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GEOGRAPHIC VARIABILITY OF CREOSOTEBUSH [*LARREA TRIDENTATA* (D.C.) Cov.] IN RESPONSE TO MOISTURE AND TEMPERATURE STRESS

by

Richard Eugene Saunier

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GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my direction by Richard E. Saunier

entitled GEOGRAPHIC VARIABILITY OF CREOSOTEBUSH [Larrea tridentata (D.C.) Cov.] IN RESPONSE TO MOISTURE AND TEMPERATURE STRESS

be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

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One of the most widespread plant species in the arid southwestern United States and northern Mexico is the creosotebush [*Larrea tridentata* (D.C.) Cov.]. To study its adaptation to the diverse environment found over its distribution, plants were collected from populations at Zapata, Texas (elevation 152 m, annual precipitation 50.18 cm), Sheffield, Texas (elevation 609.6 m, annual precipitation 33.0 cm), and Bernardo, New Mexico (elevation 1,524 m, annual precipitation 20.32 cm). These plants were subjected to different conditions of soil moisture and air temperature in a plant growth environment chamber following a one-year equilibration period in the greenhouse. The following treatments were used: temperature (38 C daytime/15.5 C nighttime, 27 C daytime/4.5 C nighttime), moisture (daily waterings during the seven day treatment period, no water during the seven day treatment period), control (plants sampled four hours after watering and previous to treatment).

Ecotypic variation appeared among the three populations in response to moisture stress. The bases of drought resistance were different. That for the Bernardo plants was one of drought tolerance, apparently an adaptation to the extreme dryness of the Bernardo area. It was shown as a loss of soluble sugars and an accumulation of

proline and other glutamic acid family amino acids under moisture stress. The Sheffield and Zapata plants were more drought avoidant. Differences in the type of drought avoidance between plants from these two areas were evident. Zapata plants had a high initial moisture status which would carry them through the short dry conditions of the Zapata area. The Sheffield plants had a comparatively slower water loss rate which was thought to be an adaptation to the erratic precipitation of the Sheffield area.

Clinal variation appeared in terms of susceptibility to lowered soil temperatures which affected the water absorption rate. The Bernardo plants were least susceptible, Sheffield plants were intermediate and the Zapata plants were most susceptible.

These ecotypic and clinal differences may be contributing factors that enable the wide distribution of creosotebush over its varied environment.

GEOGRAPHIC VARIABILITY OF CREOSOTEBUSH [*LARREA TRIDENTATA* (D.C.) Cov.] IN RESPONSE TO MOISTURE AND TEMPERATURE STRESS

INTRODUCTION

Creosotebush [*Larrea tridentata* (D.C.) Cov.] is one of the more representative and widespread shrubs inhabiting the arid regions of the southwestern United States and northern Mexico (Figure 1). In these areas it is found in at least a part of all components of the North American warm desert formation (Shelford 1963) and, through years of evolutionary history, has become well adapted to the difficult and diverse environment found there (Spalding 1904, Runyon 1934, Shreve 1936, and Axelrod 1950).

Although a preponderance of the area inhabited by creosotebush is classified as desert (Garcia, Soto and Miranda 1960), the environment is complex and variable (Hastings and Turner 1965). Mean annual rainfall and seasonal precipitation patterns vary over the region where creosotebush is found from the extremely low (3.5 in.; 8.89 cm) and erratic rainfall of the Mojave, Colorado, and Vizcaino Deserts and the winter-summer pattern of the Sonoran Desert to that of the Chihuahuan Desert where nearly 80 per cent of the 12-16 inches (30.48-40.64 cm) occurs from June through September (Shelford 1963).

An altitudinal distribution for the shrub of nearly 9000 feet (2740.32 m) from below sea level in Death Valley, California to

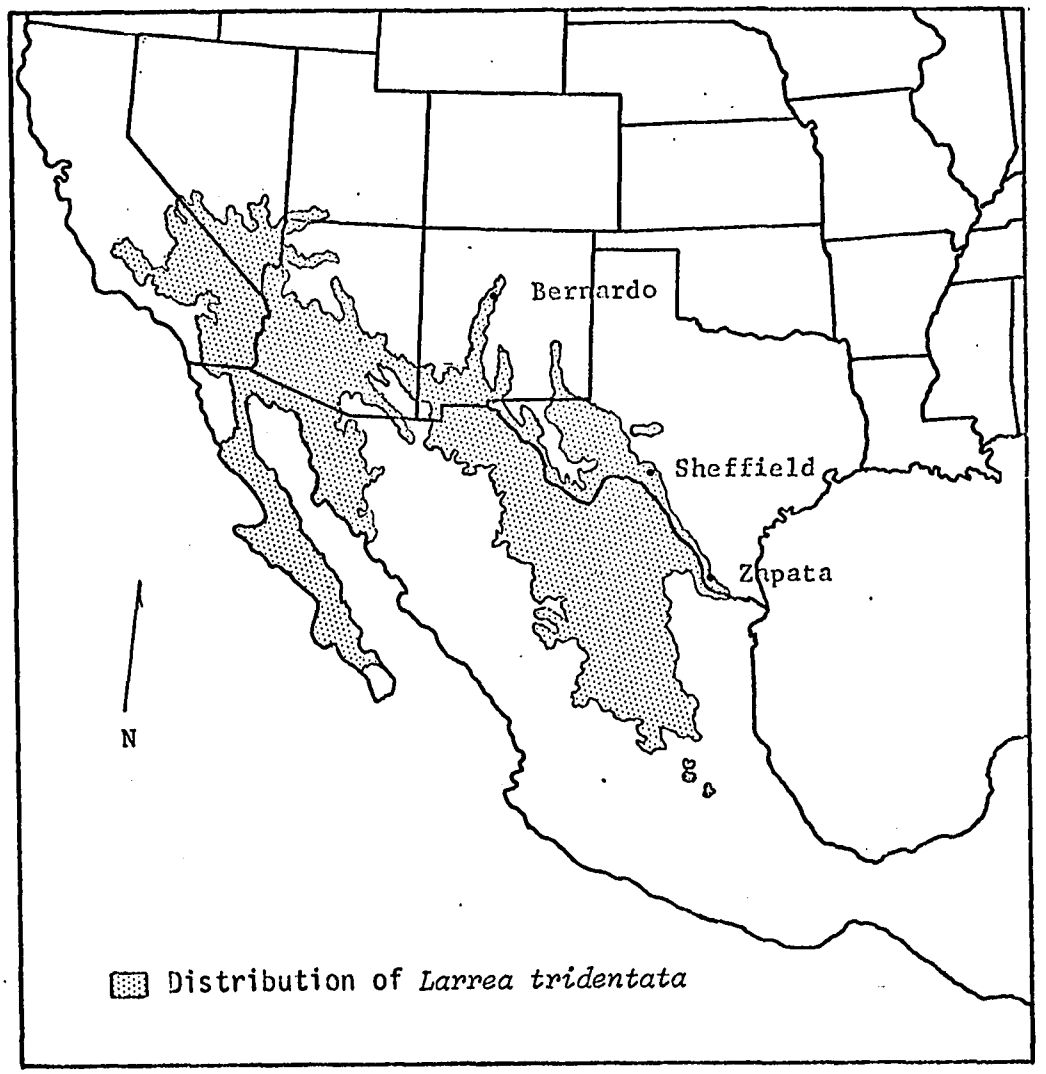

Figure 1. Distribution of *Larrea tridentata* in the southwestern United States and northern Mexico with the collection sites indicated.

8600 feet (2620.13 m) near Zacatecas, Mexico adds further variety both in precipitation and temperature to its environment (Shreve 1940). In Death Valley, temperatures have reached a high of 134 F (56.7 C) and day and night temperatures above 100 F (37.8 C) are common (Oosting 1956). At the other extreme, creosotebush occurs in areas where temperatures have reached as low as -18 F (-27.8 C) (Cottam 1937).

Creosotebush is of interest to range managers not only because of its wide distribution, almost undisputed dominance over 46.5 million acres (Platt 1959), and resiliancy of adaptation, but also because of its complete lack of forage value (Aldous and Shantz 1924), invasion into new areas of desert shrub and desert grassland types (Gardner 1951, Humphrey and Mehrhoff 1958, Yang 1961, Chew and Chew 1965), and lack of any genuinely effective and economical control.

The problems presented by creosotebush to the range manager and range scientist are numerous and difficult. To understand and deal with these problems, a basic analysis of its adaptation to its varied environment as well as applied research on control and manipulation is needed. Such an analysis requires both a knowledge of the physiological profile of the species as affected by the environment and an appreciation of the genetic structure and variability within the species.

A knowledge of the physiological profile of creosotebush will suggest possible mechanisms of adaptability that have allowed its distribution across such a difficult and varied environment (Billings 1960). Effects of environment are exerted first on internal physiological and biochemical processes (Kramer 1959). Consequently, the

ecological range of a species is a result of the evolutionary process manifest at the physiological and biochemical level. The result might be a single chemical compound having a direct relationship to the resistance or tolerance of a species as in certain disease resistant varieties (Dimond and Horsfall 1959), or as variations in physiological processes such as the timing and mechanism of flowering (Garner and Allard 1920, Borthwick and Hendricks 1960, etc.), photosynthesis and respiration (Scott and Billings 1964, etc.) and response to substrate constituents (Kruckeberg 1951).

Furthermore, any variation in physiological response populations may have to a uniform, controlled environment can help determine the type and degree of variation within a species (Grime 1965). This biosystematic approach was used for much of the above research on ecotypic variation and its success has led Mooney and Johnson (1965) to state:

> One of the most fruitful approaches to the study of the adaptations of plants to diverse environments is the comparison of different populations of the same species. Plant populations growing in dissimilar environments are subjected to different selections and, hence, may be genetically and physiologically distinct. Comparative studies of the physiological ecology of such populations yield information on the selective importance of particular components of the environmental complex. Further, these studies can show by what specific physiological mechanisms similar biotypes have been modified to meet dissimilar environmental stresses.

A knowledge of genetic structure is required because the research on ecotypic variation over the past 140 years does not allow the assumption that a species is uniform genetically, physiologically,

or even morphologically (Clausen 1951). Consequently, we have no guarantee that attempts to control and manipulate a species will be equally effective throughout its distribution. In spite of these 140 years of research, however, we know comparatively little about the ecotypic variation of range plants in general and range shrubs in particular (Tisdale 1962). The research on a number of grass species by Olmsted (1944, 1945), Larsen (1947), and McMillan (1959, 1964, 1965, etc.) plus the investigations on *Artemisia* (Ward 1953), *Purshia* (Wagle 1958, Blaisdell and Mueggler 1956) and *Prosopis* (Peacock and McMillan 1965) are noteworthy exceptions, but are only a meager beginning when one considers the vast number of important species inhabiting western rangelands.

The objective of this study, therefore, was to investigate the genetic variation of one of these species, creosotebush, in terms of physiological responses to two environmental components, soil moisture and air temperature. To accomplish this objective, plants from three local populations were grown side by side in a controlled environment and their reactions observed.

LITERATURE REVIEW

Moisture Relationships

The recognition of creosotebush's effective adaptation to arid environments initiated numerous ecological, physiological, and morphological investigations which attempted to define the nature of its drought resistance. Much of this work, like that on drought resistance in general, drew conflicting conclusions. The problems arose because of a failure to recognize the variations in adaptation that emerged through the course of evolution. Furthermore, these various adaptations could not be restricted, as was so often done, to one per species. Indeed a species may have a whole complex of these adaptations to one degree or another. Creosotebush appears to be one such species.

Drought Resistance

Xerophytism (the ability of plants to survive drought) depends on the capacity of a plant to absorb water and reduce water loss (drought avoidance), and to endure dehydration (drought tolerance) (Levitt 1963). Consequently, anatomical and morphological characteristics of leaves, stems, and roots, as well as the inherent ability of protoplasm to resist and/or survive desiccation are all involved. Kramer and Kozlowski (1960), for instance, emphasized deep and widespreading root systems, efficient conducting systems, small transpiring

surface in relation to root surface, reduction in leaf area when water deficits develop, thick layers of cutin, and few or deeply imbedded stomata as features which reduce the chances of drought injury. Although these characteristics can increase drought resistance, they are probably more xeroplastic than xeromorphic, i.e., they are more likely the results of moisture stress than inherent factors which impart drought resistance. Consequently, if these characteristics are to be used in systematic studies, comparable environments during development are a necessity.

Water Absorption

A species survival in xeric habitats is often related to the depth and spread of the plant's root systems (Oppenheimer 1960). Deep roots absorb moisture from below that reached by the drying power of the atmosphere, and an extensive surface system utilizes moisture from a wide area. Cannon (1918) and Wilson (1893) believed that the root system of creosotebush was particularly important in its drought resistance, although Duisberg (1952) doubted that it was much of an adaptation to drought.

Usually, the shrub has a tap root, although it rarely extends to any great depth (Chew and Chew 1965). Lateral roots are present and the wide, even spacing of creosotebush may be due to the efficient extraction of soil moisture by these roots. In Argentina, Morello (1956), found roots of *Larrea divaricata* extending to a depth of four meters, and observed that the widespread lateral roots could consume up to 21 per cent of a single rainfall.

Spalding (1904) studied the roots of creosotebush and found that root hairs were most abundant on plants with the least soil moisture. Fitting (1911) (according to Delf 1915) suggested that high cell sap osmotic pressures increased the suction power of a plant allowing more water to be withdrawn from the soil. Parker (1956) mentioned that this idea had fallen into disrepute, primarily because these high osmotic concentrations are the result of the environmental stresses rather than something that is "characteristic of xerophytes." However, Slatyer (1957) has indicated that the permanent wilting point is determined by osmotic concentration of the plant rather than by soil characteristics.

Reduction of Water Loss

Anatomical features which resist water movement through the stem may reduce water loss and thus be of adaptive value. For instance, ring porous woods having long, uninterrupted channels are least resistant to water translocation, diffuse-porous woods are comparatively resistant; and conifers, which lack vessels, are most resistant (Kozlowski 1964). Despite these findings, water loss from creosotebush is actually greater than loss from the mesophyte, privet, on the basis of the number of stomata (Ashby 1932).

Cannon (1905) distinguished two types of xylem tissue in creosotebush: that from "irrigated" plants and that from "non-irrigated" plants. Non-irrigated stems had more and larger ducts per equivalent area of cross section than irrigated stems. Anatomical studies of very young creosotebush plants show an early maturation of

the protoxylem vascular system, and this may have something to do with their drought resistant qualities (Shellhorn 1955).

Direct water loss from the stem to the air occurs in plants, and species vary as to their susceptability to this type of loss. *Artemisia tridentata*, when subjected to atmospheric drought, develops rings of interxylary cork between the annual growth rings, and these, since they would tend to reduce transpiration from the stem, may benefit the plant in semi-arid regions (Shields 1950). As far as is known, creosotebush does not have this anatomical modification.

Shields (1961) emphasized that leaf tissues of xerophytes are structurally altered by their environment. These alterations result in reduced external area, small cell size, thickened cell walls, a more compact network of veins, higher stomatal frequency, and palisade tissue which is strongly developed at the expense of spongy tissue. She, like Maximov (1929) and Parker (1956) supposed that leaf adaptations of this type were of secondary importance in drought resistance. Indeed, Maximov (1931) believed that most xerophytes were not structurally adapted for reducing transpiration. This appears to be true for creosotebush. For instance, the leaves, though small (6-10 mm long, 3-4 mm wide), are not exceptionally so; and stomata are numerous. Ashby (1932) gave figures of 24,000 and 36,000 per cm^2 on the upper and lower surfaces, respectively; and Warskow (1965) reported approximately 21,600 per cm^2 for both surfaces. Morello (1955a) found 13,000 cm^2 on the lower surface and 10,400 per cm^2 on the upper surface of *Larrea divaricata*.

This suggested lack of morphological adaptation to drought in the leaves of creosotebush has been questioned lately. Although Ashby (1932) stated that creosotebush stomata "show no anatomical adaptations to conditions of water shortage...," Dalton (1961) and Warskow (1965) reported the presence of a "stomatal lip" which they suggested could lower transpiration. And, although Asnby (1932) and Runyon (1934) felt that the leaves were not highly cuticularized and contained large intercellular spaces (a mesophytic characteristic), Dalton (1961) found highly cuticularized leaves and tightly packed palisade tissue which should reduce transpiration in creosotebush.

Rapid closure of the stomata under developing water stress may also be a factor in reducing transpiration (Kozlowski 1964). Morello (1955b) mentioned, however, that the stomata on creosotebush close too slowly to be of much value in the suppression of transpiration. Also, Warskow (1965) noted that stomatal movement in creosotebush probably seldom occurs under field conditions because of clogging of the stomatal chamber with debris and resins. Ashby (1932), on the other hand, believed the resins in adult leaves do not block the stomata under ordinary conditions.

The resinous leaf coating has been credited with retarding transpiration and may be of survival value (Carlock 1932, Morello 1955b). Duisberg (1952), however, reasoned that since creosotebush has high transpiration rates despite these abundant resins, they evidently do not have an appreciable effect on reducing transpiration.

Xerophytes often have trichomes which retard water loss (Shields 1961). Although shape and structure of trichomes are inherent within a species, their presence or absence is determined by the environment. In general a xerophytic form of a species produces more trichomes than mesophytic forms. Apparently drought stimulates this trichome production (Shields 1950). Dalton (1961) suggested that the many epidermal hairs on creosotebush could reduce transpiration.

A reduction in leaf area can reduce transpiration. Runyon (1934) noted the abscission of creosotebush leaves during drought, the older ones being shed first. Nevertheless, he did not believe that this reduction of transpiring surface by leaf abscission could meet the requirements for resisting extreme drought. Morello (1955a), however, felt that the leaf reduction during drought was important in the Argentina plants.

Schratz (1931) found transpiration rates of 9.7 to 12.0 mg of water per cm^2 of leaf surface per hour and Spalding (1904) calculated that, although the rate of transpiration decreased in creosotebush under drought conditions, considerable amounts of water were still transpired. Wood (1933b), after comparing a number of investigations on the transpiration rate of xerophytic, mesophytic, and shade plants from throughout the world, reasoned that the mean transpiration per unit area essentially was the same in all plants. This, he believed, disposed of both the view that xerophytes have an increased transpiration rate and the view that they have a decreased transpiration rate.

Although experiments by Boon-Long (1941) showed that increasing the sugar content of leaf cells can greatly decrease transpiration, it is now generally believed that drought resistance should be measured by the capacity to endure water loss rather than by the rate of transpiration (Shields 1961).

Resistance to Desiccation

Emphasis in the past few years has dealt with the relation between xerophytism and a plant's ability to resist desiccation. The work of Iljin (1953, 1957, etc.) established that different species of plants vary greatly in their tolerance to desiccation. Cell size, cell shape, ratio of volume to surface area, and osmotic concentration of cell sap are correlated with differences in tolerance. According to Iljin, much of the damage caused by protoplasmic distortion is avoided because of modification of the above factors. For instance, small cells confer a greater degree of resistance than large cells because there is less shrinking and expanding of the cell due to dehydration and rehydration, and consequently less mechanical damage to the cell parts.

Runyon (1936) considered this factor in creosotebush. He recognized three different leaf types: 1) fully grown leaves having a high water content and which were no more drought resistant than leaves of mesophytic plants and which abscised during drought; 2) an intermediate group; and 3) the young, partly developed leaves which had a lower water content and higher drought tolerance. The small leaves had the same arrangement and number of cells as the other

types, but they were from 38 to 78 per cent smaller. However, even at leaf moisture conditions where water content was less than 50 per cent of the dry weight, the leaves were still metabolically active. For this and other reasons, many workers have suggested that other chemical and physiological factors may be more important than morphological and anatomical factors in desiccation resistance.

High osmotic pressures favor the plant with respect to drought and should, therefore, be given consideration. Iljin (1953) showed a direct correlation between osmotic pressure of the cell sap and the preservation of life under drought conditions. Mallery (1935) reported especially high osmotic values of 55 atmospheres in the cell sap of the leaves and small twigs of creosotebush. Harris and Lawrence (1916) gave values of 33.5, 26.2, and 34.2 atmospheres for osmotic pressures of three creosotebush plants near Tucson and stated that these were "more nearly minimum than maximum values."

Duisberg, discussing a paper by Went (1953), suggested that nordihydroguaiaretic acid (NDGA), which is found only in creosotebush (Waller 1942), may be involved in the species' drought resistance. In other studies (1952), he found no indication that the NDGA was formed as a response to drought. He did observe that protein content was higher in the more desiccation-resistant plants and suggested that basic biochemical differences existed between the smaller drought-resistant and the larger non-resistant leaf types.

Effects on Metabolism

Biochemical differences arising during drying involve the carbohydrate metabolism of the plant (Wood 1932, 1933b). Levitt (1951)

stated that increased sugar content with drying causes an increased osmotic pressure. The latter is also affected by interconversion of carbohydrate. Vaadia, Raney, and Hagan (1961) reported that water deficits accelerated conversion of starch to sugars and Gates (1964) added that a change in the level of starch in the plant usually accompanies moisture shortage and leads to an increase in soluble carbohydrates. Wood (1932) found that the carbohydrate flux of plants of arid communities is diverted to form pentosans and mucilages which confer a certain amount of drought resistance on the plants. Roux-Lopez (1964) felt that mucilagenous material on creosotebush added to its resistance.

Stocker (1960) suggested two phases in response to moisture stress. The reaction phase results from decreased photosynthesis and increased respiration and oxidative-hydrolytic activity due to changes in protoplasmic viscosity and enzymatic activity. The restitution phase, brought on by a certain amount of adjustment to the stress, is accompanied by an increase in synthetic activity.

Likewise, during water stress protein synthesis is slowed and proteolysis may occur, promoting an increase in soluble nitrogen compounds such as amino acids, amides and soluble proteins (Stocker 1960). Protein synthesis is apparently impared because moisture stress causes a decreased accumulation of RNA (Gates and Bonner 1959).

Further stress causes deamination of the amino acids and other nitrogenous degradation products which allows toxic amounts of ammonia to accumulate and will ultimately cause the death of the

plant. Resistant plants retain their synthetic capacities longer under stress than non-resistant plants. The higher protein content of the more desiccation-resistant plants noted by Duisberg (1952) may have shown a result of this phenomenon. This study by Duisberg appears to be the only one attempting to relate directly the biochemistry of creosotebush to its drought resistance.

Temperature Relationships

Heat Resistance

Although no measurements of the actual temperature that creosotebush withstands have been reported, other temperature measurements are available. Strain and Chase (1966) preconditioned creosotebush plants at high, medium, and low temperatures and found that high temperature preconditioned plants still had a net photosynthesis at 40 C daytime temperature. The low temperature plants were inefficient at temperatures above 20 C and above 32 C there was a net loss of CO_2. Dalton (1961) and Shellhorn (1955) mentioned that the optimum temperature for germination of creosotebush seed were a high 35 C (95 F) and 37 C (98.6 F), respectively. Cannon (1918) found the optimum temperature for root growth of creosotebush to be 32 C (89.6 F).

Effects on Metabolism

Heat resistance, like cold resistance, is similar to drought resistance (Levitt 1956) although some would disagree (Stocker 1960). During overheating of resistant plants, metabolism and the colloidal-chemical properties of the protoplasm are modified, and various

adaptive responses are initiated. According to Henckel (1964) plants are injured and killed by high temperatures because of the decomposition of proteins and the consequent release of ammonia. Just as in drought resistant plants, temperature tolerant plants also have within them the mechanism to keep the ammonia below a toxic level.

Conclusion

Despite the occurrence of creosotebush in the hot southwestern deserts, nothing has been reported on heat resistance in creosotebush. From a review of literature concerning drought resistance as related to creosotebush, it appears that there are anatomical adaptations or physiological conditions in every part of the plant that can contribute to the overall resistance. An investigation into the variation of the drought resistant mechanisms between populations may elucidate population differences and may also give a better understanding of the biochemical aspects of creosotebush's drought and temperature resistance.

DESCRIPTIONS OF THE STUDY AREAS

Introduction

Three widely spaced sites (Figure 1) having comparatively different climates were chosen as plant collection areas: 1) Zapata, Texas, representing the southern extension of creosotebush in the United States; 2) Sheffield, Texas, representing an east-central population of creosotebush; and 3) Bernardo, New Mexico, representing a northern extension of creosotebush in the United States. Voucher specimens from collections made at these three sites are on file in the University of Arizona Herbarium.

Zapata, Texas

Zapata, Texas (26 54' N lat; 99 16' W long) is located in a ceniza-shrub area near Falcon Lake on the Rio Grande at an elevation of about 500 ft (152 m) and has the highest temperatures and annual precipitation of the three collection sites. *Leucophyllum*, *Larrea* and *Prosopis* make up a major portion of the vegetation (Küchler 1964). The plants were collected approximately 3 miles south of the town from a small isolated area of creosotebush growing on a slight rise (Figure 2). Climatic data (Figure 3) were obtained from Rio Grande City, Texas, which is approximately 30 miles southeast of Zapata (Texas State Climatologist 1966). Evapotranspiration curves were established by using the method of Palmer and Havens (1958).

Figure 2. General view of the Zapata collection site located near Zapata, Texas.

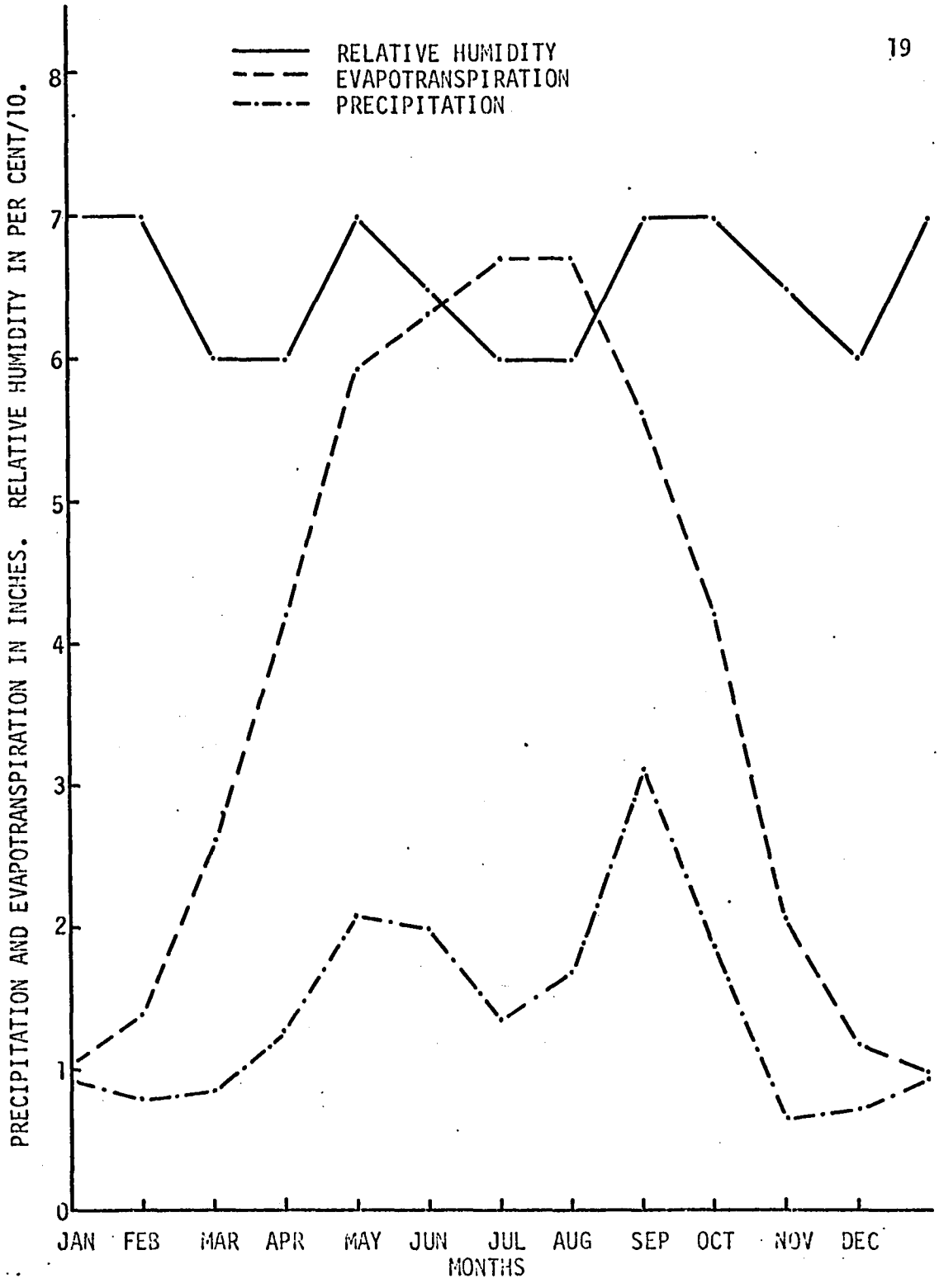

Figure 3. Climatic pattern for Zapata, Texas.

A warm-temperate subtropical climate prevails. The average annual rainfall is about 17 inches (50.18 cm), most of which falls during thunderstorms. Precipitation values of 0.10 inches (0.25 cm) or more occur on only 28 days each year.

Summer temperatures are high, reaching about 90 F (32.C). Winter temperatures are mild and the frost-free period lasts about 305 days, although in some years freezes do not occur. The record low is 10 F (-12.2 C). Snowfall rarely occurs and does not last longer than a few hours. The mean annual relative humidity is about 70%.

Sheffield, Texas

The Sheffield site (30 41' N lat; 101 50' W long) was located in the Trans-Pecos shrub savanna area of west Texas at an elevation of about 2000 ft (609.6 m). *Flourensia, Larrea* and *Juniperus* comprise the major part of the vegetation (Küchler 1964). The plants were collected approximately 10 miles northeast of Sheffield on a gently sloping bajada near the Pecos River (Figure 4). Climatic data (Figure 5) were obtained from Sheffield, Texas which is approximately 5 miles west of the site (Texas State Climatologist 1966).

The climate is predominately semi-arid and droughts occur frequently. Average annual rainfall is 13 inches (33.0 cm) and, although the monthly totals are erratic, more than 70 per cent falls from May through October. The mean annual relative humidity is about 60%.

Figure 4. General view of the Sheffield collection site located near Sheffield, Texas.

Figure 5. Climatic pattern for Sheffield, Texas.

Summers in the area are long and hot with temperatures of 90 F (32.2 C) or above occurring on about 145 days per year. The record high is 112 F (44.9 C). The winters are mild, and prolonged cold spells are infrequent, freezing temperatures occurring on about 54 days each year. The record low is -1 F (-18.3 C). Annual snowfall is usually about 4 inches (10.16 cm).

Bernardo, New Mexico

Bernardo (34 21' N lat; 106 49' W long) is located near the confluence of the Rio Puerco and the Rio Grande in central New Mexico. The collection site was at 5000 ft (1,524 m) in a creosotebush (*Larrea-Flourensia*) area about 6 miles southwest of the village of Bernardo (Figure 6). Climatic data (Figure 7) were obtained from Los Lunas, New Mexico which is about 10 miles north of Bernardo (Houghton 1966).

The area, with an average annual precipitation of less than 8 inches (20.32 cm), has an arid continental climate. Precipitation of 0.10 inches (0.25 cm) or more occurs on only 19 days each year. The relative humidity averages somewhat less than 50% for the year, with readings below 20% common during the warmer part of the day in late winter and spring.

Summers are cooler than at the previous two sites and include only a day or two when the high temperature exceeds 100 F (37.7 C) and only 84 days of 90 F (32.2 C) or above. The record high is 106 F (41.1 C). Winters are mild and dry but colder than the winters at Zapata and Sheffield. On only about two days each winter, does the

Figure 6. General view of the Bernardo collection site located near Bernardo, New Mexico.

Figure 7. Climatic pattern for Bernardo, New Mexico.

temperature ordinarily fail to rise above the freezing mark. However, on 157 days, temperatures ordinarily fall below freezing. The record low is -10 F (-23.3 C). Some snow usually falls each winter but rarely lies on the ground for more than a few hours.

Conclusion

A general comparison of the three plant collection sites reveals several important environmental differences (Table 1) which could have influenced the genetic development of their native plant populations. Therefore, since plants from the separate areas may have different genotypic adaptations to the several environmental components, the three populations have been characterized as hot-humid (Zapata), intermediate (Sheffield), and cool-dry (Bernardo). These characterizations will be used in later discussion to help clarify the plant response to moisture and temperature treatment.

Table 1. General summary of the plant collection sites.

Location	Latitude	Mean annual temperature	Mean annual precipitation	Elevation	Relative humidity	Description
Zapata, Texas	26 54' N	74 F 23.3 C	17 inches 50.18 cm	500 ft 152 m	70%	Hot-humid
Sheffield, Texas	30 41' N	66 F 18.9 C	13 inches 33.00 cm	2000 ft 609.6 m	60%	Intermediate
Bernardo, New Mexico	34 26' N	54 F 12.2 C	8 inches 20.32 cm	5000 ft 1524 m	50%	Cool-dry

METHODS

Introduction

In an attempt to determine if like physiological changes occur among plants from the three areas under similar moisture and temperature treatment, determinations included the presence of the free amino acids and soluble sugars in the ethanol extract, and the amount of ether extractables, residual nitrogen and moisture in the leaf material. Relative concentrations of protein amino acids and carbohydrates have been used in systematic studies (Alston and Turner 1963). However, since they are so closely dependent upon the physiological state at any given moment, they are almost useless in unmasking genetic differences within a species when sampled under local conditions. Nevertheless, if the plant material is subjected to homogeneous treatment, these labile metabolites may be used to investigate suspected differences (Hagan and Gunckel 1958).

Field Collections

During late September and early October, 1965, plants were collected from the sites described and were transplanted into 8-inch (20.3 cm) plastic pots containing soil from the collection site. The roots were trimmed to fit the pots and the tops of the 10-20 inch (25.4 cm-50.8 cm) plants were trimmed approximately 75% and watered with Vitamin B_1 solution[1] to enhance root growth (Hartmann and Kester 1959).

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1) A list of all chemicals used in this study is given in Appendix A.

The soil was kept wet until transported to the greenhouse in Tucson, Arizona, a maximum of four days.

One hundred forty-six plants were obtained from Zapata, 140 from Sheffield, and 127 from Bernardo. All plants were watered daily until they were established and then were watered at sufficient intervals to keep the soil material moist. Complete 65% Hogland's solution was used once a week as a nutrient source.

In December, the collections were culled to 64 plants from each area. In March, the plants were transplanted to a uniform soil mix in 8-inch (20.3 cm) pots. From these, 16 plants per area were randomly selected for treatment and 4 plants from each area were selected as controls. No original leaf material remained at the time of treatment. The interval between collection and treatment was one year.

The uniform soil mix was made up from peat, loam soil, and sand (1:1:1). Laboratory analyses established the moisture percentage of this mix at 15 atm. to be 3.5%.

All of the plants selected for treatment as well as the controls were in good condition. Only a very few of the older leaves were chlorotic, and all plants were comparable in size, vigor, and age. To find the average age, growth rings of ten of the treated plants were counted. Assuming one growth ring for each year, their ages were: three 5-year olds, three 6-year olds, one 7-year old, one 9-year old, and one 11-year old. This sample gave an average age of 6.7± 1.4 years with a .05 confidence interval.

Treatments

Plants from the three areas were submitted to two different temperature treatments [100 F (37.7 C) daytime/60 F (15.6 C) nighttime and 80 F (26.7 C) daytime/40 F (4.4 C) nighttime] and two different moisture levels [stressed (no water during the seven-day treatment period) and watered (daily waterings during the seven-day treatment period)]. At the end of the treatment period, soils under the stressed conditions were at or below the generally accepted permanent wilting point and those under the watered treatment were near field capacity.

Treatments were applied in two Sherer-Gillett plant growth chambers; one was programmed for the high-temperature regime and the other for the low-temperature regime. Light was supplied by 16-foot Sylvania VHO fluorescent lamps and eight 40-watt incandescent bulbs. These provided good spectral distribution and a minimum intensity of about 2700 foot candles two feet from the light bank. The three 24-hour time clocks that controlled the lighting were programmed to give 14 hours of light. One-half of the flourescent bulbs came on at 5:00 a.m. and turned off at 7:00 p.m. The second half came on at 6:00 a.m. and turned off at 6:00 p.m., and the incandescent bulbs came on at 5:30 a.m. and turned off at 6:30 p.m. All plants were watered at day zero; control plants were sampled at day zero (4 hours after watering). Watered plants were sampled one day after watering and seven days of treatment. Water-stressed plants were sampled seven days after watering and seven days of treatment.

Statistics

The statistical design was a 3 by 2 by 2 factorial (three areas, two temperatures, and two levels of moisture) replicated four times with the exception of amino acids. A factorial design was used because the interactions between area and temperature and area and moisture were of primary interest (Callaham 1963), since the objective of the experiment was to find, if present, differences in population response to moisture and temperature treatments. A single factor experiment would not show these interactions (Steel and Torrie 1960). The analyses of variance were of the form:

Source	df	df (amino acids)
Area	2	2
Temperature	1	1
Moisture	1	1
Area by Temperature	2	2
Area by Moisture	2	2
Temperature by Moisture	1	1
Area by Temperature by Moisture	2	2
Error	36	12
Total	47	23

Analyses of the control plants were treated as a single factor experiment since they could not be included in the factorial design. The data were further compared using Duncan's Multiple Range Test (Steel and Torrie 1960). Differences were tested at .05 and .01

for both the analyses of variance and the Duncan's test. When possible, statistical differences are discussed as "significant" differences (.05 or .01). However, since trends appear to be important, they are also discussed even though they may not be "statistically significant," particularly for amino acids where the unfortunately small sample size did not allow statistical analyses sensitive enough to pick up what seemed to be obvious differences.

Foliar Collections

At the end of the 7-day treatment period, the leaves were hand stripped from each plant and the petioles removed. The leaves were weighed immediately, placed in plastic bag, and immersed in liquid nitrogen until the next plant had been stripped, cleaned, and weighed (approximately 3 minutes). The sample was removed from the liquid nitrogen and placed on dry ice until all samples were taken. Samples were then spread out on individual pie plates and placed in a forced air oven for 5 minutes at 120 C (248 F). The temperature was then lowered to 65 C (149 F) and the samples were dried to a constant weight (Loomis and Shull 1937). After drying, the samples were ground to pass a 60-mesh screen in a Wiley mill, sealed in separate bottles and refrigerated at 30 F (-1.1 C) until extracted.

Extraction Procedure

Preliminary extraction and analyses were run on native Arizona material to develop the procedures. The flow diagram developed from these preliminary extractions is presented in Figure 8. The different steps involved are numbered and comments on each step are given below.

1) Water content of the sample was calculated using the formula, W_D = (FW-DW/DW) x 100, where W_D is the percentage moisture, FW is the fresh weight, and DW is the sample dry weight.

2) Approximately 2g of the dry, ground material were accurately weighed out and extracted. Preliminary analyses of Arizona material showed this amount would give 1 mg/ml protein equivalent which was required for calibration of the amino acid analyzer. Because the resins present in creosotebush continually interfered with the chromatography during preliminary analyses, they were removed by extracting with 150 ml of ethyl ether for 12 hours. The soxhlet extraction thimbles and samples were weighed before and after extraction. The difference between these two weighings was called "ether extractables" and included most of the resins, waxes, and NDGA (Waller 1942). Duisberg, Shires and Botkin (1949) listed several solvents which extract NDGA and other resinous material from creosotebush. Most plant amino acids and sugars are insoluble in ether. Glycine is the major exception but is only slightly soluble (Merck Index 1960).

3) The residue was extracted with 150 ml of 80% ethyl alcohol for 12 hours to remove the free amino acids and soluble

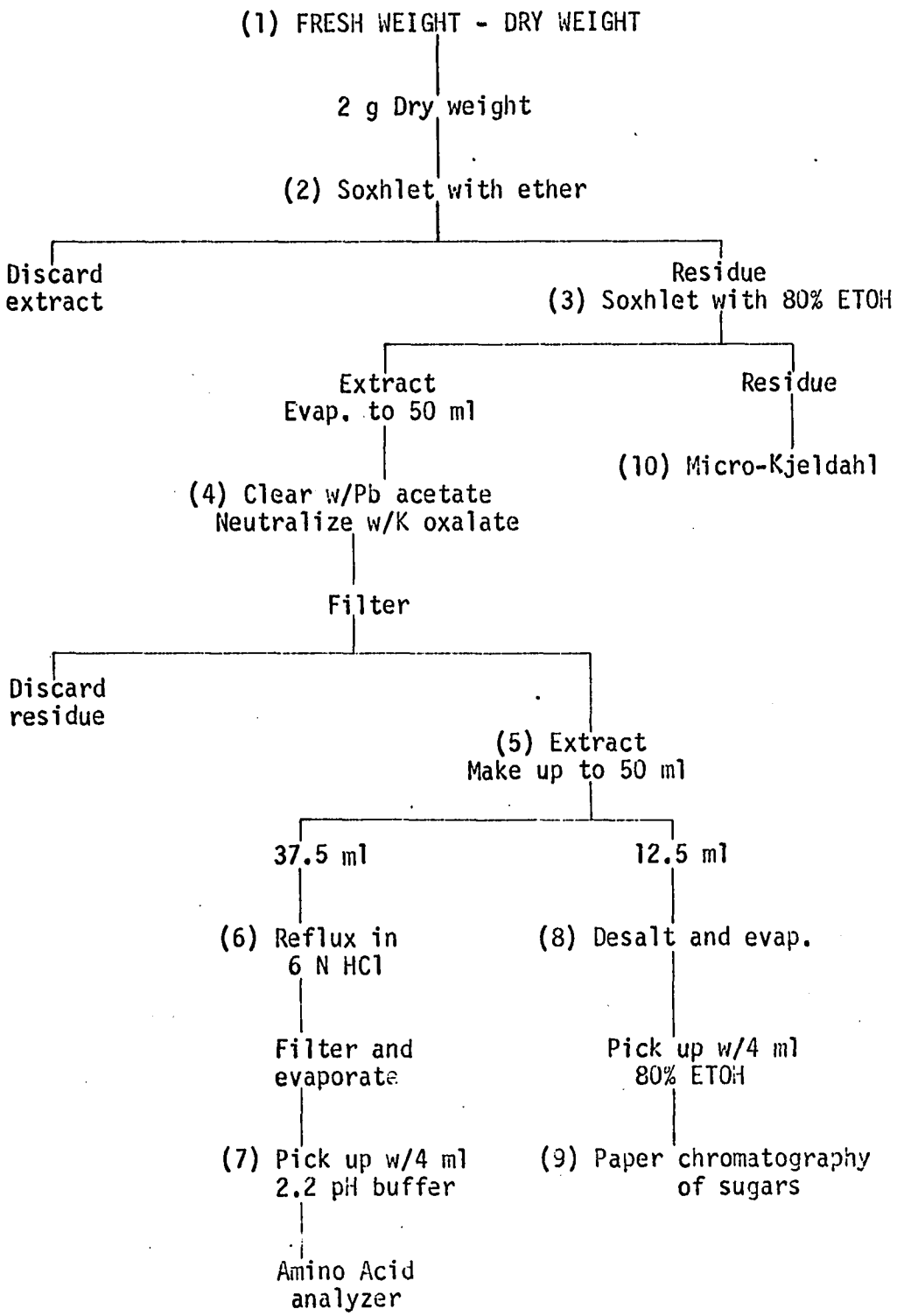


Figure 8. Flow diagram of the procedures used to study the ethanol extracts and residue of creosotebush leaves.

sugars (Loomis and Shull 1937). This extract included short chain peptides, some organic acids, tannins and inorganic salts, as well as the free amino acids and soluble sugars.

4) Saturated neutral lead acetate was added (10 drops/50 ml of extract) to remove a water soluble tannin found in creosotebush by Waller (1947) and other impurities which interfere with chromatography (Block, Durrum and Zweig 1958). Basic lead acetate precipitates NDGA which is a major constituant of the cuticular resin of creosotebush (Duisberg, Shires and Botkin 1949). Saturated anhydrous potassium oxalate was added drop-wise to precipitate the excess lead (Loomis and Shull 1937). This precipitate was filtered out and the residue discarded.

5) The extract was made to exactly 50 ml in a volumetric flask and quantitatively divided into two fractions, 37.5 ml for the amino acid analyzer and 12.5 ml for paper chromatography of the sugars.

6) Since preliminary work indicated a large amount of short chain peptides in the extract (Figure 9) the 37.5 ml of the extract were refluxed with 6 N HCl for 24 hours. Furthermore, because of distorted peaks, the amino acids present in the extract could not be quantitatively or qualitatively evaluated without refluxing. After refluxing much better chromatograms were obtained (Figure 10). Tryptophane and some serine and threonine were lost during refluxing (Sondheimer 1963). Values for glutamic acid and aspartic acid represent the combined values of both the acids and their amides, glutamine and asparigine. Also, due again to refluxing, cysteine

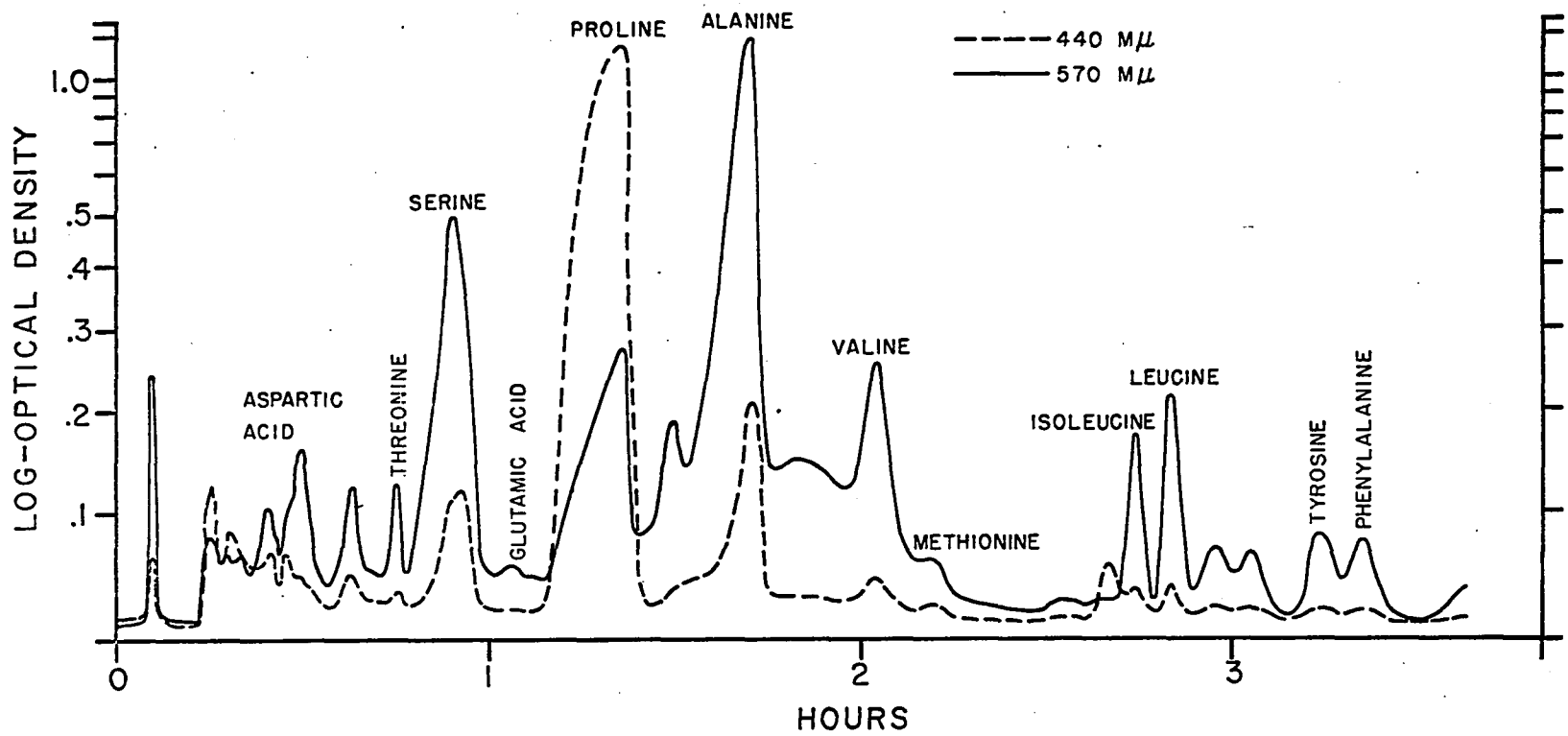


Figure 9. Preliminary chromatogram trace of the amino acids in the ethanol extract of creosotebush leaves.

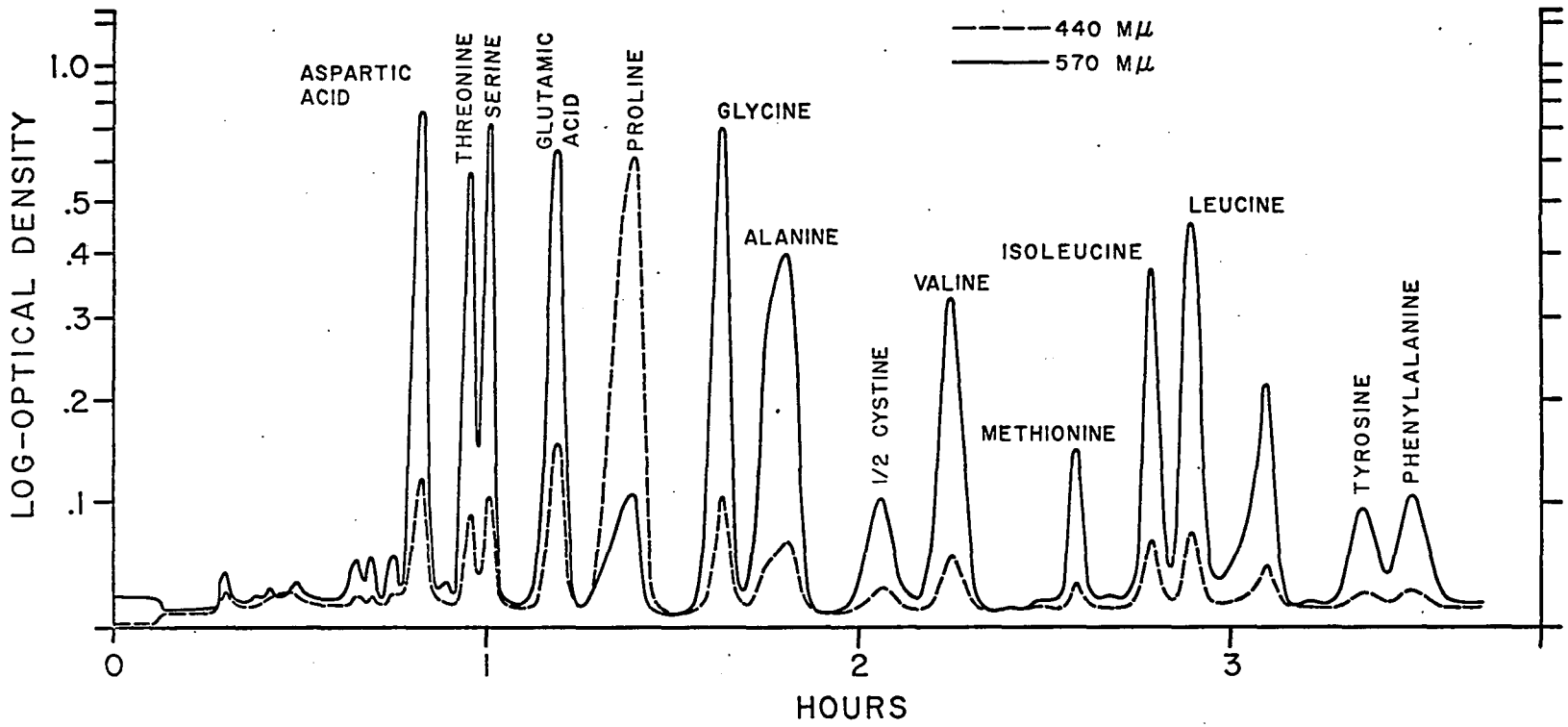


Figure 10. Chromatogram trace of the amino acids found in the refluxed ethanol extract of creosotebush leaves. (Note the increase of glutamic acid and 1/2 cystine as compared to that in Figure 9).

and cystine are presented as one value, 1/2 cystine, since cystine was readily split on hydrolysis. The increase in glutamic acid, aspartic acid and 1/2 cystine after refluxing (Figures 9 and 10) may be attributed to these reactions and to the hydrolysis of the short chain peptides.

7) After refluxing, the extract was filtered and evaporated to dryness over a steam bath. The residue was picked up with 4.0 ml of 2.2 pH sodium citrate buffer. Each sample was filtered and 1.5 ml used in the Beckman Model 120 B Amino Acid Analyzer. The amino acids were identified by comparing their time of emergence from the column with that for standards. Unfortunately, due to the tightness of scheduling and the length of time required to run each sample, only two replications were analyzed.

8) Since a large excess of inorganic ions interferes with the chromatography of sugars (Block, Durrum and Zweig 1958), the 12.5 ml of extract was desalted using a Warner-Chilcott Model 1930 electric desalter. After desalting, the extract was evaporated to dryness and picked up with exactly 4.0 ml of 80% ethyl alcohol.

9) Quantitative and qualitative analyses of the soluble fructose, glucose, and sucrose in the ethanol extract were accomplished by using a paper chromatography procedure adapted from Wilson (1959). The two reducing sugars, fructose and glucose, and sucrose were the only soluble sugars found in any measurable amounts during preliminary analyses of the alcohol extracts (Figure 11). Other spots appeared on the chromatograms in the relative vicinity of

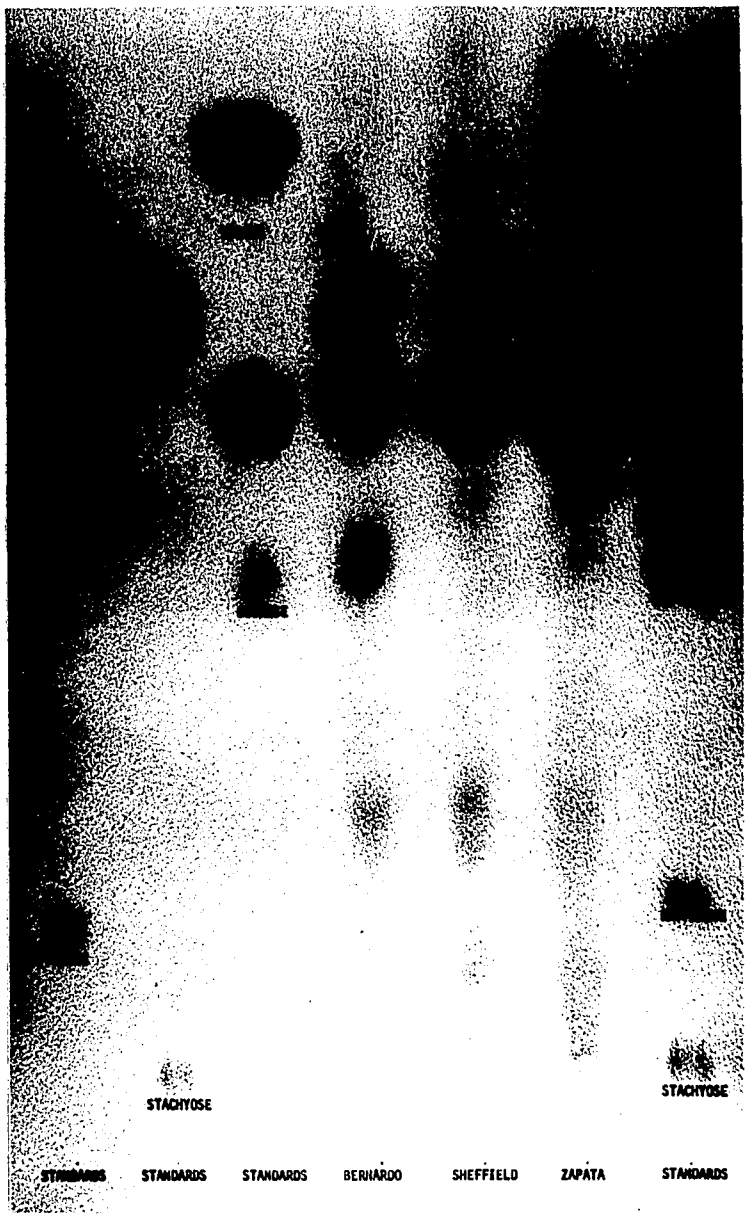


Figure 11. Preliminary chromatogram of the sugars present in the ethanol extract of creosotebush leaves.

raffinose and stachyose. Since these two polysaccharides have a role in cold tolerance (Alston and Turner 1963) (and perhaps temperature or drought tolerance), a standard sugar solution which included these sugars in addition to fructose, glucose, and sucrose was prepared in the event that some extracts would show their presence.

Whatman No. 1 filter paper, 18 by 22.5 inches, was used as the support material. The ethanol extracts and sugar standards made from commercial sugars dissolved in ethanol were applied to the paper with 50-µl micropipettes. Glucose and fructose standards were made up in a 1 µg/µl concentration. Preliminary work showed that more sucrose was required to give a spot equivalent to those of glucose and fructose; thus, the sucrose standard was made up in a concentration of 2 µg/µl.

Five sample extracts and two standard concentrations were applied at intervals of 3.0 cm along one edge of the paper. Internal standards (50 µl and 100 µl) were run on each chromatogram. The sugars were separated by three times descending development with butanol: pyridine: water (6:4:3) in a large glass chromatogram jar. After developing, the paper was air dried for at least 4 hours, dipped into 40 to 50 ml of the color reagent (1.66 g o-phthalic acid and 0.91 ml aniline dissolved in 48 ml of 1-butanol, 48 ml of ethyl ether, and 4 ml of distilled water), and dried again. The paper was then heated in an oven at 105 C (221 F) for 10 minutes to develop the spots.

Individual spots of glucose, fructose, and sucrose were cut from the chromatograms and eluted with 5 ml of the eluting reagent [0.7 N HCl and 80% ethyl alcohol (v/v)]. The absorbances of the solutions were determined on a Beckman Model B spectrophotometer using 1 cm cuvettes at 390 mμ. The amount of each sugar was determined by reference to a graph made from the standards on their particular chromatogram.

10) The nitrogen in the residue after ether and ethanol extraction was determined by a micro-Kjeldahl procedure. Samples of the residue were weighed to 0.05 ± .01 mg and placed in a micro-Kjeldahl boiling flask and washed with 5.0 ml of water. Three ml of H_2SO_4 and 0.5 g of K_2SO_4:$CuSO_4$:selenium catalyst (10:1:1) was then added and washed down with 5 ml of water.

This was distilled at approximately 200 C (392 F) for 3 to 4 hours. The samples were cooled and placed on a distilling apparatus. Concentrated NaOH was added to make the solution basic. The distillate was collected in an Ehrlenmyer flask containing 5.0 ml of boric acid and four drops of the indicator methyl red, bromcresol green. Standardized HCl was used to titrate the NH_4^+.

Residual nitrogen was calculated as mg/g of the residue. This fraction included primarily protein N since the free amino acids, amides, NH_3^+, inorganic N and nucleic acids were removed in the ethanol and ether extracts and nitrate N is not determined using the micro-Kjeldahl method.

RESULTS

Response to Moisture

Leaf Moisture Status

Soil moisture values exhibited wide variation between watered and stressed treatments, but not among area sources (Table 2). Temperature treatment also had relatively little influence on soil moisture values. Soil moisture differences between watered and stressed treatments caused significant quantitative differences in leaf moisture between watered and stressed plants. Percentage moisture (W_D) values for the control plants averaged 146% as compared to 141% for the watered plants and 79% for the stressed plants (Table 3). The hot-humid Zapata plants had a high initial (W_D) value of 174% compared with the intermediate Sheffield plants (140%) and the cool-dry Bernardo plants (123%). The hot-humid Zapata plants also had a relatively higher water loss rate than plants from the other two areas as indicated by plotting moisture percentages for the three populations under stressed and watered treatments as a percentage of their controls (Figure 12).

Leaf Soluble Sugar Status

Figure 13 is a representative chromatogram of the alcohol extract. Spots having Rf values close to those for stachyose and raffinose were evident, but these extra spots are probably artifacts separated from resins remaining in the sample. Fructose, glucose,

Table 2.† Soil moisture values as percentage of dry weight after moisture treatment.

Treatment		Cool-dry Bernardo	Intermediate Sheffield	Hot-humid Zapata
Temp. (°F)	Moisture			
80/40	watered	21.0	20.0	19.0
80/40	stressed	2.7	2.5	2.6
100/60	watered	18.0	17.4	17.8
100/60	stressed	4.0	4.2	3.0

†Absolute values for all data are presented in Appendix B.

Table 3. Leaf moisture as percentage of dry weight for the three populations of creosotebush after moisture treatment.

Population	Control	Watered (a)	Stressed (b)	Difference (a-b)
Hot-humid Zapata	174	150	68	82**
Intermediate Sheffield	140	138	91	42**
Cool-dry Bernardo	123	134	77	54**
Mean	146	141	79	

**Statistically significant at the .01 level.

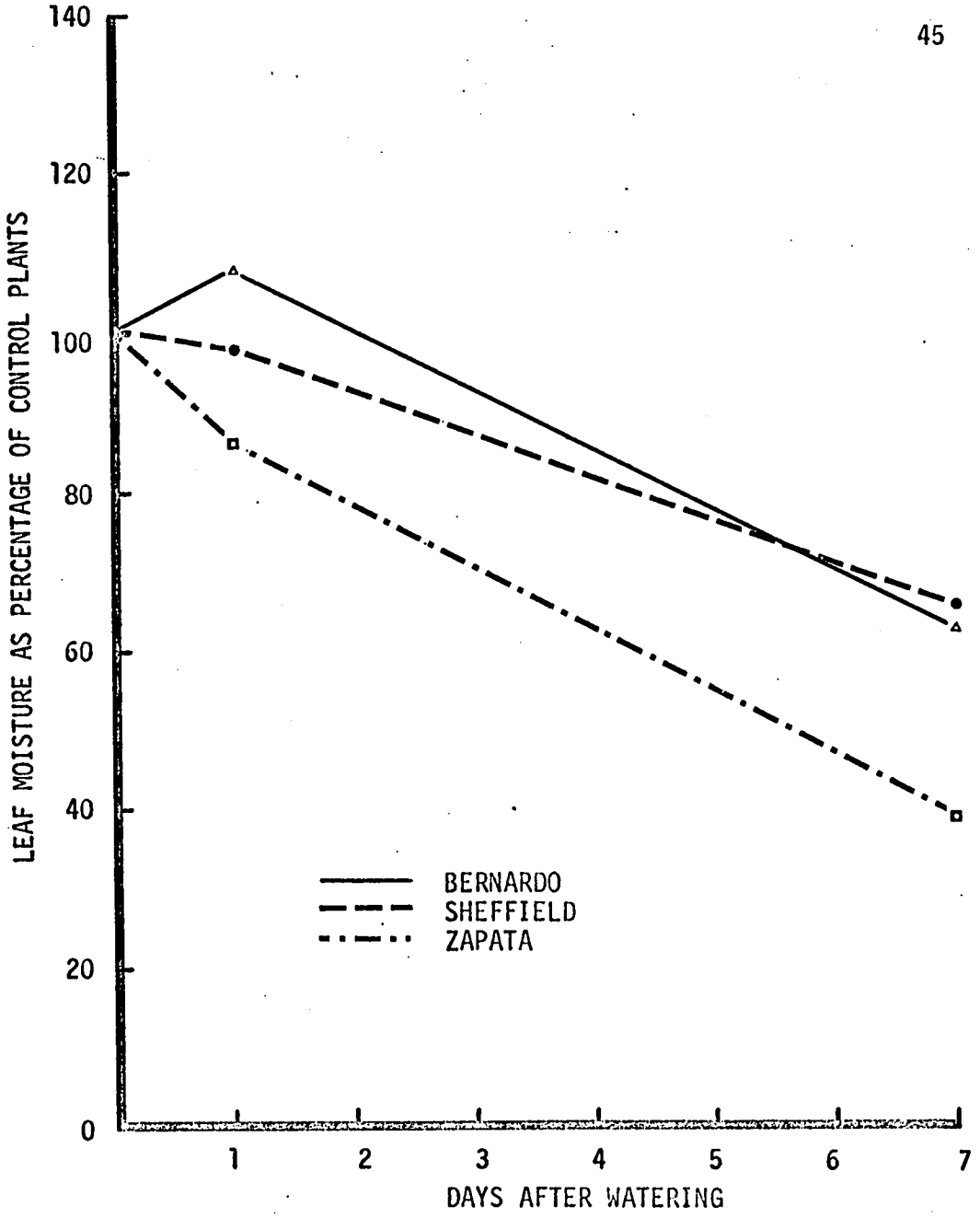


Figure 12. Leaf moisture percentages for the watered and stressed plants from the three collection sites expressed as percentages of their control plants.

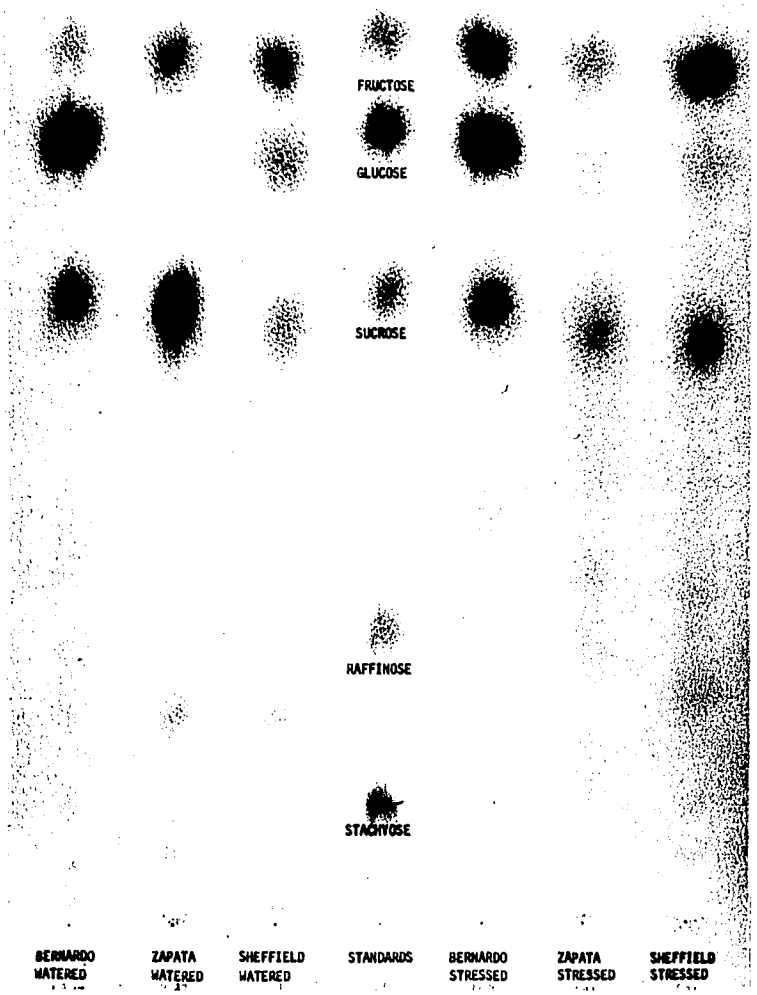


Figure 13. Chromatogram of the sugars present in ethanol extracts of three populations of creosotebush.

and sucrose were present, however, and quantitative differences due to moisture treatments were apparent.

Since metabolites may increase or decrease depending upon the degree of desiccation, the phase (reaction or restitution) existing at the time of sampling should be known. The influence of drying on the soluble sugar status may be seen if values from individual plants are grouped according to, and plotted against, their respective moisture percentages (Figure 14). With increasing degrees of desiccation, several different reactions can be identified: 1) an increase in sucrose at the expense of both glucose and fructose to a W_D of about 130%, which, according to Stocker (1960) is already in the "reaction phase," 2) hydrolysis of sucrose to the monosaccharides at W_D values of 130% to 115%, 3) an extreme reaction phase resulting in decreased photosynthesis and/or increased respiration of sucrose, fructose, and glucose, evident between 115% and 95%, 4) the "restitution phase" which is weakly evident between 95% and 70%, and 5) the drier portion below about 70% representing a complete breakdown of metabolism and rapid hydrolysis of storage and structural compounds to fructose, glucose and sucrose, and a lack of translocation of these sugars out of the leaves. The leaf moisture percentage means of 141% for all watered plants and 79% for all stressed plants indicate that the watered plants were sampled, on the average, during a slight moisture stress, and stressed plants were sampled during the early restitution phase (Figure 14).

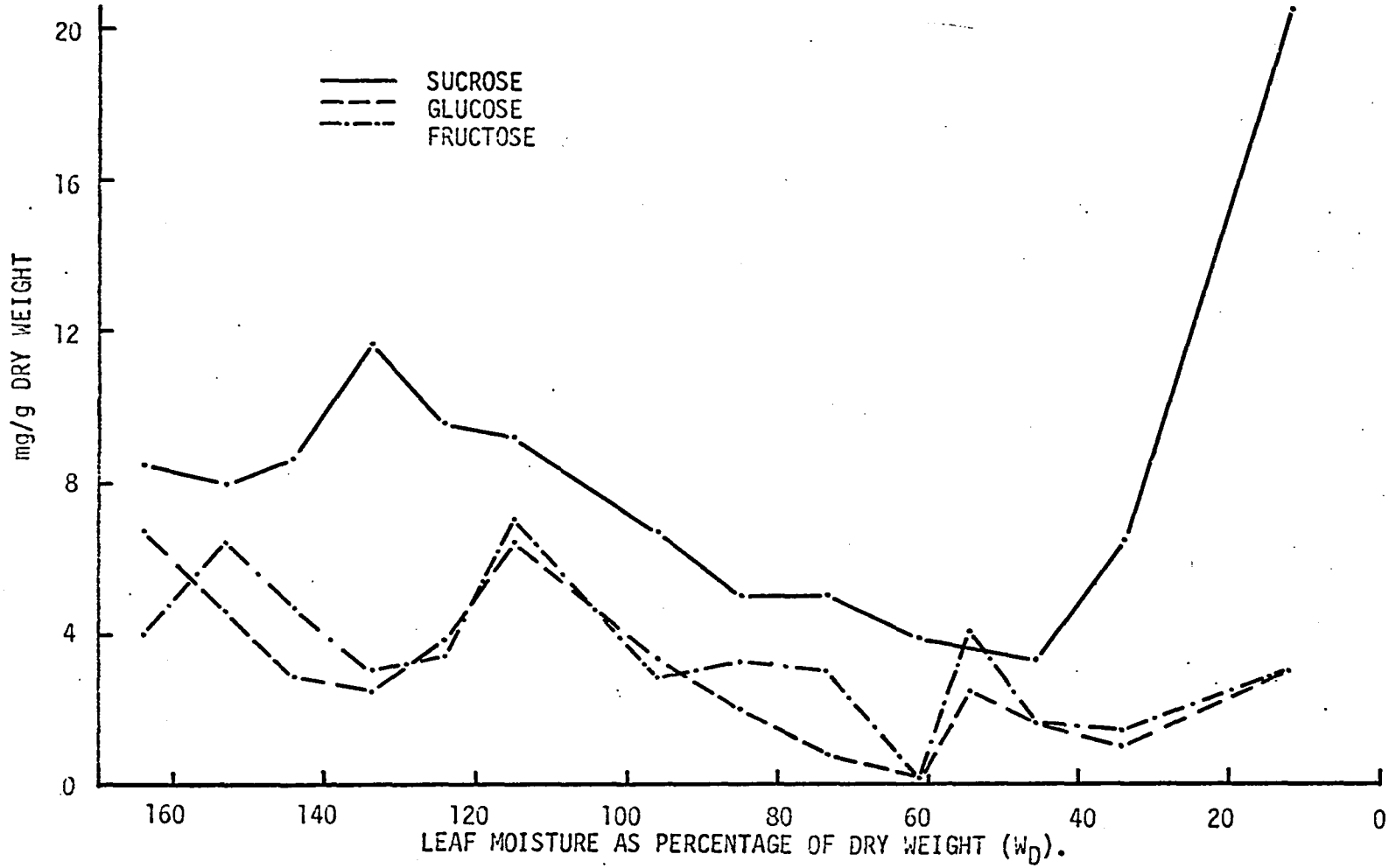


Figure 14. Response curves of fructose, glucose, and sucrose in the ethanol extracts of creosotebush having varying degrees of leaf moisture.

Expressed as a percentage of the control, fructose appeared to increase in the watered Sheffield plants and this increase was maintained under the stressed conditions (Figure 15). Both the Zapata and Bernardo plants showed less fructose under the watered and stressed treatments than for their controls. In terms of absolute values, there were no significant differences among the control plants. After treatment, however, the cool-dry Bernardo plants had significantly less fructose (1.8 mg/g) than either the hot-humid Zapata plants (3.6 mg/g) or the intermediate Sheffield plants (4.2 mg/g).

Glucose values were significantly different between watered (5.3 mg/g) and stressed (2.8 mg/g) plants. When expressed as a percentage of their control plants (Figure 16), glucose in the Bernardo and Sheffield plants increased under the watered treatment and decreased significantly upon drying. There was much less glucose in both watered and stressed Zapata plants than for the controls.

Plants from all three areas under the watered treatment demonstrated an increased sucrose content over the control plants, and a subsequent loss under the dry condition (Figure 17). The difference between the watered Bernardo plants and the stressed Bernardo plants was significant. Neither the intermediate Sheffield plants nor the hot-humid Zapata plants lost as much sucrose on drying as did those from Bernardo.

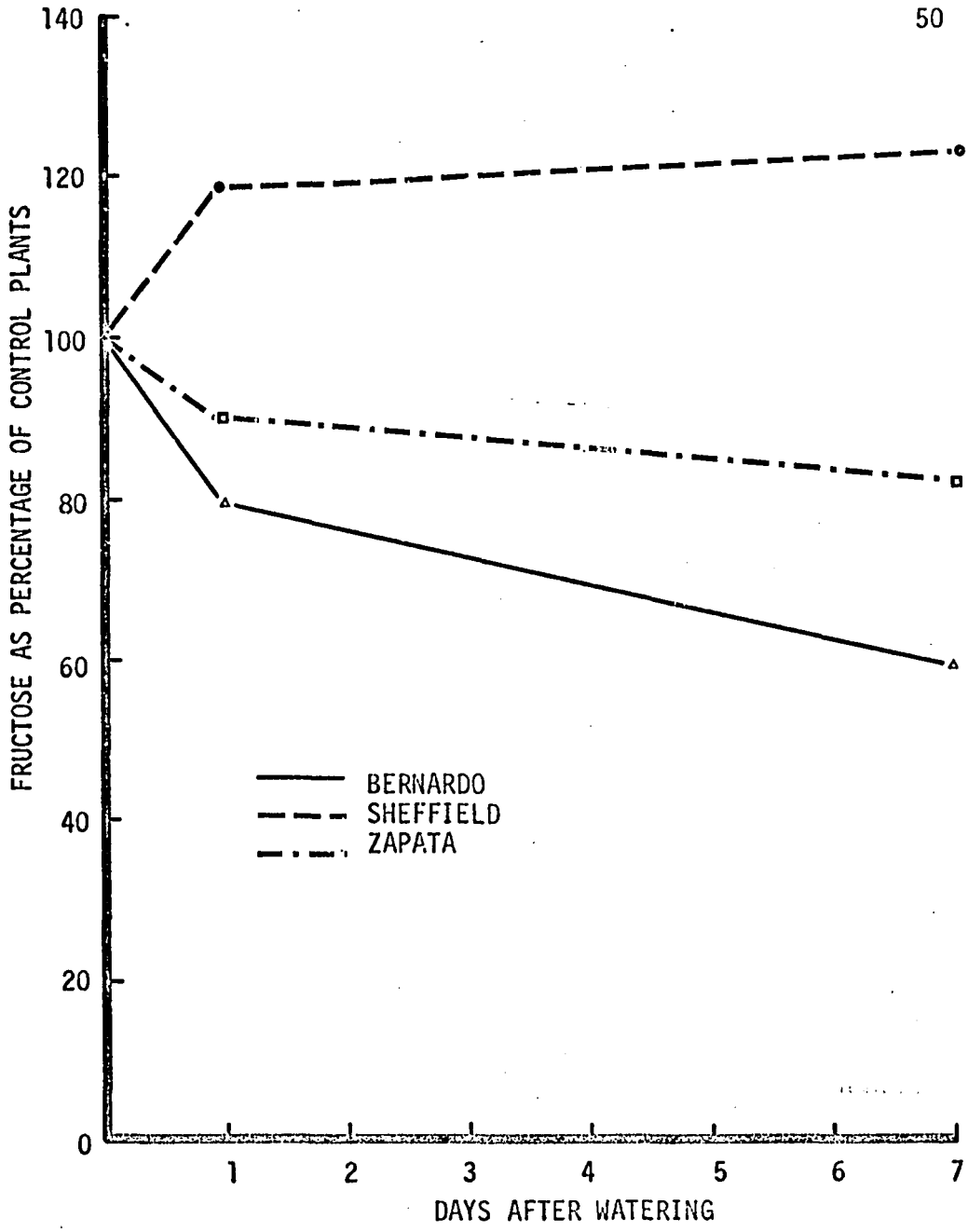


Figure 15. Fructose content of the ethanol leaf extracts of watered and stressed plants from three populations of creosotebush expressed as the percentages of their control plants.

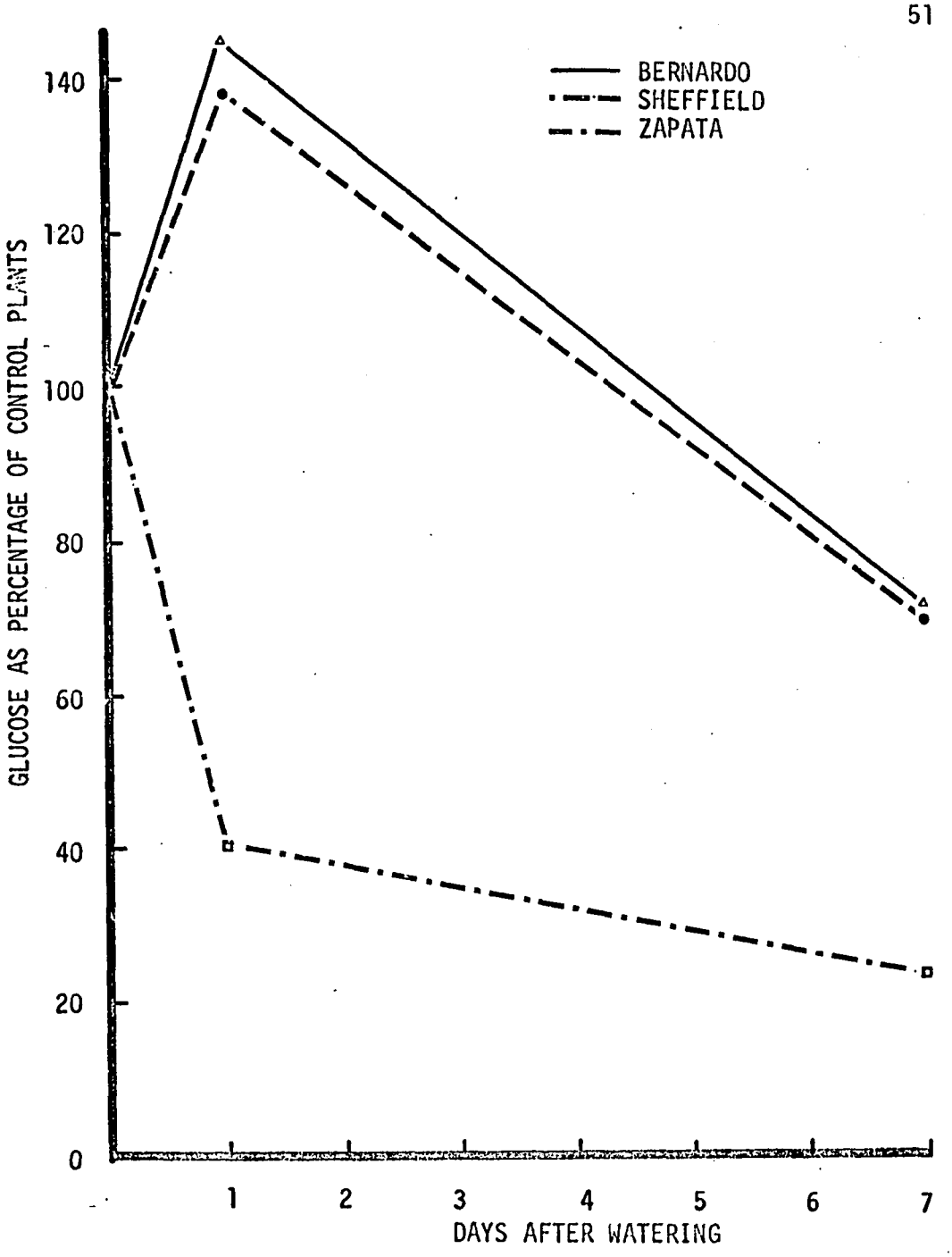


Figure 16. Glucose content of the ethanol leaf extracts of watered and stressed plants from three populations of creosotebush expressed as the percentages of their control plants.

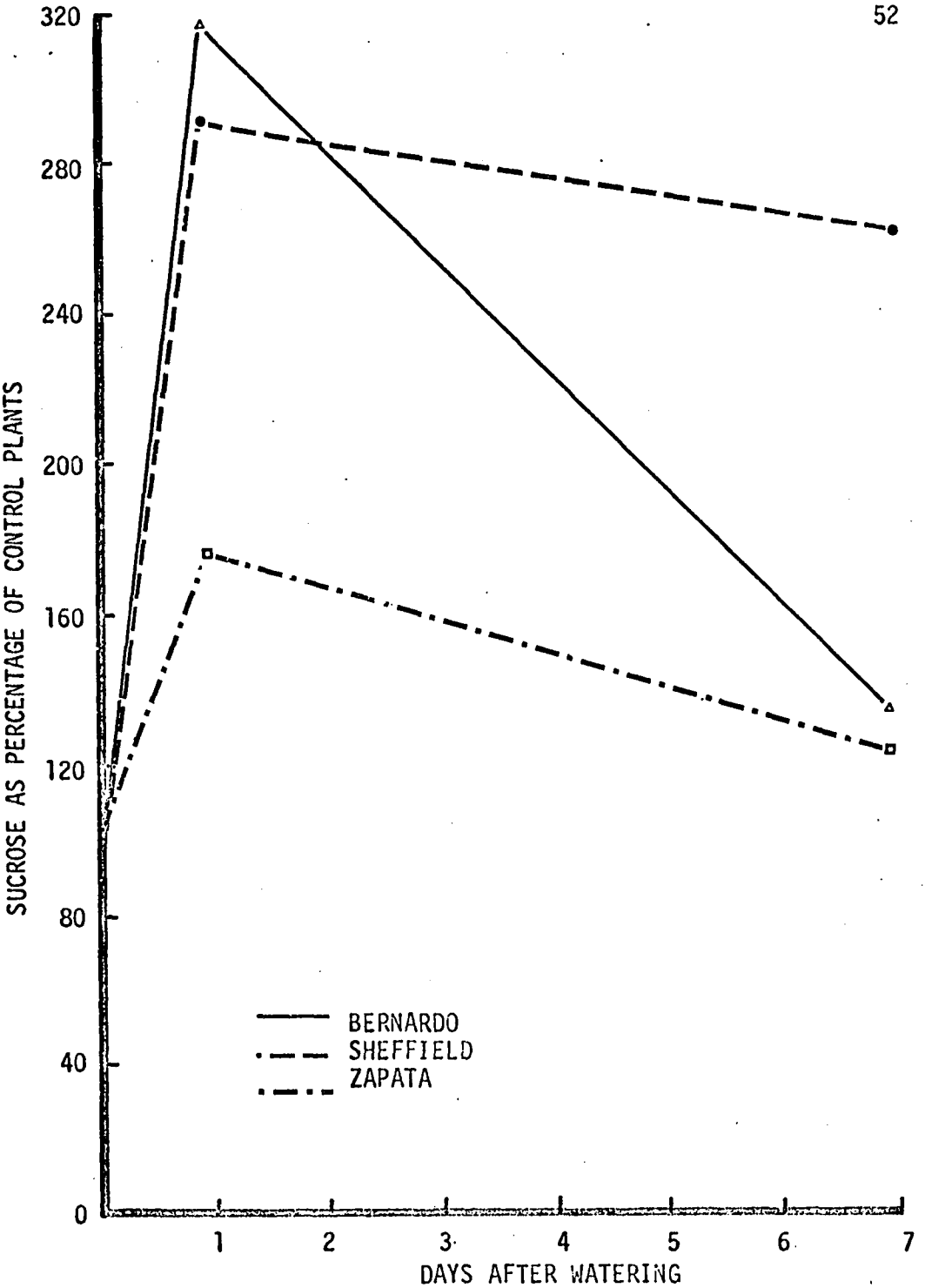


Figure 17. Sucrose content of the ethanol leaf extracts of watered and stressed plants from three populations of creosotebush expressed as the percentages of their control plants.

Figure 18 indicates a high amount of total soluble sugars in the leaf for the watered plants from Sheffield and Bernardo and a subsequent decrease with water stress. Plants from Zapata had less soluble sugars than the controls for both watered and stressed treatments. The difference between watered and stressed treatments was significant in the cool-dry Bernardo plants but not significant in plants from the other two areas.

Residual Nitrogen Status

Differences in nitrogen content of the residue appeared among the different plant populations (Table 4). For the control values, the hot-humid Zapata plants had a significantly higher value (30.1 mg/g) than the intermediate Sheffield (21.4 mg/g) and the cool-dry Bernardo plants (20.9 mg/g). The Zapata plants also had a significantly higher value for the area main effects (21.8 mg/g) than plants from Sheffield and Bernardo (18.6 mg/g and 18.9 mg/g, respectively). Residual nitrogen values for the control plants, when compared with averages for each area, showed that the cool-dry Bernardo plants lost 2.0 mg/g, the intermediate Sheffield plants, 2.8 mg/g, and the hot-humid Zapata plants, 8.3 mg/g.

No significant differences occurred between the plants from the moist treatment and those from the stressed treatment, although slight trends were indicated when expressed as a percentage of the control plants (Figure 19).

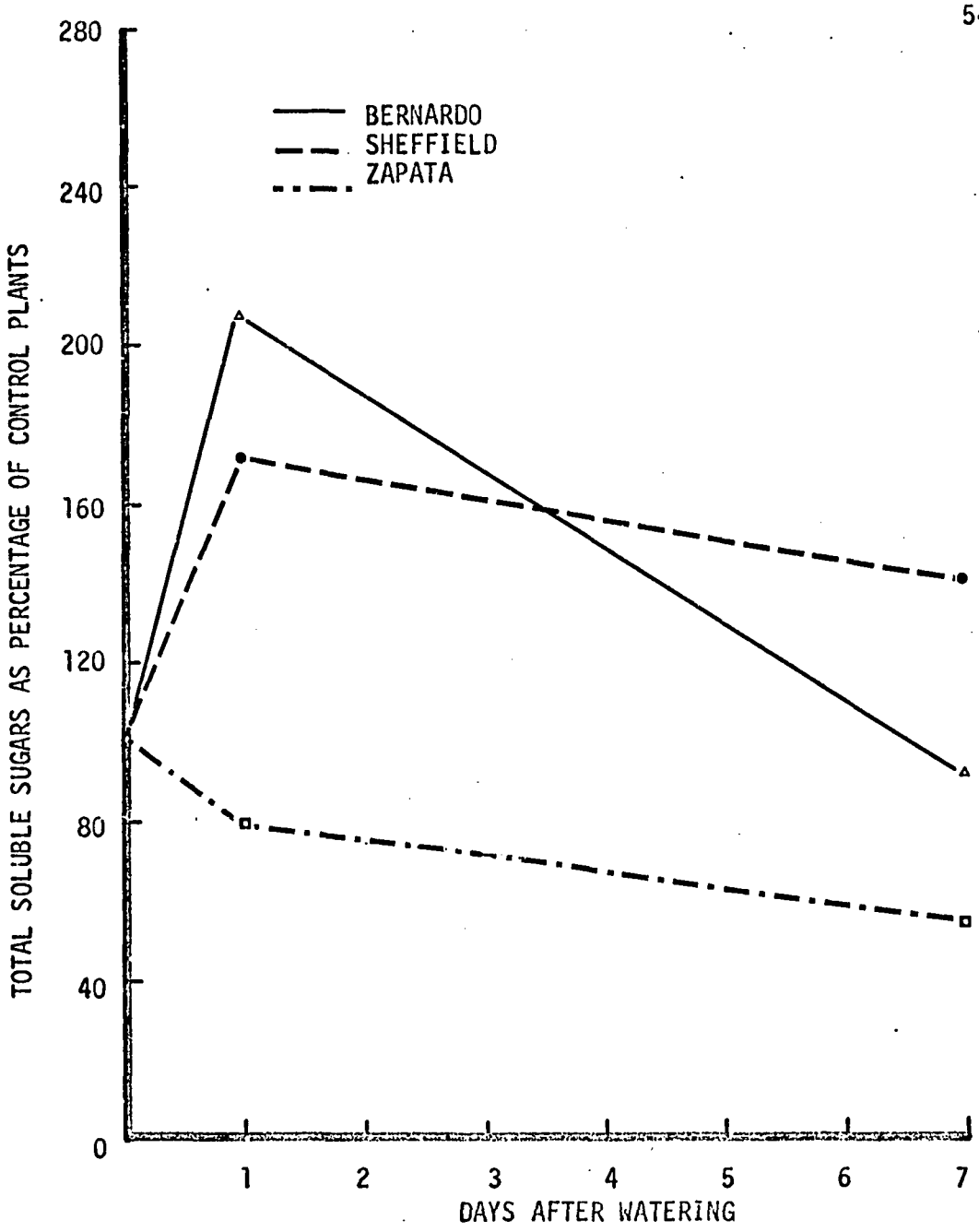


Figure 18. Total soluble sugar content of the ethanol leaf extracts of watered and stressed plants from three populations of creosotebush expressed as the percentage of their control plants.

Table 4. Residual nitrogen in mg/g dry weight of residue for the three populations of creosotebush after moisture treatment.

Population	Area Main Effects			
	Control (a)	Difference (a-b)	Watered	Stressed
Hot-humid Zapata	30.1	21.8	8.3	22.9	20.6
Intermediate Sheffield	21.4	18.5	2.8	18.0	19.0
Cool-dry Bernardo	20.9	18.9	2.0	19.4	18.4

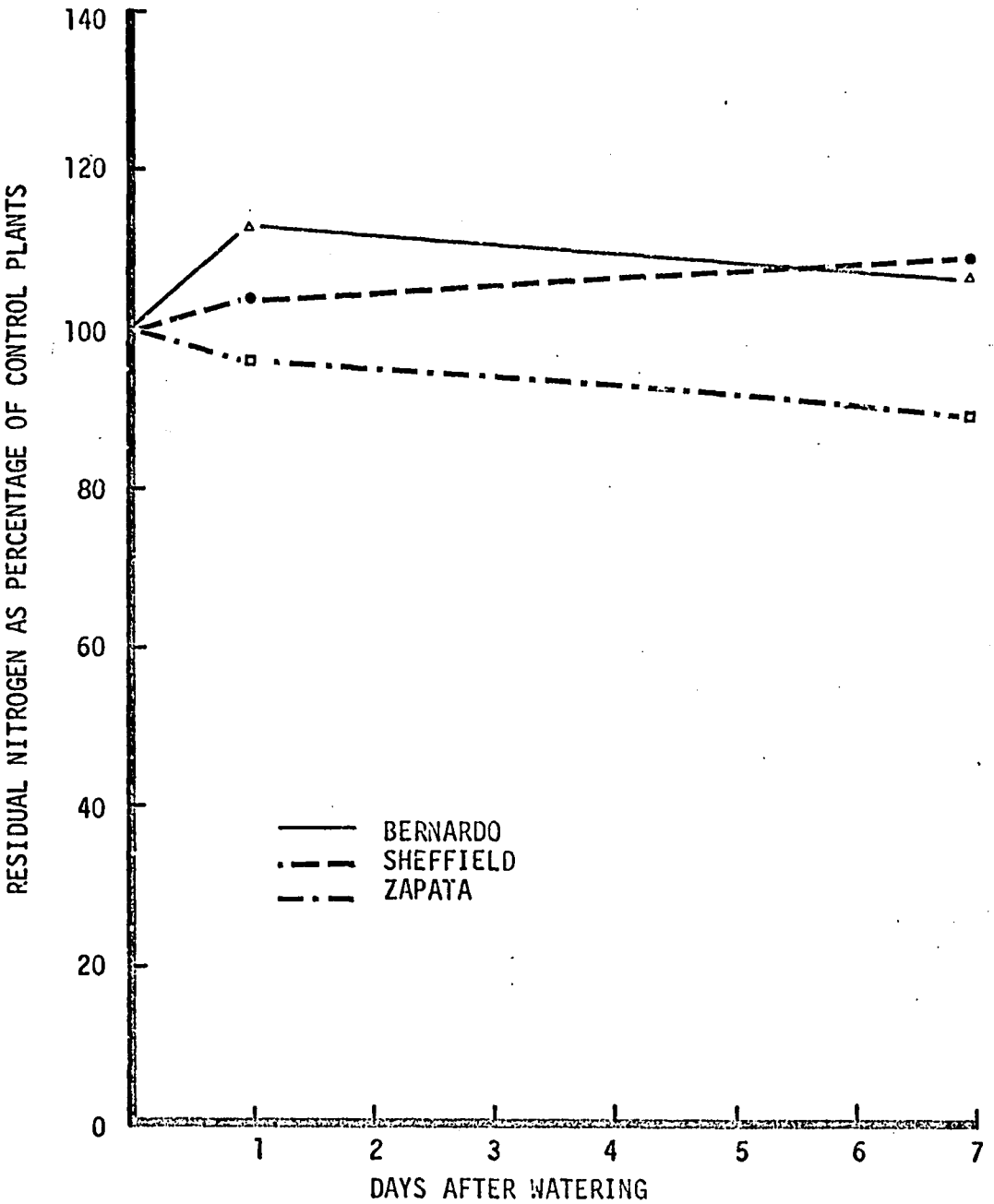


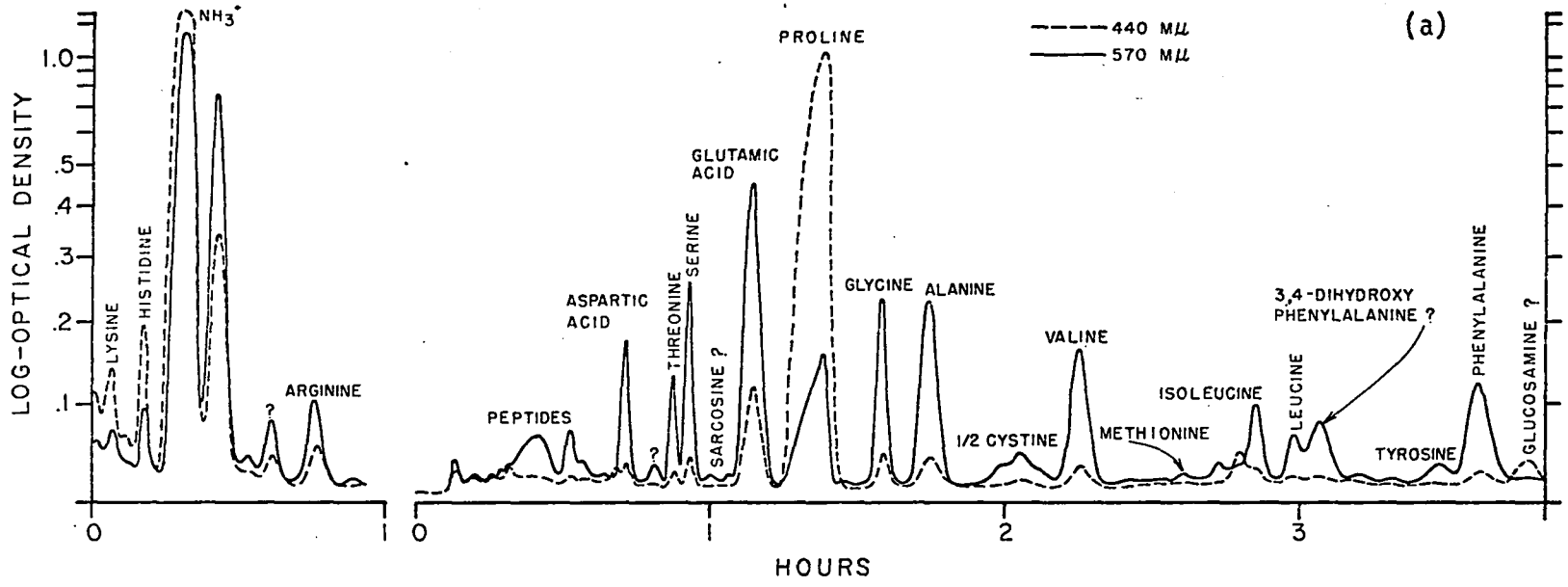
Figure 19. Residual nitrogen content of the leaf material of watered and stressed plants from three populations of creosotebush expressed as the percentages of their control plants.

Free Amino Acid Status

Several amino acids were present in the ethanol leaf extract (Figure 20). The peaks identified were lysine, histidine, arginine, aspartic acid (asparigine?), threonine, serine, glutamatic acid (glutamine?), proline, glycine, alanine, 1/2 cystine, valine, methionine, isoleucine, leucine, tyrosine, and phenylalanine. In addition to these, four other ninhydrin positive compounds appeared with some degree of consistancy. Three of these were tentatively identified by comparing them with previously run standards on file at the Agricultural Biochemistry Department, University of Arizona. The first was sarcosine which appeared as a small peak following serine, the second was 3 4-dihydroxyphenylalanine which followed leucine, and the third was glucosamine which followed phenylalanine. However, Davies, Giovanelli and ApRees (1964) have cited the difficulty in "unequivocally" demonstrating amino sugars in plant extracts, and, since, the amino sugars are generally considered to be absent from higher plants, this peak may well not have been glucosamine. The fourth compound appeared as a small peak after aspartic acid (asparagine?) but could not be given even a tentative identification.

Several statistically significant differences in amino acid content of the plants appeared among areas and between the moisture treatments. Most striking of these was the large value for total amino acids under the stressed condition (6660 µg/g) as compared to the watered condition (2880 µg/g). The amino acids which varied significantly were histidine, arginine, glutamic acid (glutamine?),

Figure 20. Representative amino acid chromatogram traces of the ethanol leaf extracts of creosotebush. (a) Sheffield plant under water-stressed treatment at 100 F daytime/60 F nighttime temperatures. (b) Sheffield plant under watered treatment at 100 F daytime/60 F nighttime temperatures.



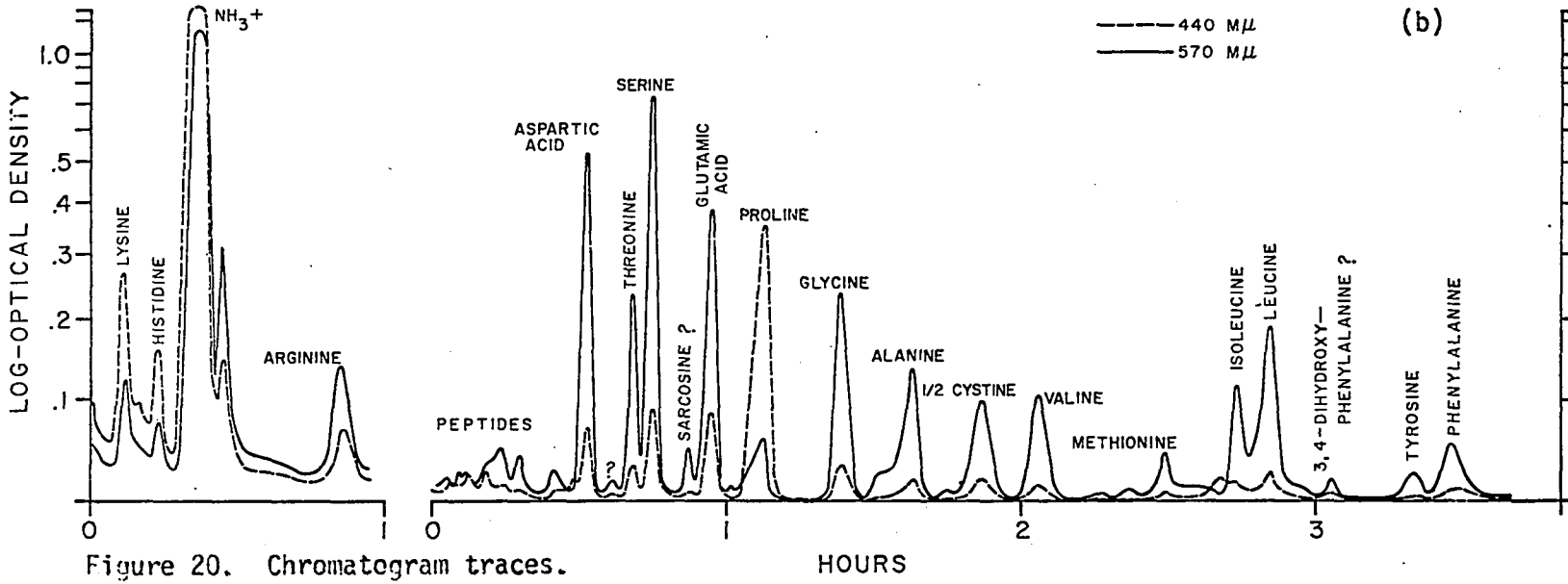


Figure 20. Chromatogram traces.

proline, alanine, valine, isoleucine, and phenylalanine. When the individual amino acid values are expressed as the ratio, W:S where "W" is the value for the watered plants and "S" is the value for the stressed plants, other trends appear (Table 5). Amino acids having ratios of from .9-1.1 were considered not to have changed, those having ratios greater than 1.1 were considered to have decreased with stress, and those having ratios of less than .9 were considered to have increased with stress. None of the amino acids that changed significantly had a ratio between .9 and 1.1.

On the basis of these ratios, seven amino acids were more abundant and five were less abundant with stress in the cool-dry Bernardo plants, 14 were more abundant in the intermediate Sheffield plants, and 11 were more abundant and one less abundant in the hot-humid Zapata plants (Table 5). Although members of the glutamic acid family had larger values in plants from all three areas under stressed conditions, they were greater in the Zapata and Bernardo plants than in the Sheffield plants (Table 5). Plants from the Sheffield area had larger amounts of serine family acids, and 1/2 cystine was the only one to have a larger value under stress in plants from Sheffield and Zapata. Significantly larger amounts occurred in members of the pyruvic acid family for the Sheffield plants and to a lesser extent in plants from Zapata. Phenylalanine was the only member of the aromatic family measured to have significantly greater values under stress in plants from Bernardo and Zapata. Large but statistically insignificant differences occurred for both tyrosine and phenylalanine in the Sheffield plants.

Table 5. Free amino acid response to moisture stress in the three populations of creosotebush.

Bernardo	Sheffield	Zapata
Increase (<.9)		
	Glutamic Acid Family	
*Glutamic acid	Glutamic acid	*Glutamic acid
†Proline	†Proline	*Proline
†Arginine	Arginine	Arginine
	Aspartic Acid Family	
	Methionine	Threonine
	*Isoleucine	Isoleucine
	Serine Family	
1/2 Cystine	Serine	1/2 Cystine
	Glycine	
	1/2 Cystine	
	Pyruvic Acid Family	
Valine	*Alanine	Alanine
	†Valine	Valine
	Leucine	
	Aromatic Acid Family	
†Phenylalanine	†Phenylalanine	*Phenylalanine
	Other	
Histidine		†Histidine
Decrease (>1.1)		
	Aspartic Acid Family	
Aspartic acid		
Methionine		
Isoleucine		
	Serine Family	
Serine		Serine
	Pyruvic Acid Family	
Leucine		

*Significantly different at .05 level (stressed>watered).
†Ratio of less than .5 (watered/stressed).

Response to Temperature

Leaf Moisture Status

No significant differences in moisture percentages appeared due to temperature. Interestingly, however, plants from Sheffield and Bernardo had smaller percentages under lower temperatures, whereas the opposite was the case for the Zapata plants (Figure 21). Moisture percentages for each temperature and area in reference to Figure 14 suggest, with the exception of the Zapata plants, that plants under the lower temperature are farther along in the reaction phase than those for the higher temperature.

Leaf Soluble Sugar Status

In general, soluble sugar values were higher under the higher temperatures (Figures 22, 23, 24, 25). Sucrose was the exception for Sheffield plants, and this difference was carried over to the total soluble sugar value (Figure 24, 25). The high value can be attributed to one Sheffield plant under the lower temperatures which had a 13% W_D value and, consequently, was in the area of complete metabolic disruption. When this one plant was discarded, average sucrose values were no longer statistically significant.

Residual Nitrogen Status

The amount of residual nitrogen was significantly greater in plants under the lower temperatures (21.2 mg/g) than under the higher temperature (18.2 mg/g), and this same trend was followed in plants from each area (Figure 26).

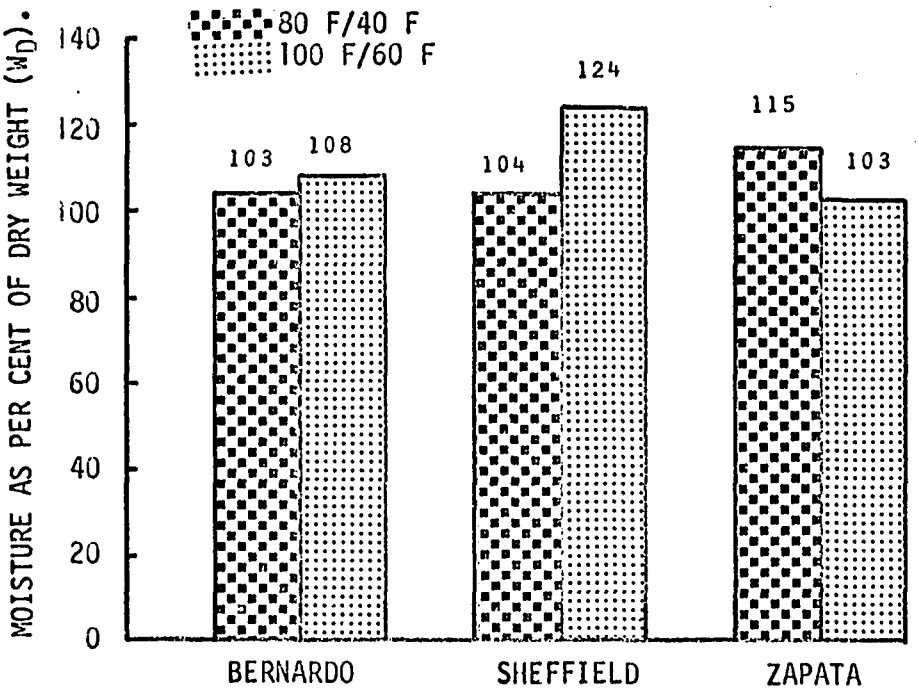


Figure 21. Percentage leaf moisture content of plants from three populations of creosotebush under two different temperature treatments.

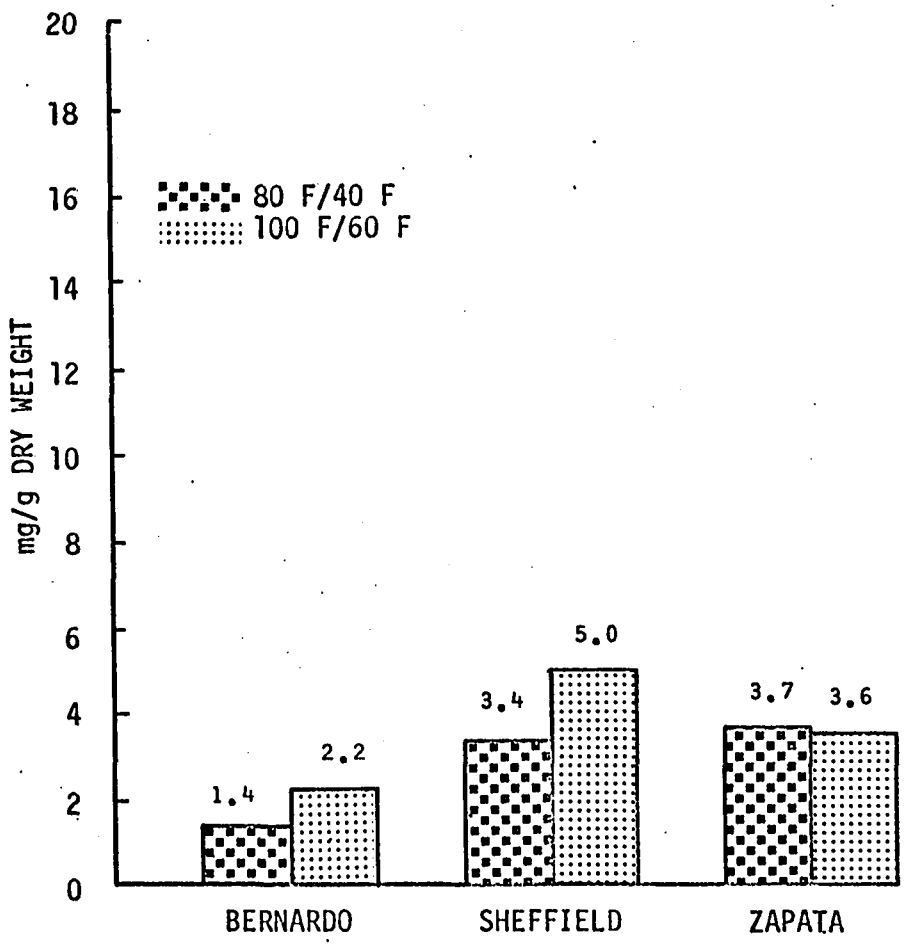


Figure 22. Fructose content of the ethanol leaf extract from three populations of creosotebush under two different temperature treatments.

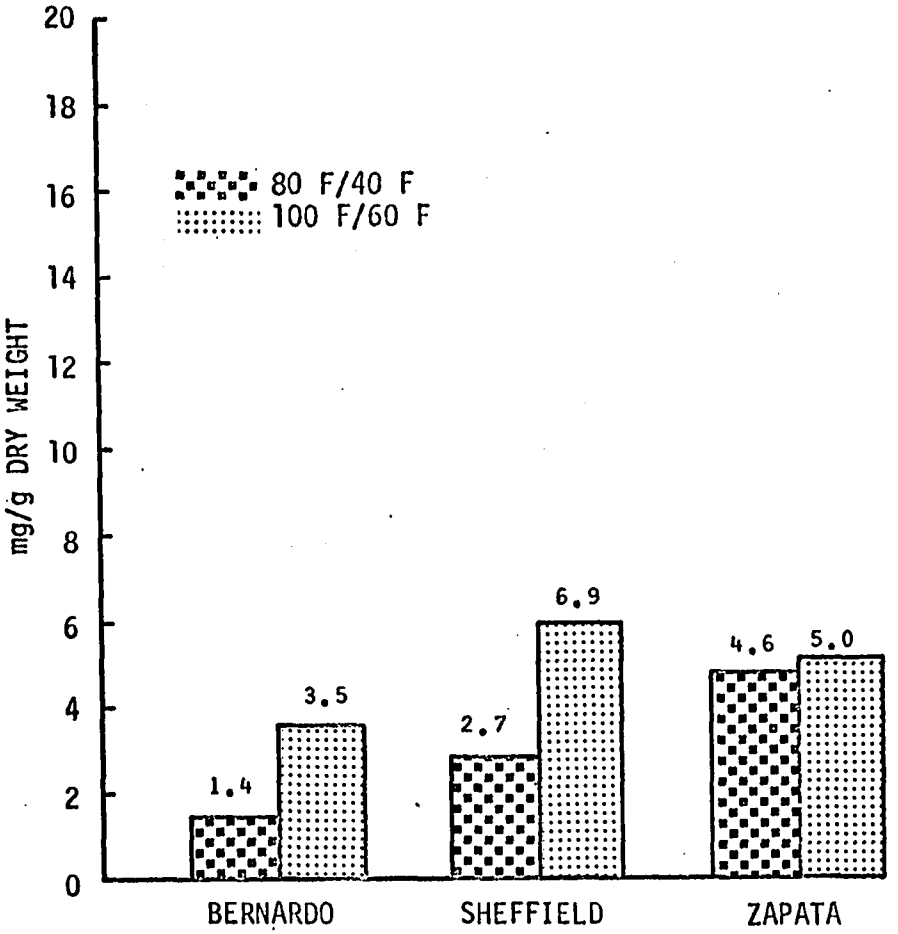


Figure 23. Glucose content of the ethanol leaf extract from three populations of creosotebush under two different temperature treatments.

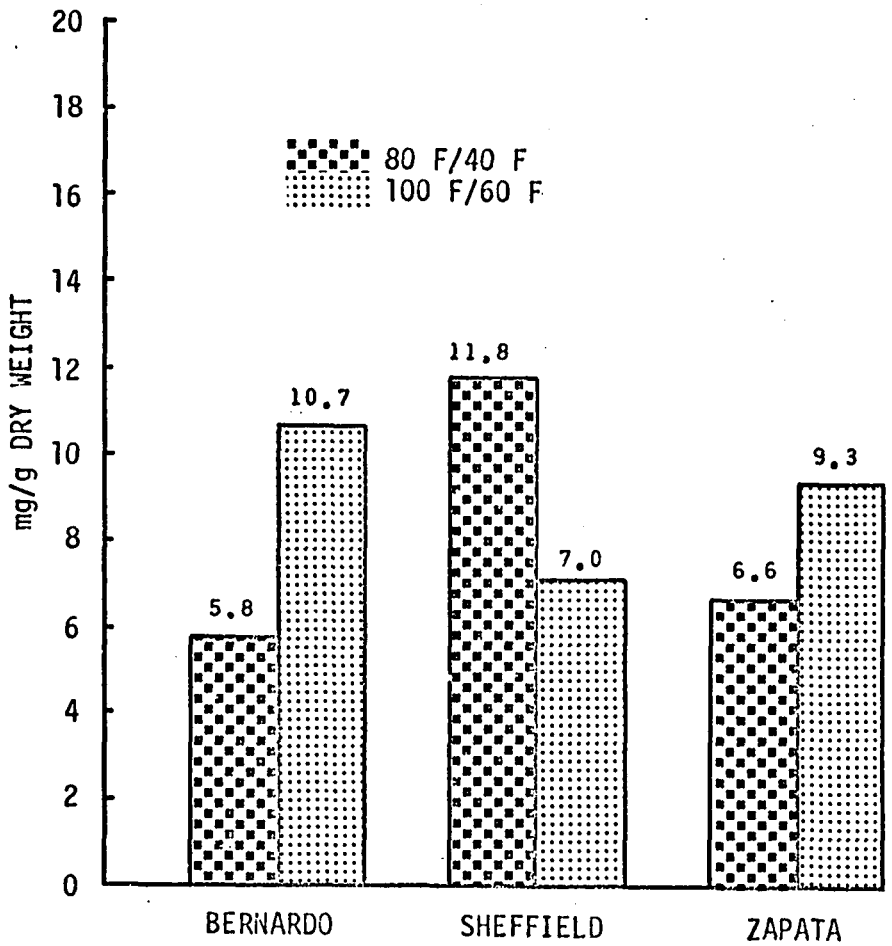


Figure 24. Sucrose content of the ethanol leaf extract from three populations of creosotebush under two different temperature treatments.

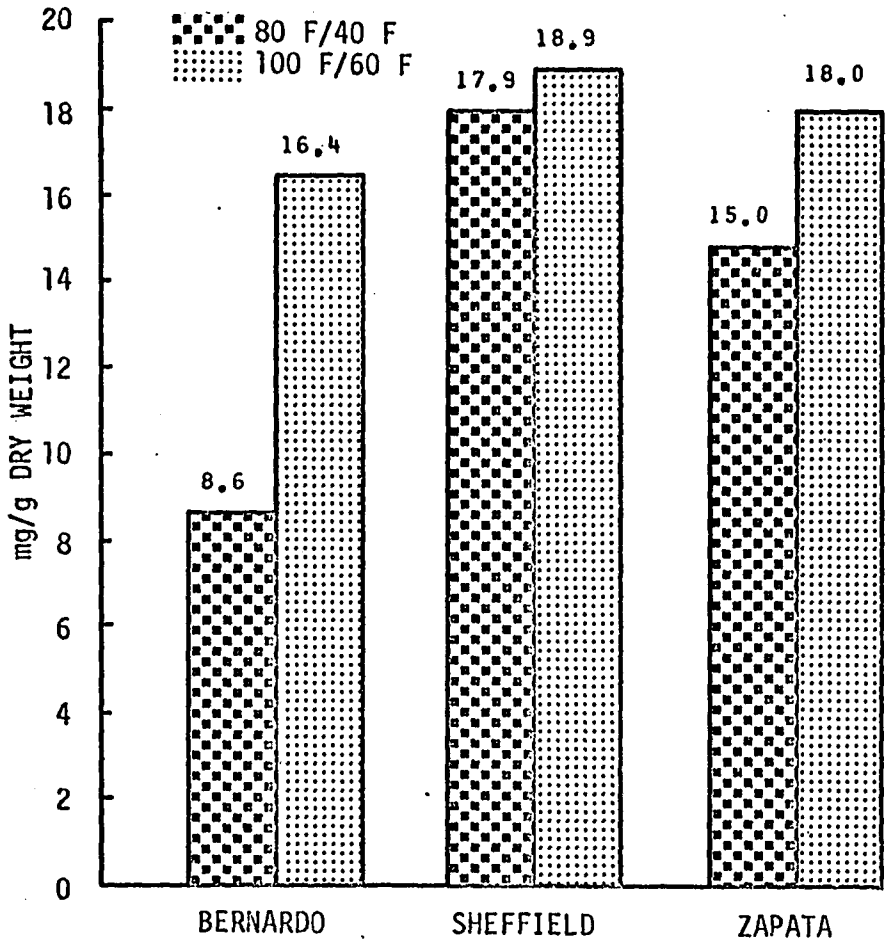


Figure 25. Total sugar content of the ethanol leaf extract from three populations of creosotebush under two different temperature treatments.

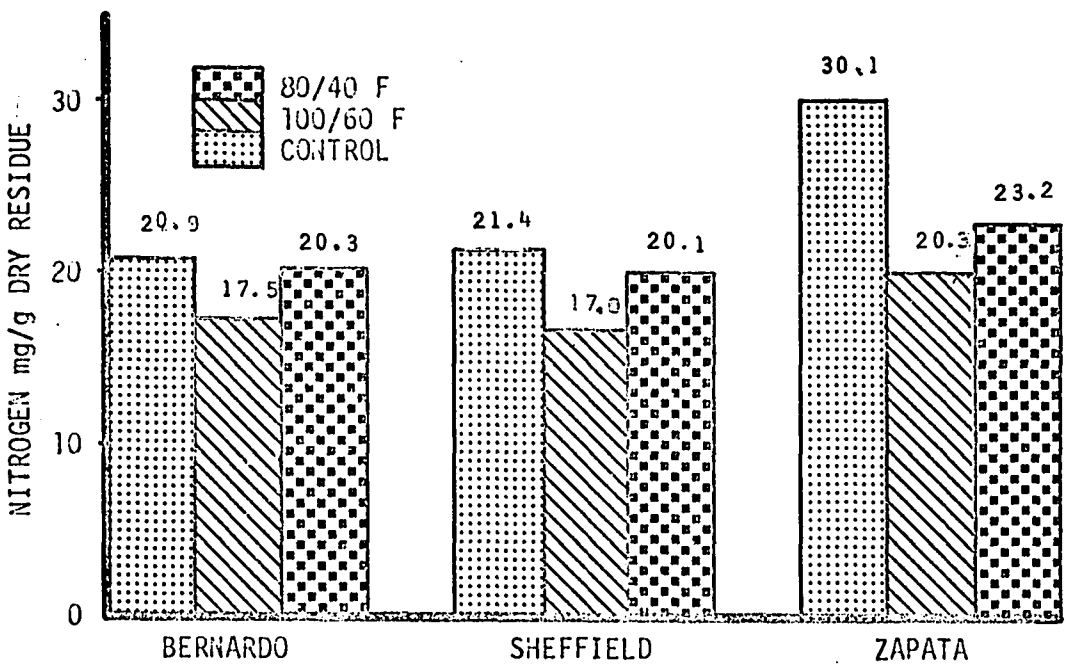


Figure 26. Residual nitrogen content of the leaf material from three populations of creosotebush under two different temperature treatments.

Free Amino Acid Status

Amino acids reacted far less to temperature treatments than to moisture treatments. Values were generally slightly higher under the lower regime, particularly for the amino acids which were high under the moisture stressed conditions. Glutamic acid (glutamine?) was significantly higher in the Sheffield plants under the lower temperatures, and a significant interaction was evident for 1/2 cystine (Figure 27). No other significant differences occurred.

Other Responses

Leaf Ether Extract

Differences of significance in amounts of ether extract were noted for the control plants with the cool-dry Bernardo (118 mg/g) and intermediate Sheffield (116 mg/g) plants having greater amounts than the hot-humid Zapata (87 mg/g) plants. These differences were generally carried through to the area main effects except for the Sheffield plants. Under the area main effects, the cool-dry Bernardo plants had a significantly higher amount of ether extract (132 mg/g) than plants from Sheffield (92 mg/g) and Zapata (89 mg/g). Differences due to temperature and moisture and area-temperature or area-moisture interaction were not found.

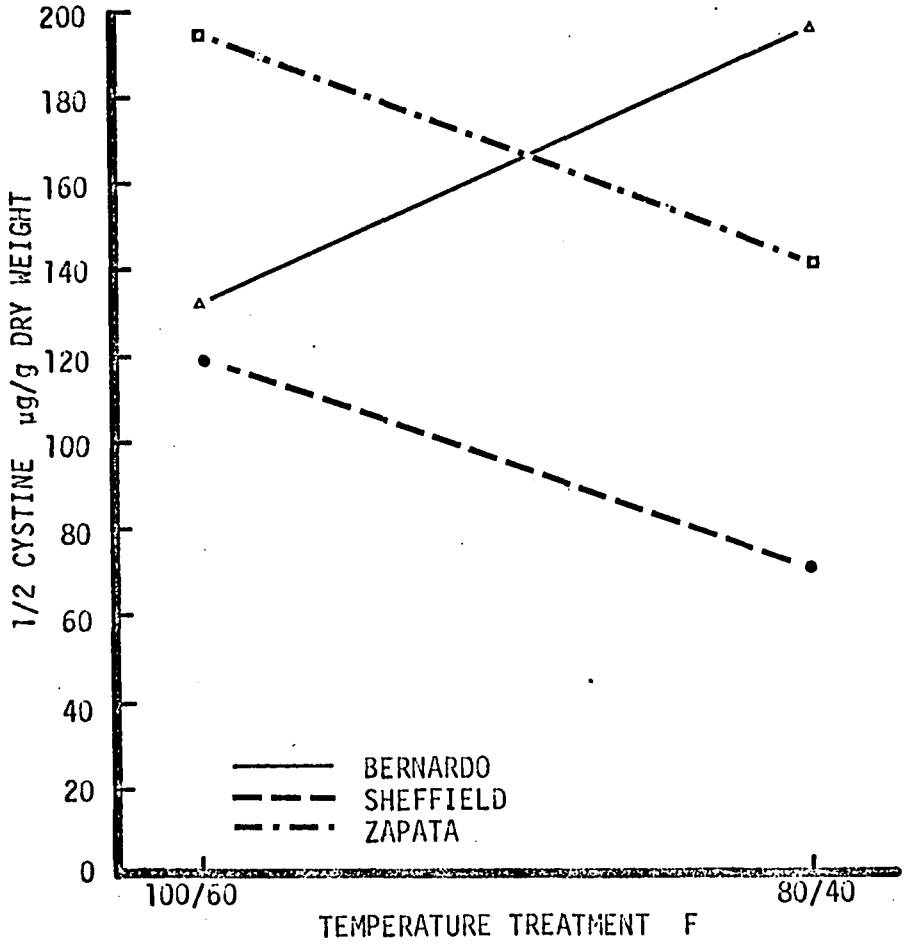


Figure 27. Content of 1/2 cystine in the ethanol leaf extract of three populations of creosotebush under two different temperature treatments, showing the area-temperature interaction.

DISCUSSION

The Response to Moisture Stress

Differences demonstrated by the three creosotebush populations can be related to the habitat of their sources. Zapata plants developed under high, fairly uniform and stable precipitation and high relative humidities of the near gulf coastal area. The high initial W_D value of the Zapata plants (Table 3) may be interpreted as drought avoidance (Chen, Kessler, and Monselise 1964) rather than drought tolerance. Despite the prodidgious water loss rate, the high hydration capacity is sufficient for dry periods in the coastal area.

The climate characterizing the Sheffield area, however, is erratic, the precipitation low and uncertain. In contrast to the Zapata population, stresses of the Sheffield area have evinced adaptations in the creosotebush plants which conserve leaf moisture. The slower rate of desiccation, as indicated by the watered and stressed W_D values (Table 3), may also be termed "drought avoidance" but of a different type than that of the Zapata plants.

Although much more uniform than Sheffield, the climate of Bernardo is characterized by even lower relative humidities and precipitation. In contrast to the hot-humid Zapata and intermediate Sheffield plants, the drought resistance of the cool-dry Bernardo plants appeared to be drought tolerance. Initial hydration of the

plants was low (Table 3) and the loss rate intermediate between the Zapata and Sheffield plants. Possible mechanisms of drought tolerance present in the Bernardo plants will be mentioned later.

Carbohydrate and Nitrogen Metabolism in Wilting Leaves

Data on the response of creosotebush to moisture stress indicated two primary trends in carbohydrate and nitrogen metabolism. One was a general loss of soluble sugars from the leaves (Figure 14), the other an increase in amino acids (Table 5) and decrease in residual nitrogen (Table 4). Figure 14 follows, in general, the graph of changes in carbohydrates in wilting leucerne leaves [Henrici (1952) as presented by Stocker (1960)] and the description of carbohydrate changes in wilting wheat leaves found by Vassiljew and Vassilieu (1936). Although the loss in soluble sugars can be attributed to translocation out of the leaves and/or synthesis of insoluble products, these workers suggest increased respiration to be the major factor.

Likewise, an accumulation of free amino acids in leaves under moisture stress can occur for a number of reasons. Petrie and Wood (1938) ascribed free amino acid increase to protein hydrolysis. Chen, Kessler, and Monselise (1964) supposed the accumulation to be nitrogen degradation products translocated from the roots. However, just a few amino acids are translocated, and these account for very little of the translocated nitrogen (Webster 1959). Amino acid synthesis is possible even during water stress (Gates 1964). For amino acid synthesis to occur, nitrogen and organic acids must be available. Protein

hydrolysis can provide the nitrogen, and the organic acids and the energy required for synthesis can be supplied by carbohydrate breakdown through Krebs cycle activity.

Glutamic acid, glutamine, proline and arginine are synthesized from α-ketoglutaric acid (Figure 28). The role of glutamine in the suppression of NH_3+ build-up in plants is well-documented (Loomis and Stumpf 1958). Accumulation of glutamic acid (glutamine?) may have alleviated the build-up of toxic amounts of NH_3+ to some degree in plants from all three areas. Proline synthesis has been found to have a role similar to that of the amides in NH_3+ storage as in perennial rye grass (Kemble and Macpherson 1954). More recently proline accumulation under moisture stress has been shown to occur in ladino clover (Routley 1966) and bermuda grass (Barnett and Naylor 1966). These workers felt that these changes were a demonstration of an adaptive response to drought by the plant. Therefore, the high amounts of glutamic acid (glutamine?) and proline under stress may be important in the drought tolerance of creosotebush. This was particularly true for the cool-dry Bernardo plants since the intermediate Sheffield plants had less accumulation of the glutamic acid family (Table 5), and the hot-humid Zapata plants apparently had more protein hydrolysis (Figure 19).

Although arginine has not been implicated as a storage product for ammonia under moisture stress, it is a possibility. More likely, however, it represents a hydrolysis product from leaf protein, or, since it is one of the amino acids which is translocated (Webster 1959), a hydrolysis product from the root.

Alanine, valine and leucine are synthesized from pyruvic acid (Figure 28). Both valine and alanine also may have an NH_3+ storage function during wilting (Tarchevskii and Siyanova 1963). The accumulation of these pyruvic acid family amino acids in the intermediate Sheffield plants and to some degree in the hot-humid Zapata plants suggests a different metabolism for these plants under moisture stress than that of the cool-dry Bernardo plants.

Isoleucine accumulation in the intermediate Sheffield and hot-humid Zapata plants may have occurred as indicated in Figure 28, but the stable values of the other amino acids synthesized through aspartic acid make this doubtful. Although isoleucine is not generally known to be translocated, it may be a hydrolysis product.

Fowden (1959) has observed that histidine is one of the amino acids that is rarely detectable unless the plant is in circumstances encouraging protein breakdown. Likewise, phenylalanine is known to increase due to hydrolysis under stress conditions (Chen, Kessler, and Monselise (1964). Protein amino acids which did not show a large increase due to hydrolysis may have been oxidized to yield the ammonia used in the synthesis of other amino acids.

The decrease in aspartic acid (asparagine?), serine, methionine, isoleucine, and leucine associated with water stress in the cool-dry Bernardo plants but not in plants from the other two areas may indicate that they were oxidized in the Bernardo plants. However, the fact that these same amino acids are, in the main, the ones that are translocated from the roots when stressed (Webster

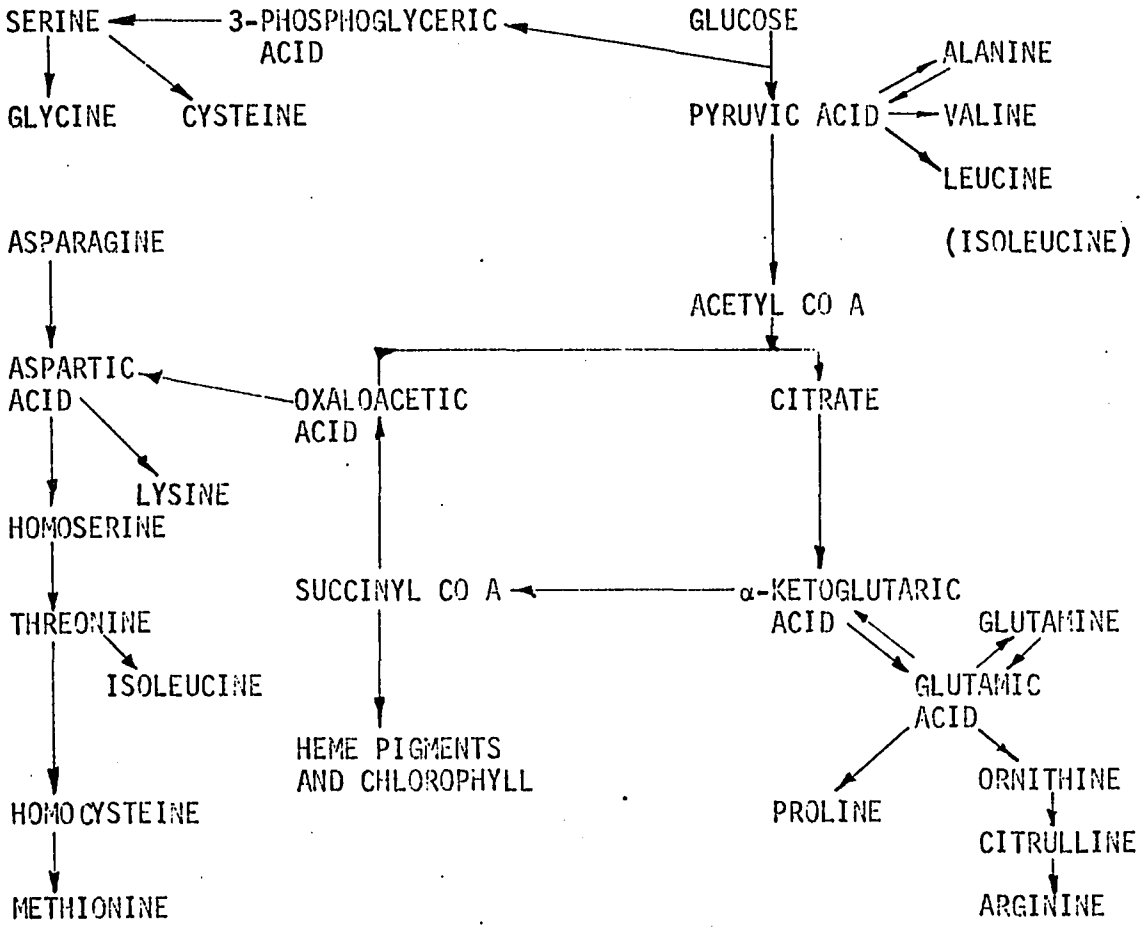


Figure 28. Relationships between carbohydrate and amino acid metabolism in plants. After Beevers, Stiller and Butt (1966).

1959) also suggests that translocation of protein degradation products from the roots could have caused the lack of their decrease in leaves of the Zapata and Sheffield plants. If so, a more stable metabolism under moisture stress was indicated for the cool-dry Bernardo plants than for the intermediate Sheffield plants and the hot-humid Zapata plants.

Response to Temperature Treatment

The effects of temperature on plant growth are difficult to separate from the effects of moisture (Henkel 1964).

The lower W_D values in plants under the cooler regime (Figure 2) merits discussion because of its conflict with what could be expected. As the air temperature rises, so does its capacity for holding water vapor. This would increase the transpiration of a plant. The internal vapor pressure of a leaf would also increase and lead to a further increase in transpiration rate (Knight 1965). Unless absorption increased to the same degree, internal water deficits would occur. The decrease of water content in plants during the day is a partial result of this phenomenon. When the rate of transpiration exceeds the absorption rate over a long period, more severe water deficits occur. One possible solution for the higher W_D values under the lower temperature treatments resides in the growth chamber used in the experiment. To produce the lower temperatures, refrigeration was required. Since the outlet for the cold air was underneath the rack which held the pots, the cold air evidently lowered the soil temperature enough to retard absorption. This caused

the greater internal deficit under the cooler treatment. A comparison of the W_D values for each area under the two temperature treatments suggested differences in the reaction of the plants to these lower soil temperatures. Under the lower temperatures, the Zapata plants had the highest net loss of leaf moisture, the Sheffield plants were intermediate, and the Bernardo plants the lowest (Figure 29). However, under the higher temperatures (Figure 30) the rates were shifted somewhat and assumed the relationships discussed above.

According to Kozlowski (1964), the capacity of water absorption by plants under low temperatures is often correlated with the temperature range of their native habitat. That is, plants from the northern latitudes and higher elevations are the least affected, and the southern ones are most affected. Apparently ecotypic differences between the creosotebush populations occurred with the cool-dry Bernardo plants being most tolerant of low temperatures, the Sheffield plants intermediate, and the hot-humid Zapata plants least tolerant.

Less residual nitrogen in plants under the higher temperatures is explained through two related lines of reasoning (Leopold 1964). Because of the lower temperature the Q_{10} would be lower resulting in lowered reaction rates of the proteases and, therefore, less protein breakdown. The other is that the lower temperature resulted in a higher concentration of CO_2 (the solubility of CO_2 is greater at lower temperatures) and, therefore, a lower pH of the cell sap (Leopold 1964). Because of this general lowering of pH, further alteration of the rates of enzymatic reaction occurred. A lower pH

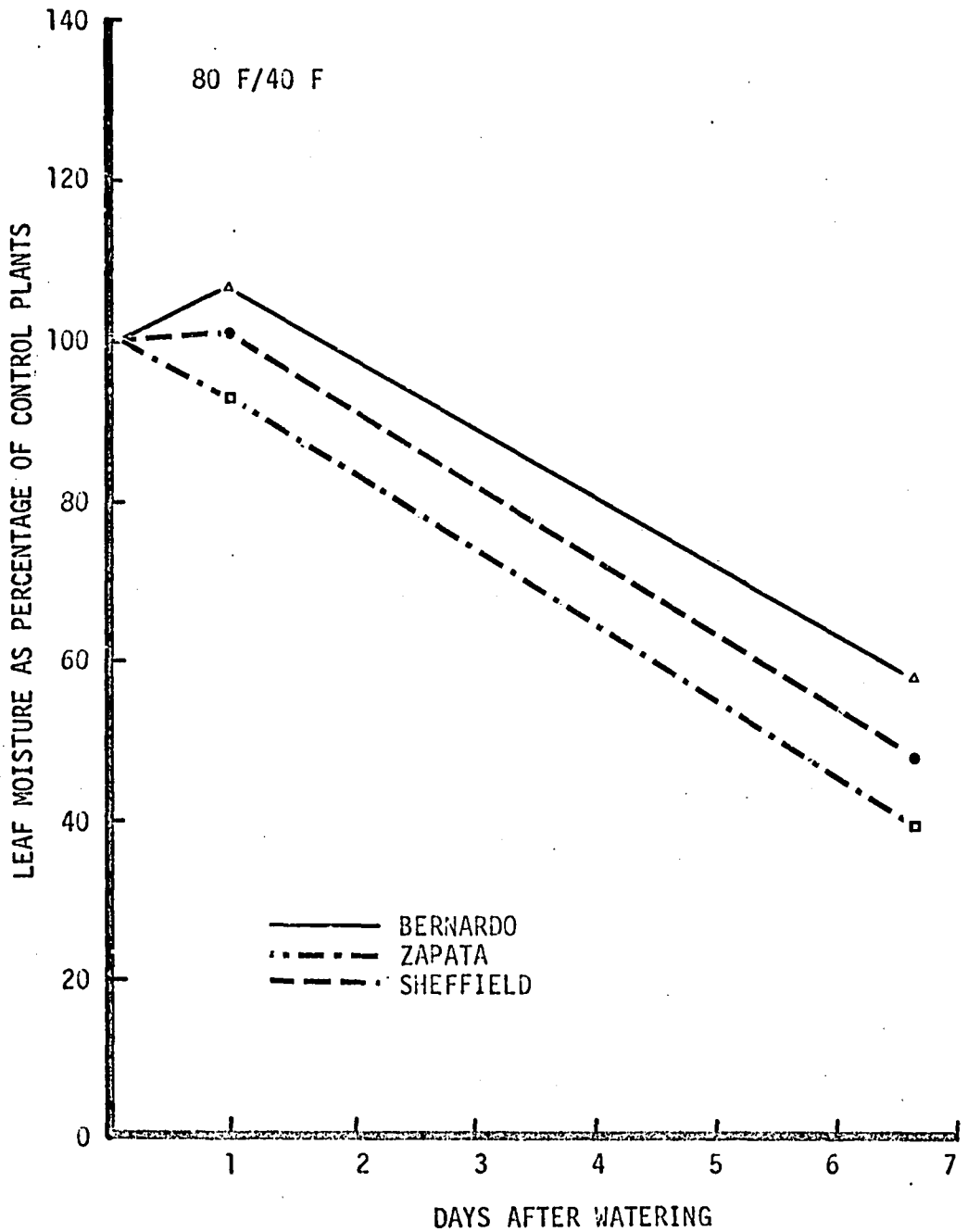


Figure 29. Leaf moisture percentages of three populations of creosotebush under watered and stressed conditions and the temperature treatment 80 F daytime/40 F nighttime.

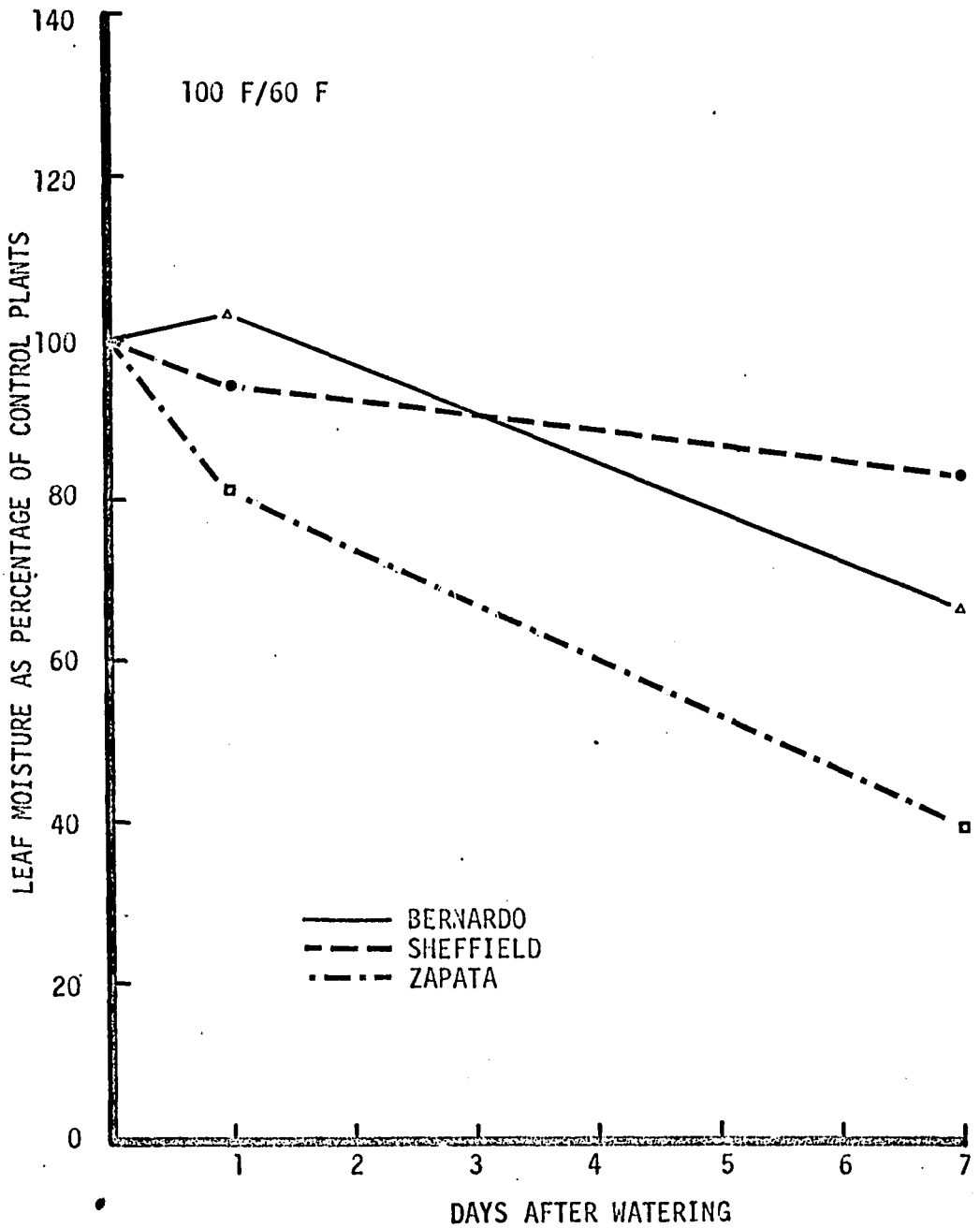


Figure 30. Leaf moisture percentages of three populations of creosotebush under watered and stressed conditions and the temperature treatment 100 F daytime/60 F nighttime.

of the cell sap results in protein conservation, and any process which would maintain the protein level in leaves, particularly under conditions of wilting, may be counted as a drought resistant feature (Wood 1933a). If this is true, and if the values were higher in the Bernardo and Sheffield plants because such mechanisms existed in these plants, then this would indicate a possible higher drought tolerance in the plants from the drier areas.

The apparent "trends" in the amino acid differences, including glutamic acid, may have been more a reaction to moisture stress than to temperature, and, therefore, may be explained as above.

The interaction for 1/2 cystine was significant because the Bernardo plants had a higher value under the cooler temperature whereas the opposite was the case for the Sheffield and Zapata plants. The value for 1/2 cystine included both cystine and cystein and its accumulation in the cool-dry Bernardo plants under the lower temperatures could represent the synthesis of cystein through 3-phosphoglycerate (Figure 28). More likely, however, it represented hydrolysis products of short chain peptides such as the tripeptide, glutathione. Values for the intermediate Sheffield plants and the hot-humid Zapata plants may have been low because of early oxidation. The phenomenon of an early increase and later disappearance of cystine under increasing drought has been cited by others (Wood and Petrie 1938). However, if these differences are due to temperature rather than to moisture, a closer relationship is indicated between

the hot-humid Zapata and intermediate Sheffield plants than between plants from either of these areas to the cool-dry Bernardo plants.

Other Responses

Since the ether extractables consist primarily of the resins, waxes, cutins, and mucilages (Bonner 1950), and since these have been implicated as having a role in drought resistance, the accumulation of ether extractables for the cool-dry Bernardo plants may indicate an adaptation to drought by these plants through a mechanism involving the ether extractable material. However, data comparing the Bernardo plants under adequate and stressed moisture conditions do not bear this out.

The design of this study did not reveal the reasons for this; indeed the design may even have obscured them. If, for instance, the mechanism were light dependent (the amount of photosynthesis and its influence on the carbohydrate flux), changing the plants from the greenhouse conditions to growth chamber conditions would tend to obscure the relationship because only one artificial light regime of lower intensity was used. Still, no matter what the reason, differences in ether extractables occurred between plants from the different areas after more than a year of equilibration under greenhouse conditions during which at least one entire set of new leaves developed. Climatological races of plants in terms of photosynthetic rate are known to occur, and these may vary with altitude (Hiesey and Millner 1965). For example, Billings, Clebsch and Mooney (1961) found that a race of *Oxyria* from 2,000 m photosynthesized

at a higher rate than a race from sea level. If such differences exist between the creosotebush populations, then light could be a factor in obscuring the ether extract data.

CONCLUSIONS

The findings of this study offer some justification for giving ecotypic status to the three populations of creosotebush. Ecotypic variation occurred primarily in response to moisture as differences in drought avoidance and drought tolerance.

Drought avoidance appeared in the hot-humid Zapata plants as a high hydration capacity which would sustain these plants through the comparatively short and rare dry periods of south Texas. Drought avoidance appeared in the intermediate Sheffield plants as a slow rate of water loss which would sustain these plants through the relatively longer periods of drought encountered at the Sheffield site. Drought tolerance appeared primarily in the cool-dry Bernardo plants as a loss of soluble sugars, which, under moisture stress, is usually ascribed to an increased respiration (Stocker 1960, Henrici 1952, Vassiljew and Vassilieu 1936, Gates, 1964). Accumulation of the glutamic acid family amino acids also occurred. These phenomena had the dual affect of conserving carbohydrate products and nitrogenous material and the suppression of toxic amounts of NH_3+. The hot-humid Zapata plants and the intermediate Sheffield plants were apparently less tolerant of moisture stress. This was indicated by a greater and earlier hydrolysis of leaf proteins, although some synthesis of the glutamic acid family and pyruvic acid family amino acids occurred.

Clinal variation appeared in terms of temperature response. Plants from the cooler Bernardo site were least susceptible to cold root temperatures, Sheffield plants were intermediate, and Zapata plants were most susceptible.

In addition, residual nitrogen was less labile in the Bernardo plants, and most labile in the Zapata plants, and the Sheffield plants were intermediate.

These differences, both ecotypic and clinal, may be contributing factors that enable the wide distribution of creosote-bush over its varied environment.

APPENDIX A

LIST OF CHEMICALS USED IN THE STUDY

Acid, glacial acidic	Mallenckrodt
Acid, hydrochloric 36%	Mallenckrodt Analytical Reagent
Acid, concentrated sulferic	Mallenckrodt
Aniline	Fisher Certified Reagent
L-Arabinose	Fisher Certified Reagent
Boric Acid 2%	
n-Butanol	Mallenckrodt
Ethanol absolute	
Ether, ethyl	Mallenckrodt Analytical Reagent
D-Fructose	Fisher Reagent Chemical
Galactose	Fisher Reagent Chemical
d-Glucose, anhydrous	Fisher Certified Reagent
Indicator, mixed Brom cresol green methyl red	
Liquid transplant shock stopper Thiamin Hydrochloride .15% a Naphthaleneacetic acid	Rolora's Laboratories
Phthalic acid	Eastman Organic Chemicals
Raffinose	Eastman Organic Chemicals
D-Ribose	Eastman Organic Chemicals

Selenium, powdered	
Sodium citrate buffer, pH 2.2	
Sodium Hydroxide, 30% solution	
Stachyose	Nutritional Biochemicals Corp.
Sucrose	Mallenckrodt Analytical Reagent
D-Xylose, purified	Fisher Laboratory Chemical

APPENDIX B

DATA TABLES

Table 1. Average values of some physiologically important properties in creosotebush.

		Control Plants			Area Main Effects		
		Bernardo	Sheffield	Zapata	Bernardo	Sheffield	Zapata
Leaf Moisture	%	123	140	174	106	114	109
Ether Extract	mg/g	118	116	87	132	92	89
Residual Nitrogen	mg/g	20.9	21.4	30.1	18.9	18.6	21.8
Fructose	mg/g	2.6	3.5	4.2	1.8	4.2	3.6
Glucose	"	2.8	4.6	14.6	7.5	4.8	4.9
Sucrose	"	3.7	3.4	5.3	8.2	9.4	8.0
Total	"	8.5	11.5	24.2	12.5	18.4	16.5
Alanine	µg/g	104	212	325	86	108	122
Arginine	"	31	102	58	81	49	113
Aspartic Acid	"	80	497	32	152	154	144
1/2 Cystine	"	trace	20	22	164	96	168
Glutamic Acid	"	30	34	99	531	384	575
Glycine	"	140	206	193	140	134	112
Histidine	"	12	50	30	40	26	37
Isoleucine	"	157	200	135	67	76	55
Leucine	"	141	246	249	134	127	118
Lysine	"	29	77	56	44	43	43
Methionine	"	14	58	81	22	20	15
Phenylalanine	"	58	108	143	165	117	138
Proline	"	1220	1060	1540	3000	1950	3570
Serine	"	101	24	20	154	159	156
Threonine	"	78	182	154	82	89	93
Tyrosine	"	25	74	79	39	45	37
Valine	"	154	245	221	107	105	125
Total	"	2380	3390	3380	5010	3670	5630

Table 2. Physiological responses of creosotebush under different temperature and moisture conditions.

		Temperature Main Effects		Moisture Main Effects	
		80/40	100/60	Watered	Stressed
Leaf Moisture	%	107	112	141	79
Ether Extract	mg/g	106	103	103	106
Residual Nitrogen	mg/g	21.2	18.2	20.1	19.4
Fructose	mg/g	2.8	3.6	3.3	3.1
Glucose	"	2.9	5.2	5.3	2.8
Sucrose	"	8.1	9.0	10.4	6.7
Total	"	13.8	17.8	19.0	12.6
Alanine	µg/g	113	98	93	118
Arginine	"	92	70	48	114
Aspartic Acid	"	145	155	153	147
1/2 Cystine	"	137	148	122	163
Glutamic Acid	"	532	461	337	656
Glycine	"	126	132	124	133
Histidine	"	38	31	21	48
Isoleucine	"	73	59	56	76
Leucine	"	120	132	121	131
Lysine	"	44	49	41	46
Methionine	"	17	20	18	20
Phenylalanine	"	158	122	77	204
Proline	"	3290	2380	1249	4380
Serine	"	152	157	161	147
Threonine	"	90	86	84	92
Tyrosine	"	42	46	40	48
Valine	"	175	100	84	141
Total	"	5300	4240	2880	6660

Table 3. Physiological responses of the three populations of the creosotebush due to moisture treatment.

		Bernardo		Sheffield		Zapata	
		watered	stressed	watered	stressed	watered	stressed
Leaf Moisture	%	134	77	138	91	150	68
Ether Extract	mg/g	135	128	90	95	83	95
Residual Nitrogen	mg/g	19.4	18.4	18.0	19.1	22.9	20.6
Fructose	mg/g	2.0	1.5	4.1	4.3	3.8	3.5
Glucose	"	3.3	1.6	6.4	3.1	6.0	3.7
Sucrose	"	11.8	4.6	9.9	8.9	9.5	6.5
Total	"	17.2	7.8	20.4	16.4	19.3	13.7
Alanine	µg/g	87	85	82	134	109	136
Arginine	"	46	116	40	59	59	166
Aspartic Acid	"	160	143	150	159	151	138
1/2 Cystine	"	152	176	90	102	123	212
Glutamic Acid	"	302	760	331	436	376	773
Glycine	"	143	138	115	152	116	108
Histidine	"	24	57	21	31	18	55
Isoleucine	"	72	62	56	95	40	69
Leucine	"	146	122	103	151	115	120
Lysine	"	42	46	44	42	37	49
Methionine	"	26	20	14	25	14	16
Phenylalanine	"	95	235	75	160	60	216
Proline	"	1480	4510	947	3000	1460	5680
Serine	"	169	139	133	174	182	129
Threonine	"	84	80	90	88	78	107
Tyrosine	"	41	38	36	58	43	48
Valine	"	98	116	66	145	89	162
Total	"	3170	6840	2390	5010	3022	8180

Table 4. Physiological responses of the three populations of creosotebush due to temperature treatment.

		Bernardo		Sheffield		Zapata	
Temperature F		80/40	100/60	80/40	100/60	80/40	100/60
Leaf Moisture	%	103	108	104	125	115	103
Ether Extract	mg/g	138	125	89	96	90	89
Residual Nitrogen	mg/g	20.4	17.5	20.1	17.0	23.2	20.3
Fructose	mg/g	1.4	2.2	3.4	5.0	3.7	3.6
Glucose	"	1.4	3.5	2.7	6.9	4.6	5.1
Sucrose	"	5.8	10.7	11.8	7.0	6.6	9.3
Total	"	8.6	16.4	17.8	18.8	15.0	18.0
Alanine	µg/g	81	92	115	100	143	102
Arginine	"	111	51	51	48	114	111
Aspartic Acid	"	145	158	162	147	129	159
1/2 Cystine	"	116	132	72	119	142	144
Glutamic Acid	"	571	491	450	313	577	573
Glycine	"	140	141	137	130	100	124
Histidine	"	39	42	32	20	44	29
Isoleucine	"	76	58	87	65	57	53
Leucine	"	145	123	117	136	97	138
Lysine	"	42	46	37	50	52	35
Methionine	"	22	23	18	21	13	16
Phenylalanine	"	157	173	138	76	179	97
Proline	"	3610	2380	2590	1300	3680	3460
Serine	"	153	155	141	166	162	150
Threonine	"	87	77	84	94	100	86
Tyrosine	"	37	42	40	54	50	40
Valine	"	123	91	120	90	132	119
Total	"	5730	4279	4393	2960	5760	5490

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