Syn 2	102	94.4	93.9	94.9	58.6
Cycle 8	Syn 3	109	99.7	100.0	98.2	61.5
Mesa-Sirsa		100	100.0	88.2	3.9	0.3
{LSD (5%)}		4	1.4	3.2	2.8	1.5

Table 12: Mean separations for dry seed weight and germination speed indices of Mesa-Sirsa and three synthetic generations of Cycle 8 under four NaCl regimes.

			Germination Speed Index			
Population	Generation	Dry weight. 50 seed (mg)	NaCl concentration (-MPa) 0	0.6	1.2	2.0
Cycle 8	Syn 1	107	98.0	94.8	72.6	28.8
Cycle 8	Syn 2	102	98.6	98.0	80.9	29.7
Cycle 8	Syn 3	109	99.9	99.6	83.1	30.8
Mesa-Sirsa		100	95.4	54.1	24.5	1.1
{LSD (5%)}		4	1.1	1.9	6.6	3.0

Table 13: Mean separations for dry seed weight and radicle length (cm) of Mesa-Sirsa and three synthetic generations of Cycle 8 under four NaCl regimes.

			Radicle Length (cm)			
Population	Generation	Dry weight. 50 seed (mg)	NaCl concentration (-MPa) 0	0.6	1.2	2.0
Cycle 8	Syn 1	107	4.74	4.34	2.29	0.32
Cycle 8	Syn 2	102	4.49	4.12	2.61	0.35
Cycle 8	Syn 3	109	4.86	4.56	2.69	0.40
Mesa-Sirsa		100	4.67	3.32	0.13	0
{LSD (5%)}		4	0.34	0.39	0.21	0.11

The phenomenon of germination salt tolerance is only valuable so long as it is genetically stable. Experiments 1 through 4 demonstrate that the factors that contribute to seed salt tolerance are stable and that the causes of this tolerance are genetic in origin.

EXPERIMENT 5: Evaluation of the effect of seedcoat scarification on Mesa-Sirsa and Cycle 8.

Scarification is commonly used to prepare alfalfa seed for tests of germination. Scarification disrupts the integrity of the testa and exposes underlying seed tissues, including the endosperm. It was important to take into account the extent of the influence of scarification in order for germination tests to be evaluated accurately. Additionally, a comparison of scarified and nonscarified seed might indicate whether physical factors contributed to seed salt tolerance.

Overall, scarification of alfalfa seed lead to increased percent germination, germination speed and radicle length in the control solution (Table 14). However, nonscarified seed performed better than the scarified treatment as measured by all three parameters in a -2.0 MPa saline solution. For percent germination there were no significant differences between scarified and nonscarified seed in the control. Scarification does not harm germination in a nonstressed environment. On the other hand, nonscarified seed germinated significantly better than scarified seed in the salt treatment. Alternatively it may be conjectured that scarification caused

Table 14: Mean separations for salt x treatment interactions of three parameters for scarified and nonscarified alfalfa seed in control and saline solutions.

	Percent Germination		Germination Speed		Radicle Length (cm)	
MPa	Scarified	Nonscarified	Scarified	Nonscarified	Scarified	Nonscarified
0	98 a	96 a	98 a	92 b	4.6 a	4.4 b
-2.0	36 b	49 a	20 a	22 a	0.3 b	0.4 a

decreased germination in the saline solution. Scarification significantly increased germination speed in the control solution. Germination speed was not improved in a saline environment. Radicle length, the most sensitive measure of seedling vigor, showed significant differences between scarified and nonscarified seed in both the control and the salt solution. Scarification appears to enhance seed vigor under control conditions but not enough to compensate for the protection against ionic stress under NaCl conditions. The intact testa and other surface tissues may offer some temporary relief to the embryo from NaCl. Alternatively, in the face of rapid water uptake tissues underlying the intact testa including mucilaginous polymers may serve to moderate powerful volumetric changes which might otherwise damage sensitive cotyledonary and radicle structures as demonstrated by Leopold (1983).

EXPERIMENT 6: Evaluation of relative fresh weight and percent germination of Mesa-Sirsa and Cycle 8.

Reid and Bewley (1979) proposed that galactomannan reduced the concentration of water in seed and also prevented water loss from the endosperm surrounding the embryo during germination. Reid and Bewley (1979) implied that there might be proportionally more galactomannan in stress tolerant seed than in a non-tolerant selection which would increase the amount of water surrounding the seed's embryo. Another possibility, given the differential hydrophilicity of polysaccharide conformations is that different proportions of galactomannan relative to other endosperm sugars

or a different ratio of the galactose and mannose units constituting the galactomannan would increase the absorptive capacity of the endosperm.

Relative Fresh Weight

The initial decline in seed fresh weight (Figure 8) was characteristic of alfalfa germination (Robinson, 1986). There were generally no differences in absorption between the control and saline treatments until 7.5 hours after planting (HAP) which represents the phase of matric water uptake. Cycle 8 absorbed less water during this period, but the differences were not consistently significant. The treatment curves diverged after 7.5 HAP. Absorption slowed in the salt treatment at 9 HAP and in the control at 11.25 HAP which represented the beginning of the second or lag phase of germination. Seed in the control treatment absorbed 18% more than the salt treatment during the lag phase. Cycle 8 had a lower relative fresh weight gain than Mesa-Sirsa during the lag phase, although the differences were not consistently significant. In the control, Cycle 8 absorbed 17.5% more than Mesa-Sirsa after 15 HAP, but both populations had achieved nearly complete germination by that point (Figure 9). Eventually there was a 26% difference in absorption between the treatments by the end of the experiment. The water activity of the first two phases of germination was crucial to radicle protrusion whereas water absorption after that may have been more important to the growth of the emergent seedling (Figure 8).

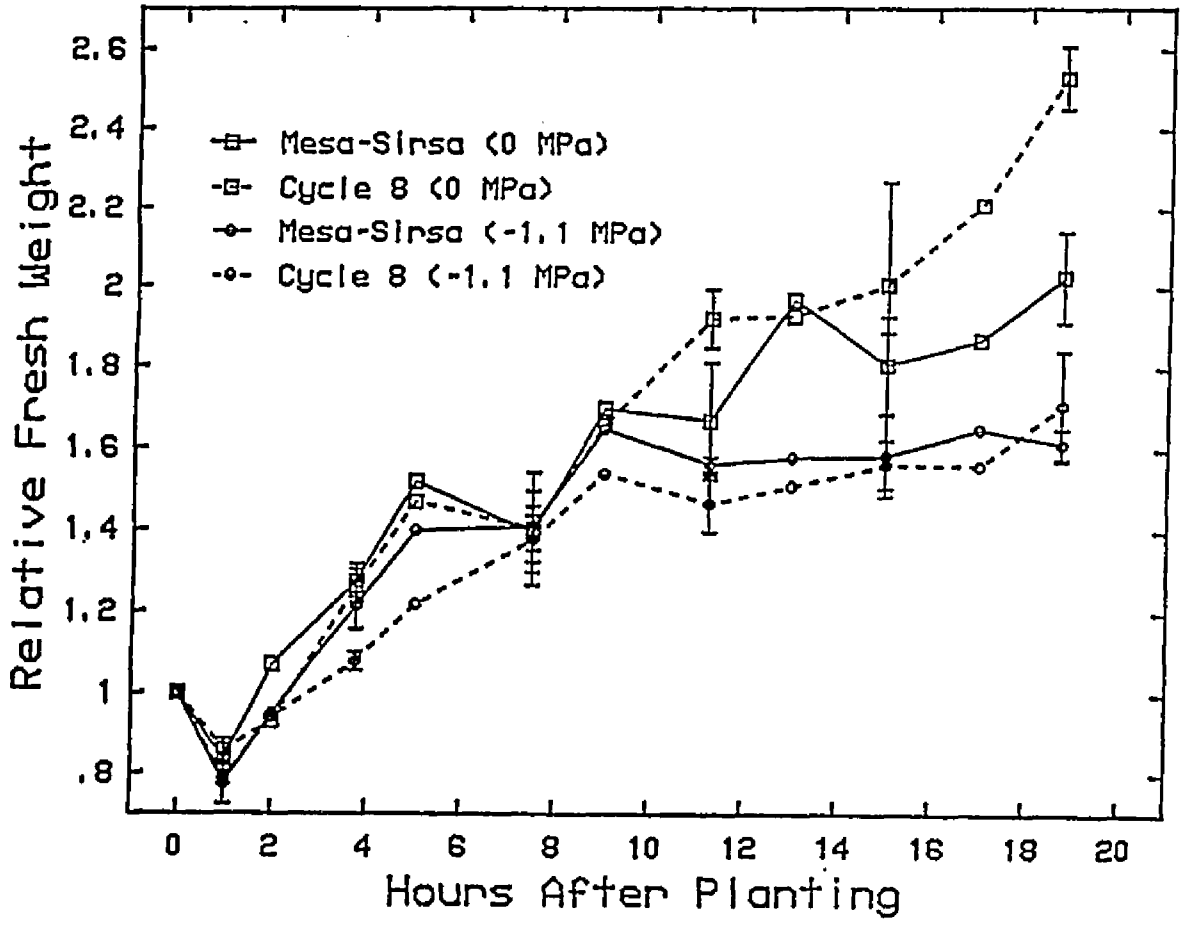

Figure 8: Relative fresh weight of Mesa-Sirsa and Cycle 8 over 18.75 hours.

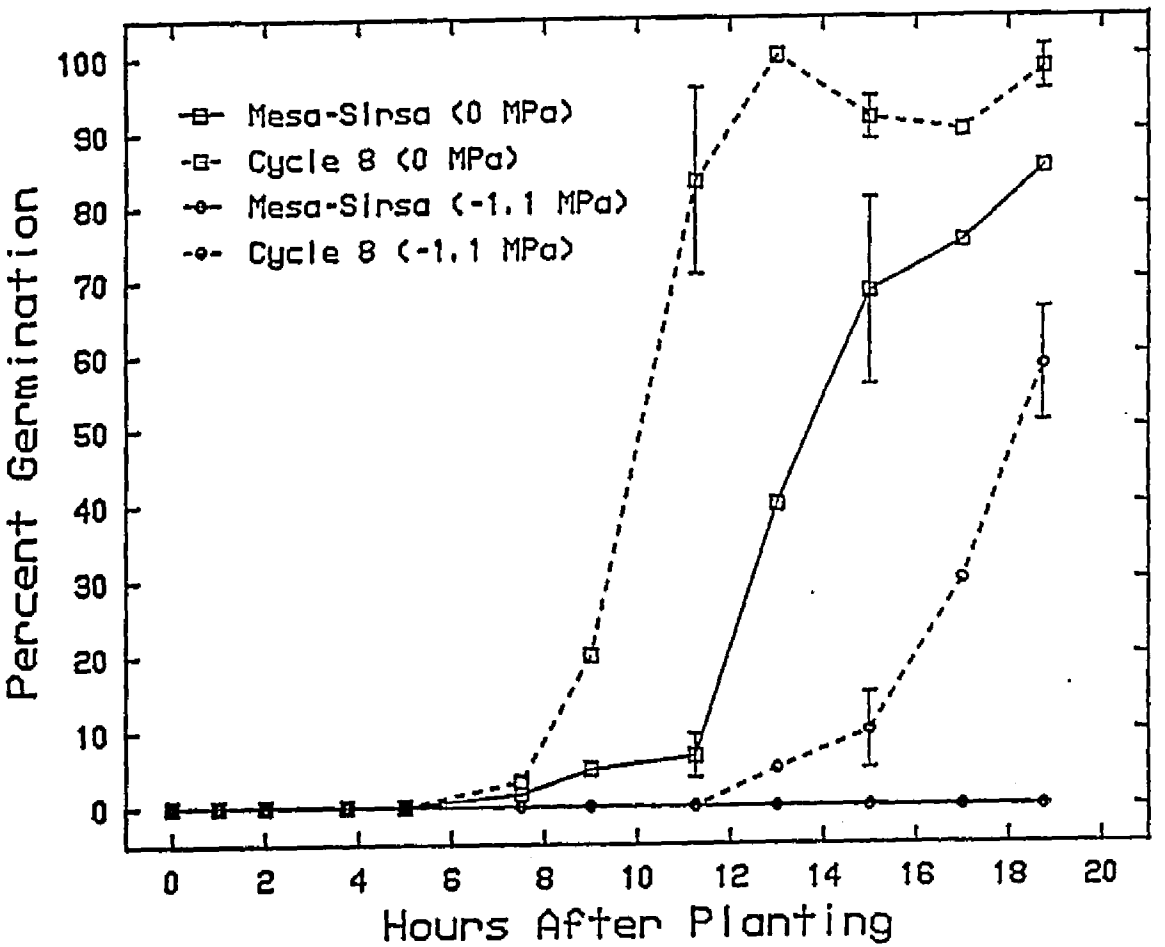

Figure 9: Percent germination of Mesa-Sirsa and Cycle 8 over 18.75 hours.

Seed selected for salt tolerance did not absorb significantly more water than non-tolerant seed during germination in both treatments. In the salt treatment Cycle 8 had a lower relative fresh weight gain than Mesa-Sirsa until 18.75 HAP. Contrary to the results of Stout et al. (1980) osmotic solutions did have an effect on seed absorption during germination. Allen (1986) suggested that increased osmotic water uptake made little difference in seed performance under stress agrees with the data in Figure 8. Germination salt tolerance in selected alfalfa germplasm does not depend on the gross imbibitional capacity of the seed. The seed's mechanism for compensating with osmotic stress does not lie in greater water absorption.

Percent Germination

Cycle 8 germinated more vigorously compared to Mesa-Sirsa in both stressed and nonstressed environments (Figure 9). Although the populations started germinating at approximately the same time, percent germination of Cycle 8 was 76% greater than Mesa-Sirsa as early as 11.25 HAP. The germination speed of Cycle 8 was diminished in the salt treatment compared to the control.

EXPERIMENT 7: Determination of the effect of selection on seed polysaccharide content.

Galactomannan was the most important component of the carbohydrates identified in the analysis of the insoluble seed residues (Figure 10A). Galactomannan demonstrated a progressive increase in terms

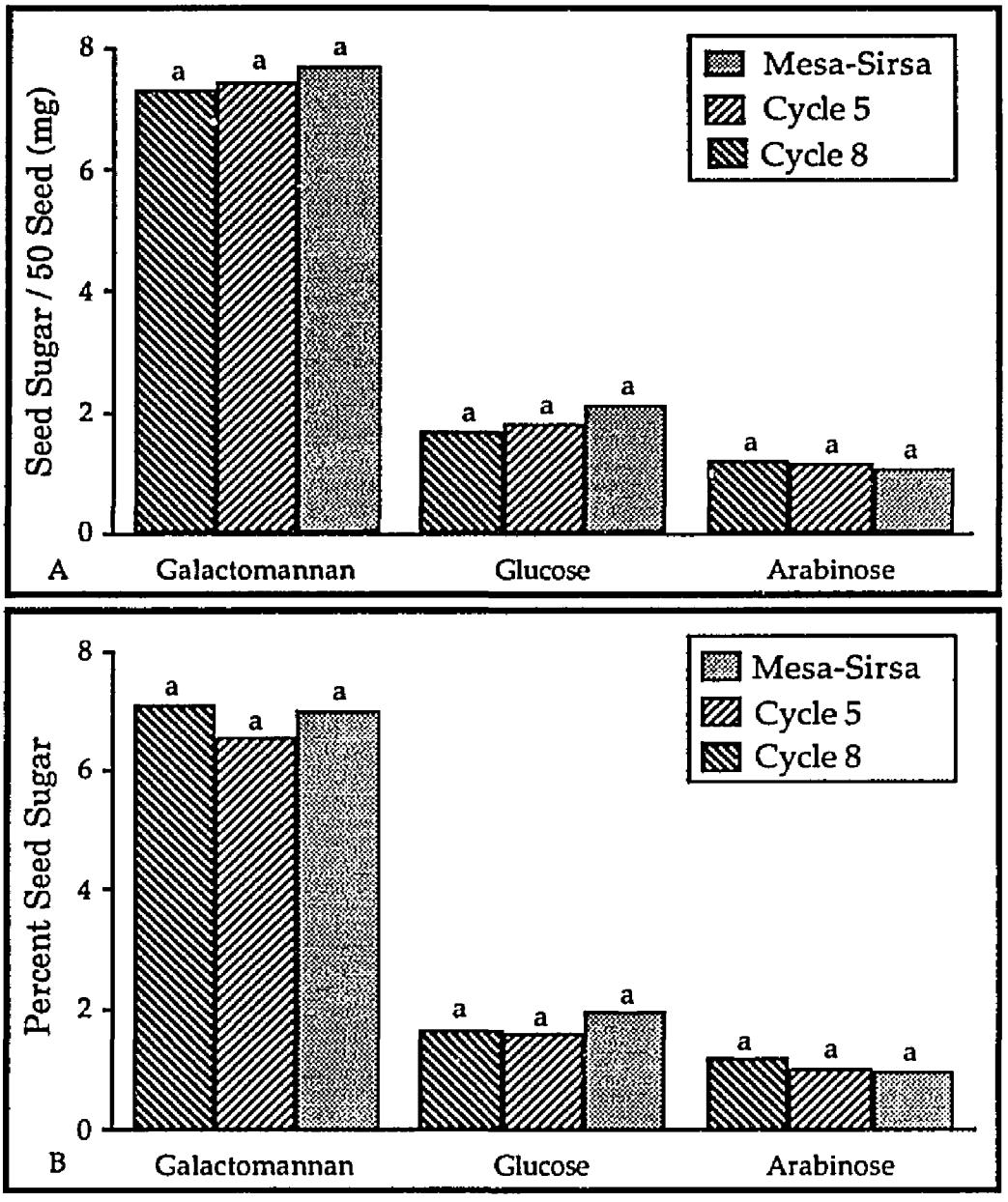

Figure 10: Seed sugar/50 seed (A) and percent seed sugar (B) of Mesa-Sirsa and Cycles 4 and 8 after acid hydrolysis. Galactomannan is taken as the composite of the galactose and mannose peaks following HPLC analysis.

of mg/50 seed but this was due to differences in seed weight. There was only about a fourth as much glucose and arabinose. Expressed as percent of the seed there were no significant differences in galactomannan between Mesa-Sirsa, Cycle 8 and Cycle 5 (Figure 9B). The amount of galactomannan falls in the mid-range of estimates made for alfalfa seed by other researchers (Dea and Morrison, 1975; McCleary and Matheson, 1975).

Reid and Bewley (1979) stated that galactomannan acted as a sponge and surrounded the embryo of the seed with water in order to protect it from dessication. A greater amount of galactomannan in the seed of one variety compared to another seed of the same variety might be expected to offer a comparative advantage in terms of dessication tolerance. This does not appear to be the case with alfalfa seed selected for germination salt tolerance. The work of Gonzalez-Murua et al. (1985) previously presented similar data. The drought and salt tolerant variety, 'Tierra de Campos' germinated better than 'Aragon' under salt stress although it contained 3% less carbohydrate in its endosperm, and 0.5% less galactomannan compared to other seed polysaccharides.

EXPERIMENT 8: Determination of the effect of selection on seed free sugar content

Stachyose was the major reserve oligomer in Mesa-Sirsa and in the cycles selected for germination salt tolerance (Figure 11A). There was

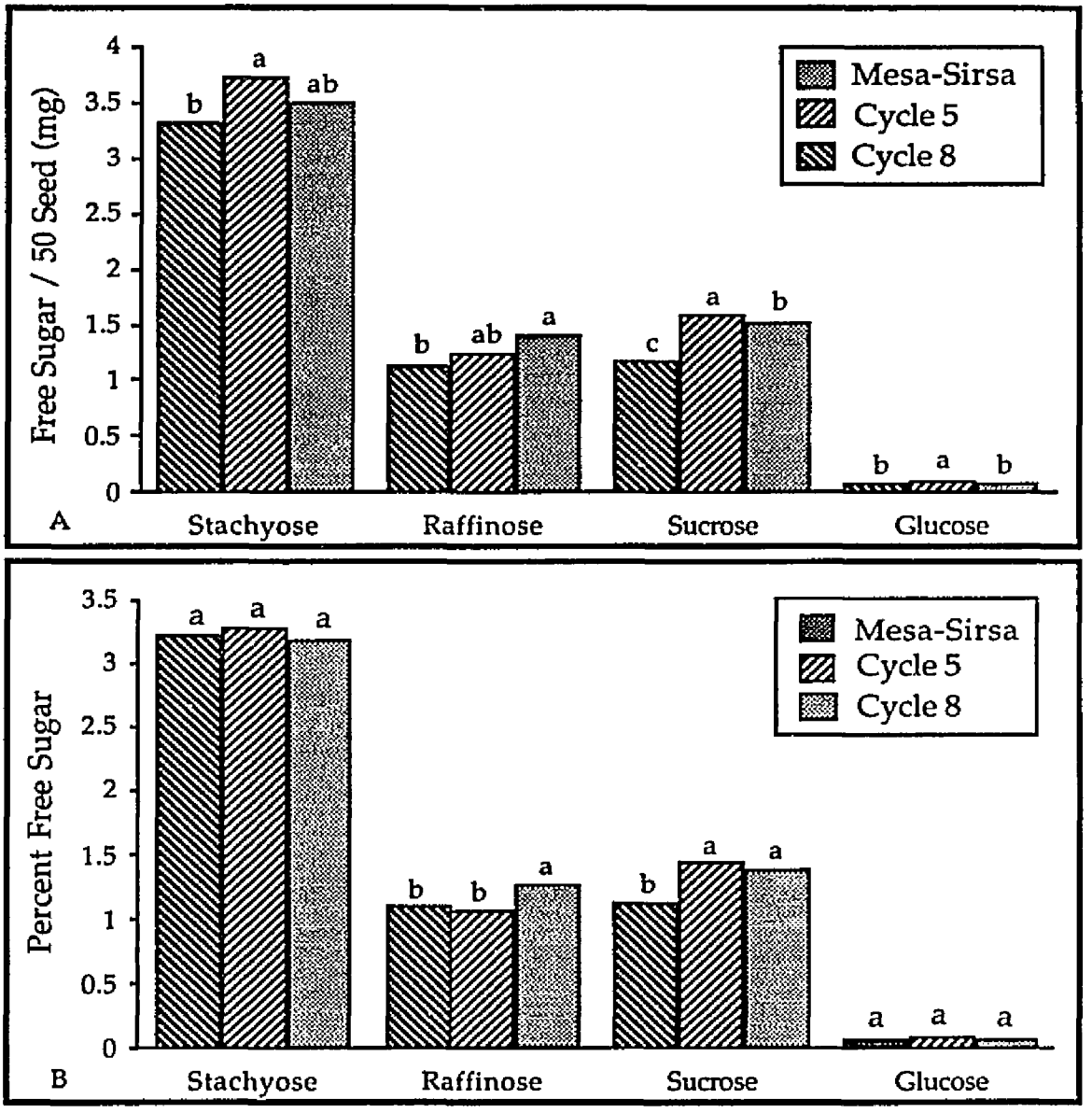

Figure 11: Total free sugar/50 seed (A) and percent free sugar (B) of Mesa-Sirsa and Cycles 4 and 8.

significantly more stachyose in Cycles 5 and 8 compared to Mesa-Sirsa but this was due to differences in seed size. Raffinose and sucrose may have been increased significantly as a consequence of selection for salt tolerance, but not glucose. Stachyose was not significantly different between the populations when expressed as percent of seed (Figure 11B). The statistically significant increase in percent raffinose probably occurred after selection for Cycle 5, so that the increase may not be a consequence of salt tolerance selection. Sucrose was significantly different as mg/seed and percent of free sugar, indicating that it may have been increased through the cycles of selection. Nevertheless, sucrose accounted for only 1.5% of the total seed weight and likely has little role in seed salt tolerance. Mercier (1985) and Gonzalez-Murua et al. (1985) suggested that hydrolyzed sugars from polysaccharides or seed monosaccharides might act as solutes and help to maintain a proper water balance in the seed. There is no evidence for this in alfalfa seed as a comparative advantage in the cycles selected for salt tolerance versus Mesa-Sirsa.

EXPERIMENT 9: Determination of the effect of selection on seed protein content.

The ten populations in this experiment were analyzed for total nitrogen per seed (Figure 12A). Mesa-Sirsa had the lowest amount of nitrogen per seed and Cycle 9 the highest, but there was no discernible trend among the other populations which would indicate that total nitrogen content was regularly affected by selection for germination salt tolerance. This was confirmed by

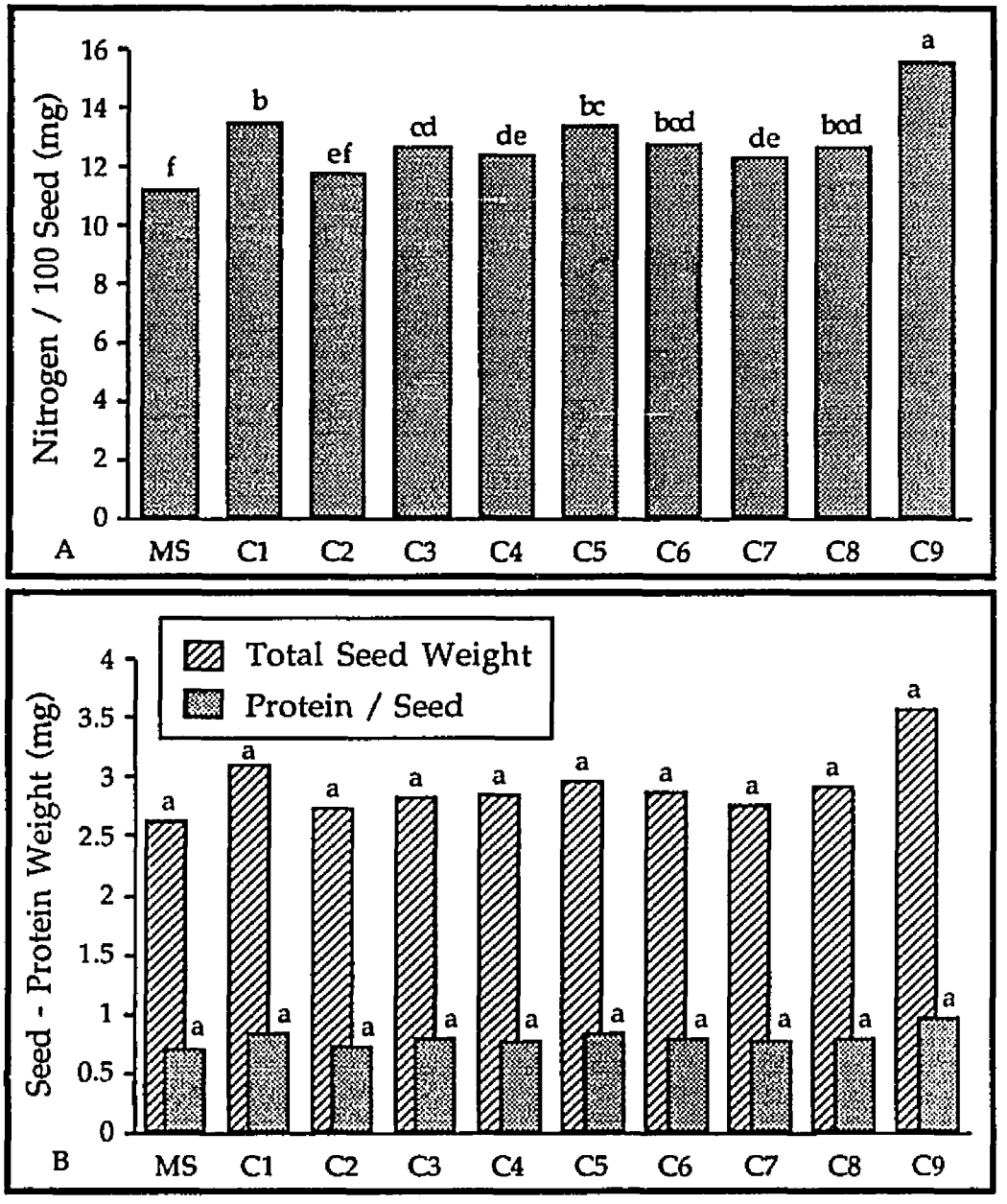

Figure 12: Total nitrogen/100 seed (A) and seed - protein weight (B) of Mesa-Sirsa and Cycles 1 through 9.

the absence of statistically significant differences in percent protein between the populations (Figure 12B). Nevertheless, Cycle 9 still had a greater amount of protein as part of the seed weight compared to Mesa-Sirsa which had the least amount of protein of any of the populations.

EXPERIMENT 10: Determination of the effect of selection on seed amino acid content.

Amino acids, expressed as μmoles per gram of seed, have been very stable throughout the process of breeding for salt tolerance (Table 15). There were no significant differences between the populations except for threonine and valine, which increased and decreased, respectively, as a result of seven cycles of recurrent selection. Valine significantly decreased in Cycles 4 and 7 compared to Mesa-Sirsa. Except for asparagine and threonine, similar decreases occurred in seven of the sixteen amino acids expressed as percent of protein (Table 16). The changes in the quantities of amino acids distinguished the selected cycles from Mesa-Sirsa. Experiment 9 showed no changes between the populations in percent protein, therefore, component materials of certain amino acids have been put to use in other molecules. There were no major changes in total amino acids which indicated that selection pressure for salt tolerance was not associated with amino acid content.

Table 15: Mean separations for µmoles of amino acids per gram of seed in Mesa-Sirsa, Cycle 4 and Cycle 7.

	µmoles of amino acids per gram of seed		
Amino Acids	Mesa-Sirsa	Cycle 4	Cycle 7
Asparagine	251.4 a	241.7 a	236.0 a
Glutamine	354.8 a	333.5 a	338.3 a
Serine	137.5 a	134.3 a	132.2 a
Histidine	51.9 a	49.5 a	50.0 a
Glycine	213.4 a	205.9 a	237.8 a
Threonine	74.5 b	73.9 b	97.3 a
Arginine	200.3 a	174.5 a	179.9 a
Alanine	151.6 a	142.6 a	139.0 a
Tyrosine	71.5 a	57.8 a	56.9 a
Cysteine	35.8 a	46.7 a	36.9 a
Methionine	29.7 a	27.9 a	28.1 a
Valine	123.4 a	114.3 b	113.5 b
Phenylalanine	81.2 a	77.9 a	77.0 a
Isoleucine	98.9 a	93.1 a	90.1 a
Leucine	185.6 a	174.1 a	169.1 a
Lysine	148.6 a	132.4 a	141.6 a

Means within a row followed by the same letter are not significantly different at the .05 probability level.

Table 16: Mean separations for amino acids as percent of seed protein in Mesa-Sirsa, Cycle 4 and Cycle 7.

	Amino acids (percent of protein)		
Amino Acids	Mesa-Sirsa	Cycle 4	Cycle 7
Asparagine	9.2 a	8.6 b	8.1 c
Glutamine	14.3 a	13.1 b	12.9 b
Serine	3.9 a	3.8 a	3.6 a
Histidine	2.2 a	2.1 a	2.0 a
Glycine	4.4 a	4.2 a	4.6 a
Threonine	2.4 b	2.4 b	2.9 a
Arginine	9.6 a	8.2 b	8.1 b
Alanine	3.7 a	3.4 b	3.2 b
Tyrosine	3.6 a	2.8 a	2.7 a
Cysteine	1.2 a	1.5 a	1.1 a
Methionine	1.2 a	1.1 b	1.1 b
Valine	3.9 a	3.6 b	3.4 b
Phenylalanine	3.7 a	3.5 a	3.3 a
Isoleucine	3.5 a	3.3 ab	3.1 b
Leucine	6.7 a	6.1 ab	5.7 b
Lysine	5.9 a	5.2 a	5.4 a

Means within a row followed by the same letter are not significantly different at the .05 probability level.

SUMMARY

Ten experiments were performed using alfalfa seed developed during a breeding program for germination salt tolerance and the non-tolerant parental cultivar, Mesa-Sirsa. With the exception of the Mesa-Sirsa used in experiment 1, the seed in the experiments was of the same age. With a few noted exceptions the seed also represented the second synthetic generation. Seed which shared the same production environment and generation may have provided more accurate comparisons of performance under stress.

Percent germination under salt stress was improved nearly 11% with every cycle of selection (experiments 1 and 2). Germination speed provided a sensitive technique with which to measure germination salt tolerance at moderate levels of salinity (experiment 1). Problems with the germination speed technique in experiment 2 demonstrated the need to simultaneously measure percent germination or use another modified index of germination speed. Seed weight was not related to performance under salt stress and was highly variable (experiments 1 and 4). The germination salt tolerance of alfalfa seed did not deteriorate significantly after one year of storage which demonstrated the stability of this trait (experiment 3). Germination salt tolerance did not decline after seed increase at non-saline sites (experiment 4). Radicle length was a very sensitive measure of seedling vigor under salt stress but was less useful in measuring germination exclusively (experiment 4).

Scarification of seed for research purposes led to decreased germination in a saline solution (experiment 5). The intact seed coat or underlying tissues may offer some physical relief from salt stress or volumetric changes. Alternatively, scarification might depress germination to some extent by damaging the embryo and have nothing to do with salinity stress.

Germination salt tolerant alfalfa and Mesa-Sirsa shared the same patterns of absorption in saline and control solutions during the first two phases of germination (experiment 6). Cycle 8 imbibed less water than Mesa-Sirsa in the salt treatment. Increased absorption of the germination medium is not part of the alfalfa seed's response to salt stress which suggests that other mechanisms are responsible for adjustment to osmotic stress. Cycle 8 germinated more vigorously than Mesa-Sirsa in the control and the saline solutions.

There was more galactomannan in alfalfa seed than any of the other sugars (*after acid* hydrolysis) or free sugars (experiments 7 and 8). Galactomannan represented 6.9% and stachyose 3.23% of the total seed weight but the percentages were not significantly different between Cycles 5, 8 and Mesa-Sirsa. Sucrose was the only sugar which showed a significant increase from Mesa-Sirsa through Cycle 8 but its impact is probably minimal due to its quantity (experiment 8). Although galactomannan and other seed sugars may play a minor role in seed water relations, they also have not decreased during the process of selection for

salt tolerance. Seed sugars do not offer the primary relief from osmotic stress in alfalfa seed. Seed galactomannan did not significantly affect germination of alfalfa seed under salt stress and did not increase after nine cycles of recurrent selection for germination salt tolerance.

The protein content of alfalfa seed did not change significantly as a result of selection for stress tolerance, although Cycle 9 had more protein per seed than the other populations (experiment 9). Differences in milligrams of nitrogen per one hundred seed were due to seed weight.

The amino acid content of alfalfa seed as measured in μmoles per gram of seed weight proved stable through the cycles of selection (experiment 10). Approximately half of the amino acids measured as percent of protein showed a significant decrease (experiment 10). The other half of the amino acids did not show a proportional increase, suggesting that the chemical constituents of the decreasing amino acids are incorporated in protein material elsewhere in the seed. It is recommended that a study be performed of the patterns of changes in amino acids over the same time course as experiment 4. Additionally, experiment 10 and evidence from other researchers suggests hormone or enzyme activity as key factors in seed stress tolerance.

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