Preliminary evaluation of selection criteria for drought resistance in alfalfa (Medicago sativa L.)
by John Robert Carlson Jr

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Philosphy in Crop and Soil Science
Montana State University
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Abstract:
Drought reduces alfalfa yield and quality. The objectives of this study were to: 1) develop screening procedures to assay alfalfa germ- plasm for drought resistance traits, and 2) determine if genetic progress could be made for these traits.

. Traits studied included stomatal density, seedling root pattern, resistance to wilting, leaf color, leaflet size, and germination salt tolerance.

The considerable amount of within-plant variability for stomatal density existing in alfalfa, necessitating the sampling of leaflets from an extremely large number of stems, prevented the use of this trait as a selection criterion for drought resistance in alfalfa.

Selection for seedling root pattern and for resistance to wilting indicated that these traits were lowly heritable. Selection for these traits will result in slow progress.

Selection for leaf color, leaflet size, and germination salt tolerance indicated that these traits were highly heritable. Selection for these traits will result in rapid progress.

PRELIMINARY EVALUATION OF SELECTION CRITERIA FOR DROUGHT RESISTANCE IN ALFALFA (*MEDICAGO SATIVA* L.)

by

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A thesis submitted in partial fulfillment
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ABSTRACT

Drought reduces alfalfa yield and quality. The objectives of this study were to: 1) develop screening procedures to assay alfalfa germplasm for drought resistance traits, and 2) determine if genetic progress could be made for these traits.

Traits studied included stomatal density, seedling root pattern, resistance to wilting, leaf color, leaflet size, and germination salt tolerance.

The considerable amount of within-plant variability for stomatal density existing in alfalfa, necessitating the sampling of leaflets from an extremely large number of stems, prevented the use of this trait as a selection criterion for drought resistance in alfalfa.

Selection for seedling root pattern and for resistance to wilting indicated that these traits were lowly heritable. Selection for these traits will result in slow progress.

Selection for leaf color, leaflet size, and germination salt tolerance indicated that these traits were highly heritable. Selection for these traits will result in rapid progress.

CHAPTER I

INTRODUCTION

Alfalfa (Medicago sativa L.) is the most important forage crop in the United States and the world. It has been cultivated in the United States for nearly 250 years and has the highest feeding value of all commonly grown hay crops. Alfalfa possesses a broad range of adaptation which has enabled its successful establishment worldwide. Although alfalfa is tetraploid and highly self-incompatible, its breeding has been highly successful.

Moisture is one of the most important factors limiting alfalfa growth. Drought reduces yield and quality. It is complex and there is little information on the factors affecting drought resistance in alfalfa. There is also a lack of efficient selection techniques to screen large populations of alfalfa required to make genetic progress for drought resistance. To develop drought-resistant alfalfa cultivars selection criteria must be identified and incorporated into alfalfa breeding programs.

The objectives of this study were to: 1) develop efficient screening procedures for physiological and morphological traits related to drought resistance of alfalfa, and 2) determine if progress through divergent selection can be made for these traits.

CHAPTER II

LITERATURE REVIEW

Alfalfa (Medicago sativa L.) was the first domesticated forage crop (34). Alfalfa, now worldwide in distribution (38, 112), probably originated in the Near East and/or Central Asia (34). It is believed to have been first cultivated in Iran, and the first documented attempt to grow alfalfa in the United States was in Georgia in 1736 (112). It is now the most important forage crop in the United States and the world, and it has the highest feeding value of all commonly grown hay crops (112).

In the semi-arid western plains, moisture is one of the most important factors limiting plant growth and breeders have been trying to develop drought-hardy forage crop cultivars for dryland production (26, 36, 91, 195, 232, 303). Drought reduces crop yield, quality, plant height, and vigor (214), and influences almost all physiological processes (207). Although alfalfa is relatively drought resistant due to its extensive root system (49) and its ability to harden during periods of drought (43), there is little information on the factors affecting drought resistance (139).

Drought resistance is complex, involving interrelationships between plant and environmental factors (117, 132, 148, 209, 237). It presents a challenge to plant breeders as drought resistance must first be identified and then transferred to high-yielding and agronomically-acceptable cultivars (27, 51). Keim and Kronstad (145) and Parsons

(209) describe the ideal cultivar as possessing a relatively high yield in dry environments without sacrificing yield in environments where adequate moisture is available. Perhaps breeders should not strive for an "ideal" cultivar, but should develop cultivars adapted for specific dryland environments.

Although alfalfa is tetraploid and highly self-incompatible, its breeding has been highly successful (33, 34, 188). Progress has been made in improving alfalfa yields, quality, longevity, seed productivity, and resistance to diseases and insects (280). Progress in any breeding program is dependent on how accurately the breeder is able to identify and select superior plants from large populations and this is determined by the genetic variability in the population and the heritability of the desired trait(s) (12). Thus, any effort to improve drought resistance through genetic manipulation will not only require the proper selection criteria but also a reliable procedure to screen available germplasm (238). The selection procedure, which would most likely include a combination of traits (191, 209, 237), should be simple, rapid, and enable the efficient screening of large populations (78, 198).

The Influence of Drought on Plant Growth

Viets (284) defines drought as a "sustained period of significantly subnormal water or soil moisture supply." Low rainfall is the basic cause of drought, but other meteorological factors (e.g., wind,

air temperature, radiation, etc.) also contribute to water deficit (256). In dryland agriculture most of the water loss is due to evaporation either directly from the ground surface or indirectly via transpiration (evapotranspiration) from plants.

Most physiological processes in plants are affected by water deficits (155). Most of a plant's fresh weight is comprised of water (288). Kramer (155) states that water serves four general functions in plants. It is 1) a major constituent of physiologically active tissue, 2) a reagent in photosynthesis and hydrolytic processes (e.g., starch digestion), 3) the solvent in which sugars, salts and other solutes move in the plant, and 4) important in maintaining turgor for cell enlargement and growth.

Water stress has its greatest influence on rapidly growing tissues (302). Germination and rate of initial seedling growth are significantly reduced by moisture stress (103, 150, 170). Germinating seeds are particularly susceptible to water stress which may alter their metabolism to the point where internal processes delay or even halt germination (265). Root initiation may be delayed and subsequent root growth inhibited by water stress (110). Species differ considerably with respect to their drought tolerance during germination (245).

Water stress may retard or stunt plant growth (3, 11, 13, 38, 164, 214, 230, 248). Drought at different stages of development affects growth in different ways (69, 212). The organ growing most rapidly at the time of stress is the one most affected (16, 92). Reduced leaf

area (1, 38, 47, 166, 220, 273, 298), fluctuations in stem diameter (45), stomatal closure (10, 11, 31, 220, 227, 259, 268), increased senescence (136, 166, 171), and associated reductions in overall yield and vigor (3, 11, 13, 38, 71, 72, 89, 92, 99, 154, 166, 179, 242, 248) may occur in the presence of water deficits. Growth may be stopped with slight drops in tissue water potential since cell division and cell expansion are affected by even a prolonged mild stress (89, 90, 95, 101, 273). Structural changes in the protoplasm (294) due to water loss in cells, inducing mechanical stresses, are believed to be a major cause of drought injury (234).

Water stress reduces both yield and quality in alfalfa (71, 99). Low yields are due to less leaf tissue, reduced leaf area, and smaller, more densely packed leaf cells (38). Drought results in fewer stems per plant, fewer internodes per stem, reduced internode length, less regrowth, and impaired root growth in alfalfa (118, 214, 217).

Drought influences many of the physiological processes in plants. Reduced efficiency and rate of photosynthesis (4, 20, 45, 104, 166, 290, 304), increased respiration (39, 84, 155, 166, 250), reduced protein synthesis (64, 116, 242, 244), altered nitrate reductase activity (4, 203, 220), increased activity of peroxidase (275) and other enzymes (8, 58, 242), and reduced nutrient uptake (3, 92, 290, 305) are characteristic of water-stressed plants. Alexander et al. (8) determined

that measuring enzyme levels alone was not a sufficiently sensitive procedure to indicate water status when water supply is altered. Measurement of several physiological processes (e.g., photosynthesis, respiration, nitrate reductase activity) might prove a better index of plant water status (4).

Internal water balance is the most important aspect of plant water relations. Internal water balance and turgidity are affected by a complex combination of environmental factors (77, 139) and are related to the rates of several physiological processes (154), which are all adversely affected by water stress (208). Maximum crop yields are dependent on the uninterrupted maintenance of adequate moisture supply throughout the growing season (256); thus, magnitude, time, and duration of water stress become important in determining how well the plant recovers (3, 54).

Blum (29) believes that present physiological knowledge of plant responses to water stress is sufficient for the initiation of breeding programs for resistance to water deficits. In order to capitalize on this knowledge, breeders must determine which physiological criteria are most important in conditioning drought resistance and how efficiently they may be implemented into breeding programs to insure significant and rapid progress.

Resistance to Drought and Its Measurement

General Forms of Drought Resistance

Many comprehensive reviews summarize the drought and drought resistance literature (17, 29, 93, 115, 119, 124, 125, 126, 135, 138, 154, 155, 164, 167, 168, 169, 180, 189, 206, 209, 234, 246, 261, 262, 276, 278, 283, 284, 302). However, there has been very limited success in breeding for drought resistance (139, 182). Genetic variability has been observed for drought resistance within almost all agronomically-important crops. Some plants can even survive after almost complete tissue dehydration without serious damage (88).

Russell (234) defined drought resistance as "the overall suitability of plants for cultivation under dry conditions." Wilson et al. (288, 299) define drought resistance as a plant obtaining "the maximum supply of water from the roots compared with that needed for survival." There are three general categories of drought resistance in plants (11, 169, 261, 264). Drought escape involves completion of the plant's life cycle during a short, favorable period when sufficient moisture is available. Drought avoidance is the ability of the plant to evade drought injury by leaf shedding, stomatal closure, development of a thick cuticle, reduced growth, or any other mechanism which "shields" the plant from desiccation. Drought tolerance (261) or hardiness (48, 234) is the ability of a plant to survive varying degrees of tissue

desiccation. All three of these mechanisms may be present to some extent in a plant (261), but avoidance is the most common type employed by agronomically-important crops (27, 28, 226, 236, 259, 263, 298, 299) so that photosynthesis and growth may proceed despite environmental water stress.

Drought resistance may be related to winterhardiness and salt tolerance since all three conditions may involve similar protoplasmic effects (165, 167, 169, 173). Resistance to one type of stress may imply resistance to the other (167). Unfortunately, there may be a negative correlation between drought resistance and yield in many instances (29) even though a plant which does not survive obviously does not yield. Smaller, low-growing plants are less affected by drying winds and have less evaporative surface area, thus avoiding desiccation. Slow regrowth after cutting and bud dormancy are contributing factors to dryland survival in alfalfa (57, 151) and several forage grass species (18, 179, 190) which may result in lower yields when moisture is available.

Morphological and Anatomical Drought Resistance Factors

Among the initial studies dealing with the problem of drought resistance in plants were those pertaining to morphological characteristics. Morphological characteristics which reduce water loss help plants avoid desiccation and could be selected to develop drought-resistant alfalfas.

1. Topgrowth Factors

Plants with thick cuticles (11, 154, 213, 253), increased pubescence (98, 300), and decreased leaf area (11, 231, 267, 268, 298) have been purported to be drought resistant. Thick, waxy cuticles resist moisture loss through epidermal cells not associated with stomata, and pubescence slows air movement and reduces transpiration. Reduced leaf area results in a smaller evaporative surface, thus conserving water. Plants with succulent leaves avoid desiccation by maintaining a high moisture content during drought (167, 213).

Leaf color may also be important in conditioning drought resistance. Dark leaves used water more efficiently than pale leaves in studies with barley (Hordeum vulgare L.) (2). Pale-leaved lines possessed lower canopy temperatures, higher albedos, higher net radiation, and greater sensible heat loss but yielded significantly less than dark-leaved lines (2, 81, 82). The cooler canopies in pale-leaved barley lines are probably due to increased reflection (81). Variability exists for this trait in alfalfa, so this trait could be implemented into a breeding program for drought resistance.

There are many studies (4, 10, 32, 41, 47, 50, 53, 59, 61, 67, 85, 87, 113, 121, 127, 128, 134, 137, 141, 166, 167, 183, 186, 191, 194, 201, 213, 219, 227, 240, 241, 247, 259, 266, 268, 269) on stomata and their relationship to drought resistance. Plants with lower stomatal densities are generally thought to be more drought resistant (67, 134).

Jones (134) suggests selection for smaller pore size and lower densities to improve drought resistance in barley. These traits are heritable in some species (32, 113, 186), but other studies (128, 191) have failed to reveal a correlation between density and/or size and transpiration.

Alfalfa leaves developed in the sun have more stomata per unit area than those developed in shade (53), and adaxial leaf surfaces have more stomata than do abaxial surfaces (50, 53). Cole and Dobrenz (50) compared stomatal densities of seven alfalfa cultivars by sampling five microscopic fields from the abaxial and adaxial surfaces of single leaflets from the top four nodes on one stem from each of two plants per cultivar in four replications. Significant differences as small as 9% were detected among cultivars for stomatal densities on the adaxial surfaces. Larger differences among cultivars were needed to detect significant differences for the abaxial surfaces. The importance of within-plant variability influencing stomatal density in alfalfa has not been determined.

Stomatal behavior is probably more important in enabling plants to avoid desiccation than either density or pore size. The ability of a plant to swiftly close its stomata during brief dry periods is a measure to conserve water even though growth may be curtailed for a short time (4, 87, 127, 166, 194, 220, 259, 268). Leaf diffusion porometers measure resistance to gas flow (4, 28, 76, 108, 249), and are used to monitor stomatal behavior. Stout and Simpson (259)

suggested that stomatal closure is only important when water stress becomes severe. Some of the drought resistance of alfalfa may be accounted for by its characteristic "midday closure" (183) of stomata, but Muenscher (191) concluded that the differential water loss observed among species is governed by a complex of factors and not just the stomata.

2. Root Factors

A large and well-developed root system is conducive to plant survival in dry areas (7, 40, 42, 62, 66, 111, 123, 124, 149, 167, 216, 221, 235, 268, 272). Extensive roots make it possible for plants to use available moisture from a large root zone (222, 272). Roots may be studied dynamically by using radioactive dyes such as P^{32} (105) or with glass-faced growth boxes (192).

Cohen and Strickling (49) found that the most important moisture for crop use in alfalfa was that in soil horizons close to the surface, so deep-rootedness might not necessarily be an advantage under certain conditions. Dittmer and Tally (65) suggest that plants possessing an extensive shallow root system supplemented by a deep, vertical one possess the greatest water use efficiency. Alfalfa has been observed to deplete moisture uniformly at several depths (79). Persistent root hairs and the ability of a plant to secrete mucilaginous substances have also been implicated in drought resistance (216). Smaller, less

extensive root systems have even been purported to be better, especially during long drought periods (156, 225).

Higher root/shoot ratios are considered to be advantageous for dryland survival (197, 200, 211, 226). Slight decreases in soil water potential may significantly increase root/shoot ratios (226). Root/shoot ratios, however, may be greatly influenced by several environmental factors (211) such as moisture, temperature, and soil type, so the heritability of this trait could be low.

Although considerable variability exists for rooting habit in alfalfa (243, 254), observation of root growth is difficult, tedious, and little is still known about what constitutes an effective root system (228). Drought resistance is most likely not simply related to differences in rooting depth or other rooting characteristics as has been suggested by Steckel and Gray (257) for potatoes (Solanum tuberosum L.).

3. Anatomical Factors

Anatomical characteristics in plants have also been studied (28, 38, 56, 80, 100, 152, 167, 199, 234). Drought-tolerant plants have been purported to have smaller, more densely-packed cells (38, 56, 65, 167, 234) since smaller cells, when desiccated, undergo a smaller proportionate reduction in volume (234). Cell wall elasticity may also be important in preventing desiccation injury (28). Modified vascular anatomy in the roots such as increased wall thickening in the endodermal

cells, well-developed root xylem, wider Casparian strips, and a reduced cortex (80, 100) would most likely increase the efficiency of water transport (199). A well-developed vascular system allows for better translocation of photosynthates and water (38).

Morphological and/or anatomical characters are hard to directly relate to drought resistance or yield (32). Since drought resistance is governed by a complex of characters (237, 234), it would probably not be advantageous simply to rigidly select for one or more morphological and/or anatomical traits to isolate superior germplasm (68) but to use these traits in conjunction with drought resistance screening and evaluation techniques. Once heritable, adaptive morphological plant characters are identified, they can be implemented into breeding programs (81).

Physiological Drought Resistance Factors and Selection Techniques

Elaborate procedures to measure drought resistance in crop plants have been described (15, 27, 28, 35, 76, 146, 156, 157, 159, 181, 286). Although these procedures are useful in identifying drought-resistant plants, simpler techniques are needed to more rapidly assay large, segregating populations (35, 78, 198). Seedling screening techniques that correlate to mature plant resistance would be very valuable since a minimum of time and space would be involved (237).

1. Germination Under Stress

Several attempts have been made to relate germination in sodium chloride, mannitol (163), polyethylene glycol, and similar solutes to drought resistance or winterhardiness. The most common selection technique has been to place seed on blotters or filter paper in closed containers and to moisten with salt solution (44, 73, 282, 305). Others (14, 174) have used saline soil or water cultures to measure salt tolerance of germinating seeds. Workman and West (301) used agar containing NaCl to test germination in winterfat (Eurotia lanata (Pursch.) Moq.). They observed genetic differences within the species which enabled certain strains to better germinate at higher NaCl levels.

Croughan et al. (55) studied cell growth of alfalfa grown in agar containing 1% (w/v) NaCl. They observed differences in growth between a salt-tolerant line of alfalfa and a nonselected line from the same original population. They suggested increased NaCl tolerance in the selected line due to its better relative growth at high levels of NaCl. Since the salt-tolerant line performed poorly in the absence of NaCl, they hypothesized that a substantial amount of NaCl was required for optimal growth.

Plants are usually most sensitive to osmotic stress during germination and early seedling development (14). Osmotic stress, induced by saline conditions, affects plant growth similarly to drought stress such as increasing root/shoot ratios (44), decreasing yields (14, 74,

86, 109, 130, 160, 196, 208, 288), and delaying or reducing germination (14, 52, 70, 73, 114, 150, 174, 175, 245, 260, 282, 295, 297). Although genetic variability exists for salt tolerance (24, 83, 144, 245, 298), the relationship between salt tolerance and drought tolerance is controversial (178). No correlation has been found between ability to germinate in osmotic solutions and drought tolerance in mature plants (172, 245, 305) probably because osmotic substrates induce effects, including toxicity, more complex than simple drought (130, 184, 282). Thus, even though alfalfa is relatively salt tolerant (37, 153), the same mechanisms may not condition drought tolerance.

2. Water Potential Measurements

Water retention of plant tissues is one of the simplest measurements used in assessing a plant's ability to avoid desiccation (158). Hydrophillic colloids in the plant cytoplasm are purported to be the most significant factor in water retention (195). Dedio (60) observed genotypic differences in wheat (Triticum aestivum L.) for water retention in excised, drying leaves. Martin (179) found that stalks of sorghum (Sorghum bicolor L.), a relatively drought-resistant crop, dried more slowly than those of corn. Teoh (270) suggests that drought-tolerant plants lose water less readily than susceptible plants when they are cut. However, Salim and Stutte (236) and Sandhu and Laude (239) suggest that water retention may provide only a "fair" means of assessing drought tolerance.

Leaf water potential measurements are more complex and time-consuming. Instruments such as pressure bombs (35, 63, 291), psychrometers (35, 152, 176, 177, 187, 224), and beta gauges (181, 193) measure water potential. Many studies (6, 27, 28, 31 35, 76, 143, 148, 152, 210, 292, 293) relate water potential and related factors (matric potential, turgor pressure, suction tension, and relative water content) to plant water status and drought resistance in plants. Blum (27) measured leaf water potential in sorghum periodically during the growing season and found genotypic differences in drought resistance. Al-Saadi and Wiebe (6) determined that different species had different capacities to bind water by matric forces, probably due to different properties of colloidal materials. However, the correlation between drought tolerance and matric potential was low and variable. Water potential measurements provide useful and accurate estimates of plant water status, but are too time-consuming to use for the screening of large, heterogeneous plant populations.

3. Concentration of Ethylene and Abscissic Acid

Increased concentrations of ethylene and abscissic acid initiate leaf abscission and may be an index of drought resistance. The role of ethylene and abscissic acid has been studied in many species (5, 21, 23, 25, 75, 102, 162, 215, 218, 223, 229, 289) and their accumulation often results from water stress (22, 136, 171, 229, 289). Ben-Yehoshua and

Alohi (22) found that as water stress increased, more ethylene was produced in 'Valencia' oranges (Citrus simensis Osbeck.). McMichael et al. (171) evaluated the effects of water deficits in cotton (Gossypium hirsutum L.) and found a linear relationship between ethylene production, leaf abscission, and water deficit. Water stress was also related to a decrease in auxins and enhanced production of hydrolytic enzymes in the abscission zone. Walton et al. (289) discovered an association between water stress and abscissic acid levels in bean roots (Phaseolus vulgaris L.).

Both ethylene and abscissic acid can be readily measured. Assessment of their concentration following drought stress may be useful in a breeding program for drought resistance.

4. Proline Accumulation

Free proline accumulates in the leaves of many species following water stress (19, 30, 106, 107, 118, 120, 204, 205, 233, 251, 277, 279, 285, 286). Large proline accumulations following water stress may be a positive index of drought tolerance (19, 30, 261, 285). However, Waldren et al. (286) point out that proline accumulation is not a very sensitive indicator of drought stress, since it only accumulated after yield was severely affected in sorghum and soybeans (Glycine max Merr.). Tymms and Goff (279) found that proline accumulation was not related to the extreme drought tolerance of "resurrection" plants. Hanson et al.

(106, 107) suggest that proline-accumulating potential should not be used as a positive index of drought tolerance as the massive proline accumulation in barley is merely a symptom of severe water stress and has no survival value during drought. They further suggest that selection for high proline accumulation may even result in a shift in the population in the direction of drought susceptibility. Even though proline-accumulating ability may be a heritable trait, isolation procedures currently in use are too time-consuming for use in initial germplasm screening. Measurement of proline accumulation may be useful for evaluation of prospective drought-tolerant cultivars selected by other methods.

5. Other Techniques

Recovery following drought stress, whether artificially imposed or in the field, has been used to assess drought tolerance (13, 42, 54, 116, 122, 156, 167, 185, 196, 198, 202, 274, 298). In 1936, Hunter et al. (122) simulated drought under laboratory conditions by withholding water. They were able to distinguish among eight corn (Zea mays L.) inbred lines differing in drought resistance and field data confirmed the validity of their procedure. Todd and Webster (274) simulated repeated drought periods in the greenhouse and results correlated well with known field drought resistance for several cereals.

O'Toole et al. (202) withheld water for 10 days from rice (Oryza sativa L.) seedlings in a growth chamber and found differences in survival among plants. Nour et al. (198) subjected sorghum seedlings to four successive cycles of water stress in a growth chamber and measured percent survival. They concluded their technique to be a simple and effective screening method. However, Chinoy (46) points out that severe stress may not be useful in showing cultivar differences.

Osmotic substrates, such as polyethylene glycol (108, 129, 131, 142, 144, 161, 207, 208, 271) are used to simulate drought in the laboratory or greenhouse. Johnson and Asay (131) tested emergence of 120 crested wheatgrass (Agropyron spp.) lines under polyethylene glycol-induced drought stress. Cellulose acetate membranes were used to avoid direct contact between growth media and osmoticum. They found differences among lines for emergence under the imposed stress. However, selection under artificially-imposed stress in the greenhouse resulted in low correlations with field drought resistance (133) since field conditions were more complex and variable (137).

Kaloyereas (140) proposed the chlorophyll stability index (CSI) as a possible measure of drought resistance of pine (Pinus taeda L.) seedlings. The less readily chlorophyll degraded under heat stress, which often accompanies drought (94), the higher the drought resistance.

Kilen and Andrew (147) measured CSI to assess drought tolerance in sweet corn (Zea mays L.). Susceptible lines had an index of 5.6, while resistant lines had an index of 1.6, in a test involving 12 dent and sweet corn lines differing in phenotypic response to field drought. Chlorophyll concentration can be easily measured with portable reflectance meters (287) so this characteristic may warrant further consideration as a screening tool.

Genkel et al. (96) and Genkel and Schelamova (97) believe that starch hydrolysis in the roots of many species is negatively correlated with drought resistance. Excised root tips were stained with Lugol's solution following desiccation and were then rated on a 1-4 scale under the microscope for starch hydrolysis. Seedlings in which less than 30% starch hydrolysis took place following an artificial desiccation stress were considered drought tolerant. The authors suggest that this method is reliable for selecting drought-tolerant cultivars.

Kessler (46) described the relationships between cell size, nucleic acid fraction and the tolerance of bean and pea plants to heat and water stress. He concluded that the plants with greater heat and drought tolerance had more adenine. The DNA to RNA ratio also increased with greater drought tolerance.

The problem of drought has presented a challenge to plant breeders in obtaining yield improvement in drought-stricken regions without

irrigation (27, 124). Drought resistance is very complex and little progress has been made through breeding. In order to be successful in developing a dryland alfalfa, heritable characteristics conditioning drought resistance must first be identified and later incorporated into high-yielding and agronomically acceptable cultivars.

CHAPTER III

SAMPLING STOMATAL DENSITY IN ALFALFA

The relative importance of within-plant variability influencing stomatal density in alfalfa has not been determined and sample size requirements are not documented. The objectives of this study were to determine 1) the magnitude of within-plant variability for stomatal density associated with stems, leaf position, and microscopic fields in alfalfa; 2) optimum allocation of sampling resources to obtain maximum precision for detecting differences among individual genotypes; and 3) whether within-plant variability was too large to use stomatal density as a possible rapid screening criterion in breeding for drought resistance in alfalfa.

Materials and Methods

Five alfalfa plants were randomly chosen in 1978 from a 1977, Bozeman, Montana planting of diverse progeny rows. The plants had been harvested once and were at the early bud stage. Leaf samples were taken from 10 stems per plant from three successive nodes. Node 1 was the first node from the top of the plant that appeared to have fully expanded leaves. Nodes 2 and 3 were immediately below node 1. The two lateral leaflets from each trifoliolate leaf were removed and the adaxial surface of one and the abaxial surface of the other were sprayed with acrylic Tuffilm. The spray was allowed to dry for

5 minutes before applying clear plastic tape to the sprayed leaf surface. The hardened Tuffilm was peeled from the leaflet and taped to a microscope slide.

A microprojector was used to count stomata from 10 random microscopic fields (0.128 mm^2 per field), 5 on each side of the midvein. If at least one-half of a stomate was in view, it was counted as in the field. Fields obscured with vascular tissue, epidermal tears or other obstructions were not sampled. Epidermal cells were counted simultaneously from a 0.03 mm^2 pie-shaped section from the same fields used for the stomatal counts. Portions of epidermal cells within the field were rounded to the nearest one-fourth and were added to the number of whole cells present in the field to arrive at total epidermal cells per 0.03 mm^2 field.

Analyses of variance were conducted for stomatal density at each node and then combined over nodes. Stems and fields were considered as random and plants and nodes as fixed effects in the analysis. Variance component estimates for within-plant sources of variation, stems, and fields, were obtained by equating observed mean squares to their expectations and solving for the appropriate variance components.

Various sampling combinations were compared to the specific case of sampling 10 fields per leaflet, 3 nodes per stem, and 10 stems per plant. Relative efficiency was defined to be (255):

$$\hat{\sigma}^2_{\bar{y}} / \left(\frac{\hat{\sigma}^2 S/P/N}{rn} + \frac{\hat{\sigma}^2 F/S/P/N}{rnm}\right)$$

where: $\hat{\sigma}^2_{\bar{y}}$ = variance of a plant mean with 10 stems per plant and 10 fields per node on each of 3 nodes;

$\hat{\sigma}^2 S/P/N$ = variance component for stems from the combined analysis of variance;

$\hat{\sigma}^2 F/S/P/N$ = variance component for fields from the combined analysis of variance;

m = number of fields per node;

n = number of stems per plant; and

r = number of nodes per stem.

The within-plant variance components were also used to estimate sampling requirements needed to detect pre-chosen differences between plants fulfilling given probability levels for Type I (α) and Type II (β) errors using the formula

$$n = 2(z_\alpha + z_\beta)^2 S^2 / d^2$$

where: n = number of stems;

z_α = the z value corresponding to the two-tailed significance level α for a normal variable; and

z_β = the t value corresponding to the desired probability $1-\beta$ of detecting a significant result if the true difference, expressed as a percent of the mean, is d.

Results and Discussion

Significant differences were observed among plants for stomatal density at each node and combined over nodes on both the abaxial and adaxial leaflet surfaces (Table III-1). Stomatal density decreased from node 1 to node 3. Although data are not presented, epidermal cells were counted along with stomata. The ratio of stomata to epidermal cells increased from upper to lower nodes (0.225, 0.239, and 0.250 for abaxial leaflet surfaces and 0.242, 0.254, and 0.267 for adaxial leaflet surfaces at nodes 1, 2, and 3, respectively). Plant x node interaction was not significant for either leaf surface indicating that relative differences among plants are similar provided that leaflets from the same nodes are sampled on all genotypes.

The two within-plant variance components were always less for the abaxial leaflet surface at any given node (Table III-2). In general, within-plant variability declined from node 1 to node 3.

Because efficiency of different sampling schemes was nearly identical for both leaflet surfaces, only data for the abaxial surface are plotted in Figure III-1. An efficiency of 100 percent has been arbitrarily defined to be the sampling of 10 fields on leaflets from 3 nodes on each of 10 stems. Increasing the number of fields sampled from leaflets on a given stem has little effect on increasing efficiency compared to sampling leaflets from more stems. In practice one should

Table III-1. Mean stomatal densities (stomata per mm^2) of five alfalfa plants at three successive nodes.

Plant	Node 1		Node 2		Node 3		Mean	
	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial
1	196.4	240.3	192.0	236.9	177.4	217.3	188.6	231.5
2	155.1	192.3	157.3	186.0	147.1	175.3	153.2	184.5
3	167.6	234.6	166.6	225.7	161.5	217.7	165.2	226.0
4	180.6	221.3	173.5	215.3	167.1	204.4	173.7	213.8
5	206.8	238.3	191.9	234.3	181.1	219.5	192.3	230.7
LSD (0.05)	16.3	22.0	16.1	18.2	14.8	17.5	13.0	15.8
Mean	181.3	225.4	176.3	219.6	166.8	206.8	174.6	217.3

Table III-2. Within-plant components of variance for alfalfa stomatal density (stomata per mm^2) for three successive nodes.

Node	Stems $\hat{\sigma}_S^2$		Microscopic fields $\hat{\sigma}_F^2$	
	Abaxial	Adaxial	Abaxial	Adaxial
1	318±74	593±131	276±18	365±24
2	316±70	394± 90	212±14	375±25
3	261±59	372± 83	238±16	274±18
Combined	298±39	453± 59	242± 9	338±13

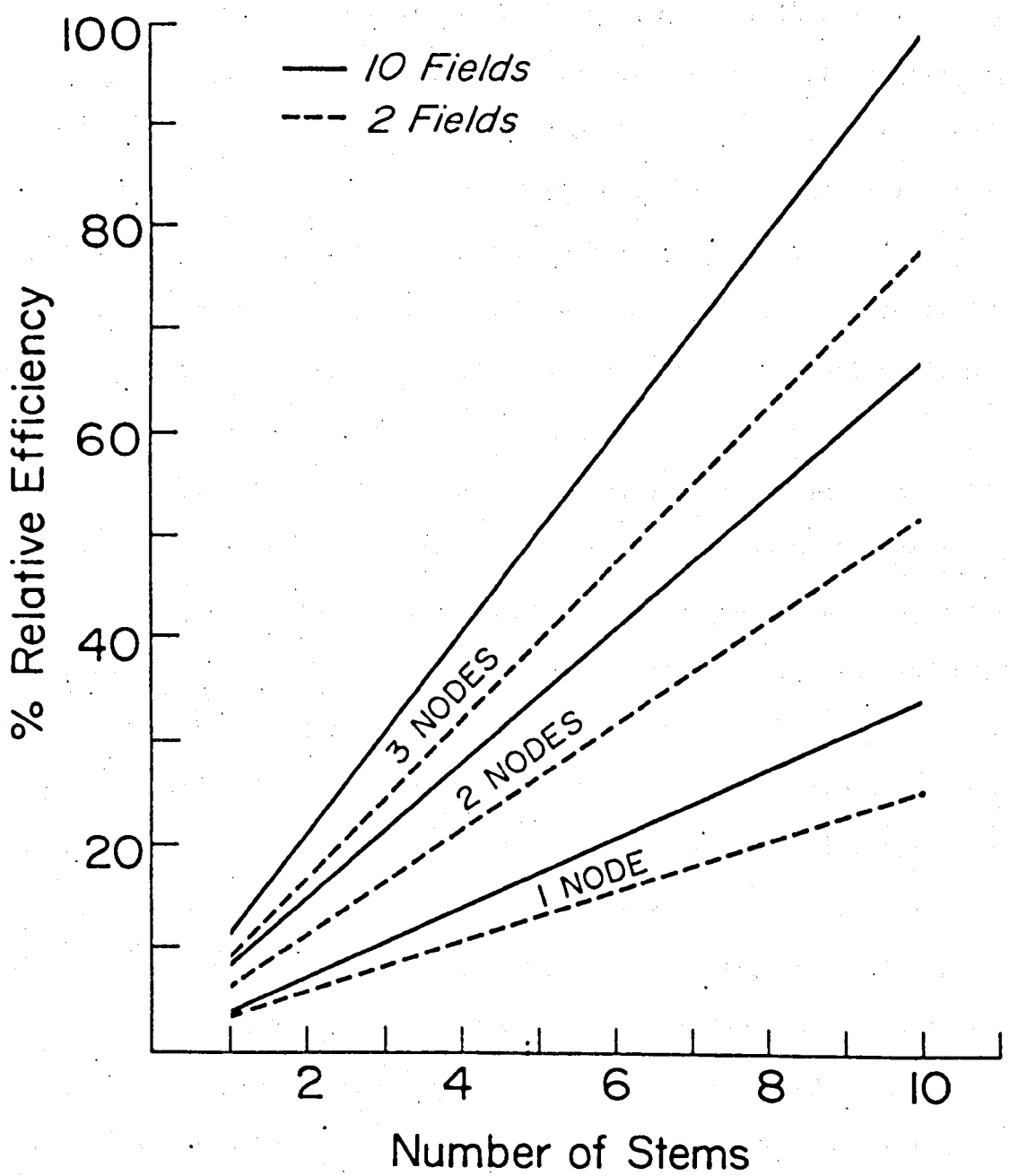

Figure III-1. Relative efficiencies for sampling stomata in alfalfa for one, two, and three nodes when 2 and 10 fields are counted on leaflets from 1 to 10 stems.

allocate samples to leaflets from more stems as opposed to more nodes on the same stem to insure that all leaflets are of similar age.

Sampling requirements needed to detect differences between plants, holding Type I and II errors at 0.05 and 0.10, respectively, are presented in Table III-3. Because node 3 is less variable and more mature, only stem and field combinations for node 3 are shown. These data again corroborate that sampling resources should be allocated to leaflets from additional stems as opposed to more fields on leaflets from the same stem. Sample sizes could be reduced if one is willing to accept larger Type I and Type II errors. However, for selection purposes the plant breeder is concerned with minimizing both Type I and Type II errors, since it is important to detect differences among genotypes when they do exist and to have those differences be real and not due to random chance.

Genetic variability exists for stomatal density; however, the considerable amount of within-plant variability necessitates the sampling of leaflets from an extremely large number of stems to discriminate among plants differing by less than 10%. As a result, stomatal density was not used as a selection criterion in breeding for drought resistance in alfalfa. Many factors affect stomatal density and this study was conducted at only one location and in one year. However, testing plants in more environments would probably further increase the number of total samples required.