FACTORS AFFECTING STOMATAL OPENING OF CREOSOTEBUSH (*LARREA TRIDENTATA* (DC.) COV.) AND THEIR COMBINED EFFECT ON HERBICIDE ACTIVITY

by

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APPROVAL BY THESIS DIRECTOR

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Edwin M Schmutz — April 19, 1965

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W. L. Warskow

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ABSTRACT

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Results of the field and growth-chamber studies initiated in 1962 to determine the effects of environmental factors on stomatal movement in creosotebush were erratic. Subsequent detailed anatomical studies revealed that the collodion technique used was inadequate to determine stomatal opening in creosotebush. This invalidated the stomatal data obtained during the initial field and growth-chamber studies.

The detailed anatomical studies of creosotebush stomata also confirmed previous reports of cuticle lips on the guard cells and further revealed that the guard cells have very thick cell walls which resisted chemically-induced plasmolysis. However, under field conditions, no plasmolysis was observed in guard or epidermal cells. Also, debris was found in the aperture formed by the cuticle lips largely blocking the opening. From these studies it was concluded that mature creosotebush guard cells are essentially incapable of producing stomatal movement; that stomatal movement probably does not occur under field conditions; and, that the effect of any stomatal movement that may occur is reduced or nullified by the cuticle lips and aperture debris. This ineffective stomatal movement combined with lack of consistent daily

response to herbicide treatment led to the conclusion that the stomata of creosotebush do not affect herbicide toxicity.

Supplemental studies showed that the moisture content of creosotebush leaves varied directly with relative humidity and inversely with air temperature and light intensity.

INTRODUCTION

Creosotebush (Larrea tridentata (DC.) Cov.) is classified as a noxious shrub, worthless for livestock forage (U.S. Forest Service, 1937). It is the dominant or associate dominant species on approximately 17.5 million acres in Arizona (Nichol, 1952; Dalton, 1961). The total area covered by creosotebush in the Southwestern United States and northern Mexico is estimated to be in excess of 35 million acres (Botkin and Duisberg, 1949).

Most of the area covered by creosotebush has a climate which is marginal with respect to range forage production. However, creosotebush also covers thousands of acres of once-productive desert grassland (Mehrhoff, 1955; Humphrey and Mehrhoff, 1958; Dalton, 1961; Yang, 1961). The forage production and carrying capacity of these former grasslands can be partly or wholly restored by control of the invasion stands of creosotebush.

At the present time, the use of herbicides appears to be a feasible means of controlling large stands of creosotebush. Intensive studies on the chemical control of creosotebush on Arizona rangelands (Schmutz, 1963) indicated a seasonal effect and possibly a time-of-day effect on the absorption and translocation of herbicides. Generally, greater kills were obtained with herbicides applied in August one month after initial summer rains exceeding one-half inch. Also, in some preliminary studies, greater absorption and translocation of herbicides and

plant kills resulted from applications of herbicides made during the middle of the day. This suggested a correlation with some light-influenced factor such as stomatal opening.

Opening of stomata has been correlated with light intensity by Loftfield (1921), Ashby (1932), Wilson (1948), Daubenmire (1958), Dybing (1958), Ketellapper (1959, 1963) and many other writers. Thus, under field conditions, stomatal opening in many plants would be greatest during daylight hours.

Stomata were considered to be a major pathway for entry of herbicides into plants by Skoss (1955), Leonard and Crafts (1956), Dybing and Currier (1959) and Pallas (1960). They reported greater absorption of herbicides into plants when stomata were open than when stomata were closed.

Because of the above findings, field and growth-chamber studies were carried out to determine the factors affecting stomatal opening in creosotebush and their combined effects on herbicide activity in creosotebush. The results of these studies, in turn, led to a more detailed anatomical study of creosotebush stomata.

METHODS

Field Studies

Study Area

Field studies on the factors affecting stomatal opening were conducted in a creosotebush desert shrub type (Figure 1) 15 miles southeast of Tucson, Arizona, and one-half mile northwest of the abandoned railroad stop of Esmond. The soil on the study area is a shallow to moderately deep, reddish-brown, sandy-loam member of the Continental series developed from dissected alluvium.[1] Schmutz (1963) estimated from U.S. Weather Bureau records that the mean annual rainfall on the study site is 11.5 inches, half of which falls during July, August and September. Temperatures at the study site are very similar to those occurring at Tucson, where summer temperatures frequently exceed 100° F and winter temperatures rarely drop below 10° F (Sellers, 1960). Sellers also reports that Tucson has an average frost-free season of 250 days extending from March 15 to November 20.

Collection of Environmental Data

Environmental, leaf moisture and stomatal opening data were collected over two 24-hour periods on August 13-14 and 20-21, 1962.

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1. Personal communication to Dr. Ervin M. Schmutz from Dr. Stanley W. Buol, Department of Agricultural Chemistry and Soils, University of Arizona, Tucson.

Figure 1. General view of the creosotebush desert shrub type occurring at the Esmond study site 15 miles southeast of Tucson, Arizona.

Environmental data included soil temperature, soil moisture, light intensity, wind velocity, air temperature and relative humidity.

Soil Temperature. Soil temperatures in bare soil and soil shaded by creosotebushes were recorded by two thermometers, one placed in the middle of a bare, open spot and the other under the north side of a creosotebush. The thermometers were inserted 5 inches into the soil in holes made with a screwdriver so as to disturb the soil as little as possible. The soil was packed around the thermometers to insure good contact between the thermometer bulbs and the soil. The bulb depth of 5 inches was chosen to place the bulbs in moist soil since the top 4 inches of soil were dry. A depth greater than 5 inches would have resulted in greater soil disturbance and possible mixing of the moist and dry soil layers when the holes for the thermometers were made. Soil temperatures were read 12 minutes before the hour.

Soil Moisture. Soil samples were collected from a depth of 4 to 7 inches and placed into sealed soil-sample cans for soil moisture determination by the gravimetric method. Samples were collected both in the open and under a creosotebush. The samples in the open were collected from a barren area 15 x 48 inches on a radius of 10 feet from the nearest plants. The samples under the creosotebush were collected 5 to 8 inches from the center of the bush on the sides of the bush away from the sun. All samples were collected about 20 minutes after the hour.

Light Intensity. Both reflected and incident light measurements were made with a Weston Master IV exposure meter. Readings were recorded in light meter units which were later converted into foot-candles by multiplying by 4. The two readings were taken 1 to 2 minutes before the hour. The reflected light readings were taken with the photoelectric cell held 7.5 inches from two thicknesses of $8\frac{1}{2}$- x 11-inch white bond paper lying on the ground. The light meter was held to prevent shadows falling on the paper and to obtain maximum reflected light readings. Incident light readings were taken with the Invercone in place and the photoelectric cell directly facing the light source (sun or moon).

Wind Velocity. Wind velocity readings were made 5 minutes to the hour with a Dwyer wind meter. Wind velocities below 2 miles per hour were not readable with the wind meter and were recorded as zero miles per hour. All readings were taken with the wind meter orifices facing the direction of strongest wind and by reading the maximum value at which the ball remained steady for a period of several seconds. On the afternoon of August 14, 1962, winds greater than 12 miles per hour were observed during a thunderstorm, but none of these velocities occurred at the times when experimental readings were taken.

Temperature and Relative Humidity. Temperature and relative humidity data were determined by using an Abbeon sling psychrometer. Wet- and dry-bulb temperatures were taken 3 minutes to the hour. The psychrometer was swung at waist height in the shadow of the user and away from surrounding obstructions. Using these data, the relative

humidity was read from a wet-bulb temperature depression table using a standard corrected pressure of 29.9 inches of mercury.

Analysis of Leaf Moisture

Two methods of collecting leaves for leaf-moisture studies were used. The first method consisted of collecting ten leaves at random from each of ten plants randomly selected at each collection period and sealing the leaves in a marked can. Leaf moisture was determined on a dry-weight basis by drying the leaves at 105° C for 24 hours. This method was discarded after the first day in favor of a second method which was much faster and gave more reliable results.

In the second method, leaf samples were obtained by removing intact the terminal 2.75 inches of newly produced branchlets. At each collection period, two branchlets were taken from each of five creosote-bushes chosen at random before the start of the 24-hour study period. The leaves and the terminal branchlets were dried at 105° C for 24 hours and leaf moisture was calculated on the basis of dry weight.

Stomatal Analysis

Determination of Stomatal Opening. A perusal of the literature on stomata revealed a number of chemicals which affect stomatal opening. Stålfelt (1957) reported that a 0.01 molar solution of sodium azide (NaN_3) inhibited the passive, photoactive and hydroactive water output of turgid guard cells. The effect of the azide was correlated with the degree of turgidity. Thus, open stomata remained open and, if moisture

was available, were forced even further open. Conversely, Zelitch (1961) obtained 76 to 100% stomatal closure with 0.01 molar sodium bisulfite ($NaHSO_3$).

Using the above information, the appearance of open creosotebush stomata was determined by immersing a creosotebush branchlet in 0.01 molar sodium azide in the light for one-half hour. Immediately upon removal from the sodium azide solution, the leaves on the branchlet were blotted dry. Collodion imprints of the stomata were made using the technique developed by Long and Clements (1934). The resulting collodion strips were mounted on a slide, and the appearance of open stomata as imprinted on collodion films was carefully studied.

The appearance of closed creosotebush stomata was determined by immersing a creosotebush branchlet in distilled water and holding it in the dark for one-half hour. This was followed by immersion in 0.01 molar sodium bisulfite for one-half hour in the dark. Collodion imprints of the stomata were made, mounted and studied as described above in the sodium azide treatment.

Open and closed creosotebush stomata were also studied directly by mounting creosotebush leaves in sodium azide and sodium bisulfite solutions and observing the stomata with a microscope.

Collection of Stomatal Imprints. Stomatal imprints were collected from three plants which had about the same leaf size and the same relative degree of leaf development. Plant one was about 1 meter tall, plant two was about 2 meters tall and plant three was about 1.5 meters tall. At hourly intervals, one branch was selected on the side of each

plant facing the light source and marked with black tape to aid in relocating the collodion-treated leaflets. The first pair of mature leaves back from the tip of the branch was used for imprints. Exactly on the hour, and as rapidly as possible, Merck collodion was applied with one or two strokes of a camel's-hair brush to the upper surface of two leaflets of the same leaf and to the bottom surface of the two leaflets of the opposite leaf on plants one, two and three, in that order. The branchlets on which the leaves grew were then plucked. The collodion on the leaflets was allowed to dry for a few minutes before it was stripped off. The imprints from the top leaf surfaces from plants one, two and three were mounted in sequence along the top edge of a glass slide. The corresponding imprints from the bottom leaf surfaces were mounted in sequence along the bottom edge of the same slide. A second glass slide was used as a cover glass. The two glass slides were taped together at the ends and the mounts stored for future analysis.

Analysis of Stomatal Opening. The degree of stomatal opening was estimated by classifying the stomatal imprints, as observed in a ten-celled microscope grid, into one of five different percentage classes of stomatal opening. At a magnification of 150X, each cell of the grid covered an actual leaf area of 0.01 mm^2. A total area of 0.1 mm^2 was analyzed per leaf surface.

The stomatal-opening classes and the mathematical procedure for estimating the average degree of stomatal opening on either leaf surface are shown in Table 1. To obtain the average stomatal opening for both leaf surfaces of a particular plant, the numbers of stomata occurring in

Table 1. Stomatal-opening classes and mathematical procedure for estimating the average stomatal opening on each leaf surface

Stomatal-opening classes	Midpoint of each class	No. of stomata in each class*	Percentage of stomata in each class	Weighted class percentages (Column 2 x 4)
0 - 20	10	0	0.0	0.0
20 - 40	30	3	12.5	3.8
40 - 60	50	14	58.3	29.2
60 - 80	70	3	12.5	8.8
80 -100	90	4	16.7	15.0
Total	—	24	100.0	56.8**

* Data for top leaf surface of plant number 1 at 9 p.m. on August 13, 1962.

** Average degree of stomatal opening.

each stomatal-opening class on top and bottom leaf surfaces were first combined. The average degree of stomatal opening for each leaf was then calculated as shown in Table 1. The average stomatal opening for one surface on three plants (or both surfaces on three plants) was similarly calculated.

Pilot Growth-Chamber Studies

Design and Construction of Growth Chambers

Pilot growth-chamber studies on creosotebush were carried out in three makeshift growth chambers designed by Dr. Ervin M. Schmutz of the Department of Watershed Management, College of Agriculture, University of Arizona. The design and construction of the growth chambers can be seen in Figures 2 and 3.

Light Control. Light was provided by a light bank, the construction of which can be seen in Figures 2 and 3. Each of the three light units in the light bank contained one 150-watt incandescent bulb plus one 22-watt and one 32-watt circular fluorescent tube. Each light unit and the glass plate below it was removable to permit easy access to the growth chamber below. The light bank was cooled with a 1-ton Carrier air refrigeration unit. Light intensity was considered a constant throughout the growth-chamber studies. Table 2 shows the maximum incident light intensities which occurred at different levels in the growth chambers.

Figure 2. Growth chambers showing plant chambers (lower left) with temperature and humidity gauges, refrigeration unit (far end), light bank (top left) and auxiliary heater (top, right foreground).

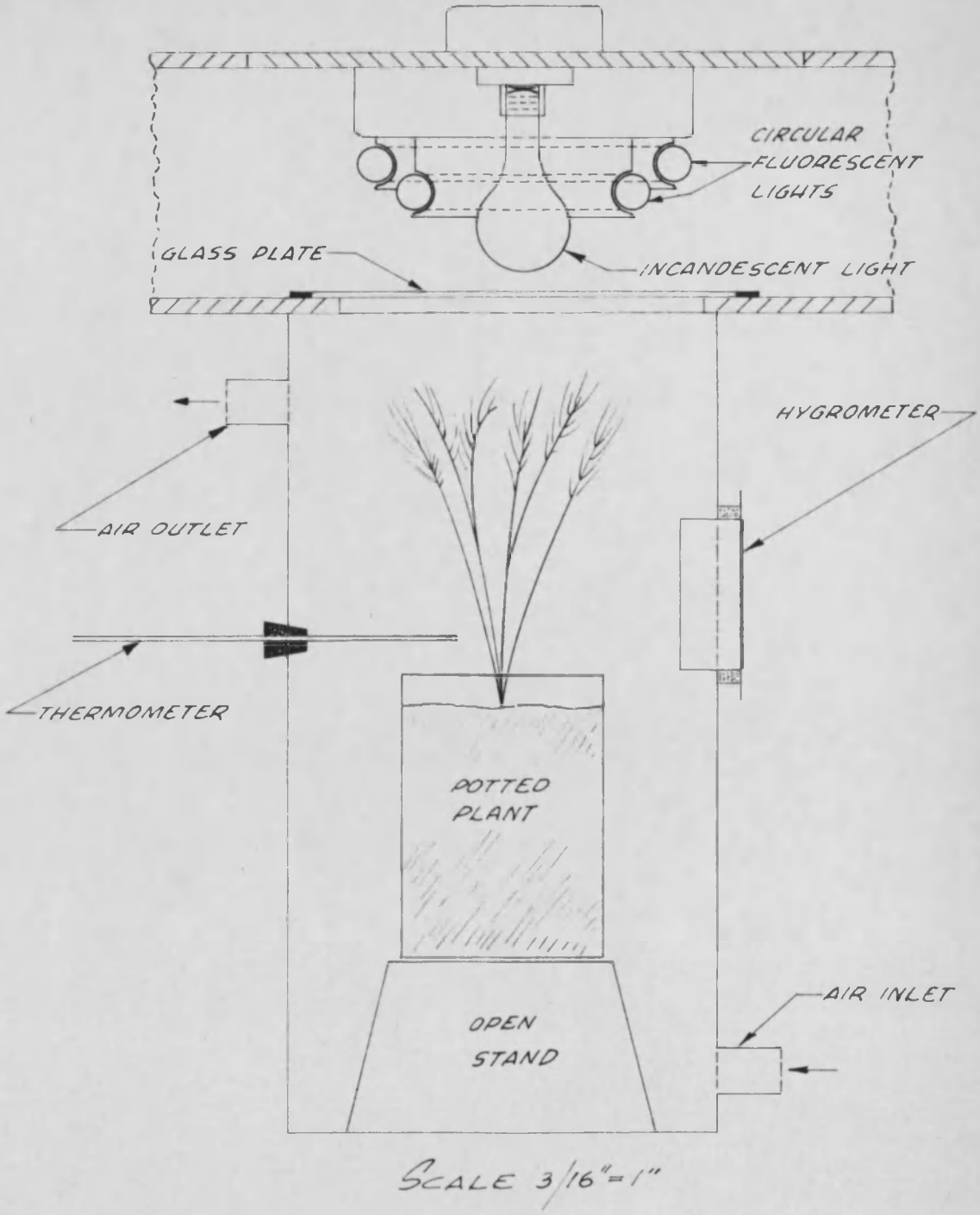


Figure 3. Cross-sectional diagram of one growth chamber and its segment of the light bank.

Table 2. Incident light intensities in foot-candles at different levels in the growth chambers as read with a Weston Master IV light meter using the Invercone attachment

Distance of reading in inches from top of growth chamber	Incident light intensity in foot-candles
0.00	800
2.75	360
10.50	200
14.00	100

Temperature Control. Since previous creosotebush studies had indicated that under certain conditions greater absorption and translocation of herbicides and plant kills resulted from midday applications of herbicides made during the high-temperature period from mid-August to the first week in September,[2] and since the average of the noon temperatures recorded with hygrothermographs at the Esmond study site during this period in 1959, 1960 and 1961 was 30.5° C, a temperature of 30° C was selected as the optimum temperature for herbicide absorption and translocation in the growth-chamber studies. The effects of lower and higher temperatures on herbicide absorption and translocation were tested by using temperatures of 22 and 38° C, respectively.

Temperature control in the growth chambers was accomplished by cycling part of the air from the light bank through the growth chambers

2. Personal communication from Dr. E. M. Schmutz, Department of Watershed Management, University of Arizona, Tucson, prior to its later publication in 1963.

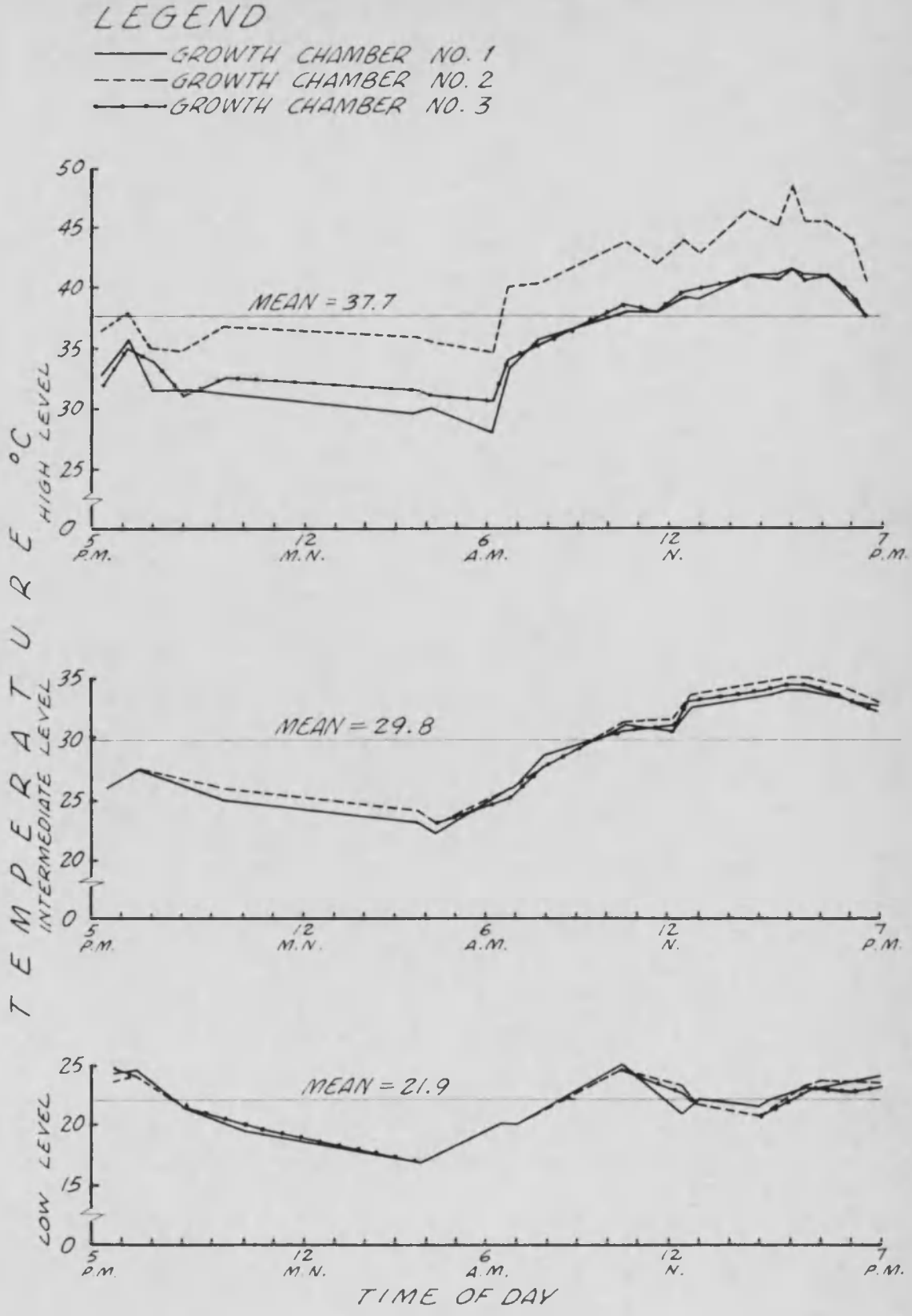
(Figure 2). The coldest growth-chamber temperature was produced by placing the refrigeration unit on the coldest setting. The intermediate temperature was obtained by reducing the degree of refrigeration on the lights and allowing them to warm the growth chambers and the air flowing to them. The highest growth-chamber temperature was produced by blowing room-temperature air through the light bank to the growth chambers and using a small auxiliary electric heater (Figure 2) with a fan to regulate the temperature level by increasing, as needed, the temperature of the air flowing to the growth chambers.

Growth-chamber temperatures were held as constant as possible, but they varied as a result of temperature changes in the room in which the chambers were set up. The temperature also varied from one chamber to another as a result of unequal cooling of the lights and unequal flow of air to the chambers. Figure 4 shows the temperature variations for each growth chamber at each temperature level and the mean temperature for each run.

Humidity Control. Relative humidity was not controllable in the growth chambers and, as a result, varied with growth-chamber temperature and room humidity. The relative humidity varied from 36.0 to 57.0% during the low temperature run, 20.8 to 31.5% during the intermediate temperature run and 15.4 to 26.8% during the high temperature run.

Air Velocity Control. The velocity of the air passing through each growth chamber varied slightly but was considered a constant in the

Figure 4. Deviation of individual growth-chamber temperatures from each temperature mean studied.


LEGEND
GROWTH CHAMBER NO. 1
GROWTH CHAMBER NO. 2
GROWTH CHAMBER NO. 3
TEMPERATURE °C
HIGH LEVEL
MEAN = 37.7
50
45
40
35
30
25
0
INTERMEDIATE LEVEL
MEAN = 29.8
35
30
25
20
0
LOW LEVEL
MEAN = 21.9
25
20
15
0
5 P.M.
12 M.N.
6 A.M.
12 N.
7 P.M.
TIME OF DAY

statistical analysis of the growth-chamber data. The average air velocity measured at the air outlets was 3 miles per hour.

Instrumentation. The instrumentation in each growth chamber consisted of a -20 to 100° C laboratory thermometer and an Abbeon hygrometer (Figures 2 and 3). The hygrometers were calibrated against a sling psychrometer at 20% relative humidity and synchronized with each other. Inherent variability between hygrometers resulted in slight to moderate variations in readings at higher and lower relative humidities.

Collection, Establishment, Selection and Conditioning of Growth-Chamber Plants

Collection and Establishment of Growth-Chamber Plants. Sixty creosotebush plants (6 to 12 inches tall) were collected on the Downtown Tucson Airport property south of East 36th Street, Tucson, Arizona. The soil on the collection area was similar throughout in texture, degree of slope, depth and rockiness and had a caliche layer at a depth of 14 inches or less.

The plants were collected with care being taken to preserve as much of the root system intact as possible. They were immediately transplanted into 5-quart oil cans in the same kind of soil in which they were growing. The soil was damp due to rains which fell in the area 2 and 3 days before collection. The plants were transferred to the University of Arizona where they were placed in the shade. Each plant was given 1 pint of water containing about 0.5 cc of Superthrive, which contains .06% Vitamin B_1 and .024% 1-naphthyl acetic acid, per gallon

of water. This treatment was repeated one week later. The plants were watered with tap water until water dripped from the holes in the bottoms of the cans. To reduce transpiration, each plant was pruned to the five branches needed for growth-chamber studies. The plants were kept well-watered until they were moved into full sunlight two weeks after collection. They were then watered just enough to keep them healthy until used in the growth-chamber tests.

Selection of Growth-Chamber Plants. Nine of the sixty plants were selected for growth-chamber studies on the basis of similarity in height and degree of leaf development. The nine plants were randomized as to the growth chamber in which they would be placed and the temperature at which they would be studied. One branch on each plant was used for collodion imprints of stomata. The other four branches were randomized with respect to the time of herbicide application.

Pre-treatment Conditioning of Growth-Chamber Plants. The plants to be studied at each temperature in the growth chambers were thoroughly watered at 2:00 p.m. on the day before the studies began. Between 5:30 p.m. and 5:45 p.m., the plants were placed inside the lighted growth chambers which had already been brought to the study temperature. At 6:30 p.m., the lights were turned off or, at the higher temperatures, blacked out with aluminum foil. A 12-hour dark period was used to approximate the length of night occurring in mid- to late-August at Tucson. The dark period was also used to condition the plants to the study temperature.

Herbicide Treatments Applied in Growth-Chamber Studies

The effects of temperature and length of photoperiod on herbicide absorption and translocation were studied using radioactive 2,4-D (designated 2,4-D*).

A stock solution was prepared to contain 5.58 micrograms (µg) of 2,4-D* and a radioactivity of 0.025 microcuries (µc) per 0.01 ml of solution. This was accomplished by dissolving 2.23 mg of the acid form of 2,4-D-2-C^{14} with a radioactivity of 10 µc in 4 ml of a 1:1 water-ethanol solution. A total of 0.04 ml of stock solution was applied with a micropipette to the first and second pairs of mature leaves on each treated branch. The application of the 2,4-D* was followed by 0.04 ml of a 2% diesel oil-water emulsion to act as a surfactant and to retard evaporation and drying of the herbicide.

Four branches per plant were treated, one each at the four application times. The four application times were 4:00 a.m. (two hours before light), 8:00 a.m., 12:00 noon and 4:00 p.m.

Three hours after application of the herbicide, the treated branches were cut off at the soil level. The treated branchlets and sections of bark stripped from one side of the supporting branches were glued onto heavy mounting paper 14 x 17 inches in size. The number of leaves on the branchlets was thinned before mounting to prevent overlapping and consequent incomplete contact with the X-ray film during autoradiographing. The sections of bark were mounted, phloem side up, in columns approximating the length of the branches from which they were taken. The branchlets and bark sections from each plant were mounted on

a single sheet of paper. After each temperature run, the mounts were pressed and air dried.

The relative degree of 2,4-D* absorption and translocation was determined by autoradiographing the mounted plants using the autoradiography techniques developed by Leonard and Crafts (1956) and Yamaguchi and Crafts (1958) and modified by Schmutz (1963).

Stomatal Analysis

The degree of stomatal opening at the time of herbicide application was determined from the collodion imprints made of stomata on a pair of leaves on the fifth branch of each plant each time one of the other four branches was treated with 2,4-D*. The collodion imprints were mounted and analyzed exactly like those obtained during field studies.

Anatomical Laboratory Studies

Equipment

Anatomical studies of creosotebush stomata were carried out with the aid of a Zeiss Standard GFL Routine and Research Microscope fitted with a Zeiss attachment camera and 24 x 36 mm miniature camera adapter. Panchromatic studies and photography of creosotebush stomata were done with light from either the microscope substage lamp or Zeiss epi-illuminator passed through a blue filter. Transmitted light from the substage lamp projected through either a 29F or an 88A filter was used for infrared studies and photography. Epi-illuminating ultraviolet light from a

Zeiss Multipurpose Microscope Lamp utilizing a maximum pressure HBO 200 mercury-vapor lamp was used for fluorescent studies and photography. A 220 volt Vorschaltgerät u. Drossel transformer provided power for the mercury-vapor lamp. A variable voltage (0-8 volts) Regel-Transformator provided power for the substage and epi-illuminator lamps. A light meter utilizing a cadmium probe inserted between the ocular lens and the camera body was used to indicate relative light intensity.

Plant Material Preparation

Cross sections of creosotebush leaves prepared by S. J. Shellhorn using a six-dye staining technique (Shellhorn and Hull, 1961) were used to study the gross cross-sectional anatomy of creosotebush stomata and guard cells.

Studies of the external features of creosotebush stomata and guard cells were carried out on living creosotebush leaves. Creosotebush branches 10 to 12 inches long were collected from creosotebushes growing adjacent to the Tumamoc Hill laboratory. The branches were trimmed to a single pair of leaflets and immediately placed in a beaker of water. The leaflets were carefully taped, abaxial side up, to a slide. Microscopic examination and photography of the stomata were immediately initiated and carried to completion as rapidly as possible.

The effect of sodium azide on stomatal movement was studied by collecting creosotebush branches as above and immersing them in a 0.01 molar solution of sodium azide. The immersed branches were kept in the light for one-half hour prior to examination. The effect of sodium

bisulfite on stomatal movement was similarly studied by immersing creosotebush branches in a 0.01 molar solution of sodium bisulfite and leaving them in the dark for one hour prior to examination.

Photographic Techniques

Fluorescent Photography. Ultraviolet light with a wavelength of 330 to 500 millimicrons was obtained by placing a blue Zeiss BG12 exciter filter between the HBO 200 mercury-vapor lamp and the epi-illuminator. The observer's eyes were protected from ultraviolet radiation by placing a yellow Zeiss OG4 barrier filter between the objective and the ocular lenses of the microscope. Photographs of creosotebush leaves fluorescing under ultraviolet light were made with Kodak PX-135 film having an ASA rating of 125. No light meter readings were made because the cadmium probe was not sensitive to ultraviolet light. A time exposure series of 2, 3, 4 and 6 minutes was used to determine the exposure required to obtain photographs showing the greatest amount of stomatal detail. The best photographs were obtained with exposures of 2 and 3 minutes.

Infrared Photography. Infrared photography of creosotebush stomata was accomplished by first bringing the desired detail into sharp focus using visible light transmitted through the leaf and then placing either a 29F or an 88A filter over the substage lamp prior to each photographic exposure. The cadmium probe of the light meter was sensitive to infrared light and gave light meter readings of 10 and 5 on the 100 scale for the 29F and 88A filters, respectively. Exposures of 0.2, 0.5,

1.0, 2.5 and 5.0 seconds were made using the 29F filter. Exposures of 0.5, 1.0, 2.5, 5.0, 10.0, 20.0, 40.0 and 80.0 seconds were made using the 88A filter. All infrared photography was done with Kodak IR-135 film having a tungsten ASA rating of 20.

Panchromatic Photography. Minute details of living creosotebush stomata which were obscured by the diffusion of transmitted light as it passed through the leaf tissue were clearly revealed when the leaf was illuminated from above. For this reason, epi-illumination was used for all panchromatic studies of living creosotebush stomata. Resolution of stomatal detail was further improved by reducing the diameter of the epi-illuminated area to one-third of the diameter of the microscope field.

All panchromatic photography of prepared cross sections of creosotebush leaves was done with transmitted light.

The quality of photographs taken with panchromatic light was found to be improved by placing a blue filter in the epi-illuminator or between the substage lamp and the substage condenser to remove the red portion of the spectrum. Thus, the light striking the film was not, in the strictest technical meaning, fully panchromatic.

Light meter readings for epi- and substage illumination were 120 on the 1000 scale and 7 on the 100 scale, respectively. To obtain photographs of varying intensity, exposures of 0.2, 0.5, 1.0, 2.0 and 5.0 seconds were made at these light meter readings using Kodak PX-135 film having an ASA rating of 125.

Film Processing and Printing

The Kodak IR-135 film exposed during the infrared studies was processed commercially by Palms Photo Lab of Phoenix, Arizona. The processed negatives were contact printed on glossy paper.

The Kodak PX-135 film exposed during anatomical studies of creosotebush stomata was developed in Acufine. A stock solution of Acufine was prepared, as directed on the label, by dissolving 2-1/10 ounces of Acufine in 1 liter of distilled water. The film was developed for 10 seconds at 24° C in two parts of the stock solution diluted with three parts of distilled water. Developing was completed with standard stopping and fixing baths. The developed negatives were contact printed on glossy, single-weight Velox paper. Photographs selected for this thesis were enlarged and printed on glossy, double-weight Polycontrast paper.

Statistical Analysis

Data collected in the field and growth chambers were coded and then analyzed by the University of Arizona Numerical Analysis Laboratory using an electronic computer. Analysis consisted of individual correlations and multiple regressions comparing the stomatal opening of creosotebush leaves with the factors and interactions of factors studied in the field and in the growth chambers. Factors affecting leaf moisture were also analyzed.

RESULTS AND DISCUSSION

Environmental Effects on Stomatal Opening

The data obtained during the field and growth-chamber studies are shown in Figures 5, 6 and 7. Statistical analyses of these data showed no significant correlations between stomatal opening, as determined by collodion imprints, and the environmental factors studied. Comparisons of stomatal opening with interactions of the environmental factors also showed no significant correlations. The correlations (Tables 3 and 4) varied erratically from leaf surface to leaf surface and plant to plant and raised doubts as to the accuracy of the collodion method in measuring the response of creosotebush stomata to environmental factors. As a consequence, detailed anatomical studies of creosotebush stomata were subsequently initiated to investigate questions arising from the analysis of the field and growth-chamber data.

Anatomical Studies

Results

Results of the detailed anatomical studies made on the structure and extent of the cuticle of creosotebush leaves appeared to conflict. A photograph of a leaf fluorescing under ultraviolet light (Figure 8) showed that the cuticle was fragmented and that the stomata appeared to occur below openings in the cuticle. Photographs taken during infrared

Figure 5. Relationships of diurnal changes in field environmental factors and leaf moisture to average stomatal opening on top and bottom leaf surfaces of creosote-bush during August 13 and 14, 1962.

PLANT MEASUREMENTS
SOIL FACTORS
ATMOSPHERIC CHARACTERISTICS
WIND VELOCITY - M.P.H.
REFLECTED LIGHT INTENSITY-FT. CA.
MILES PER HOUR
FOOT-CANDLES THOUSANDS
AIR TEMPERATURE - °C
RELATIVE HUMIDITY - %
PERCENT
DEGREES C
SOIL MOISTURE - %
SOIL TEMPERATURE - °C
STOMATAL OPENING ON TOP LEAF SURFACE - %
STOMATAL OPENING ON BOTTOM LEAF SURFACE-%
LEAF MOISTURE - %
TIME OF DAY
M.N.
N.

Figure 6. Relationships of diurnal changes in field environmental factors and leaf moisture to average stomatal opening on top and bottom leaf surfaces of creosote-bush during August 20 and 21, 1962.

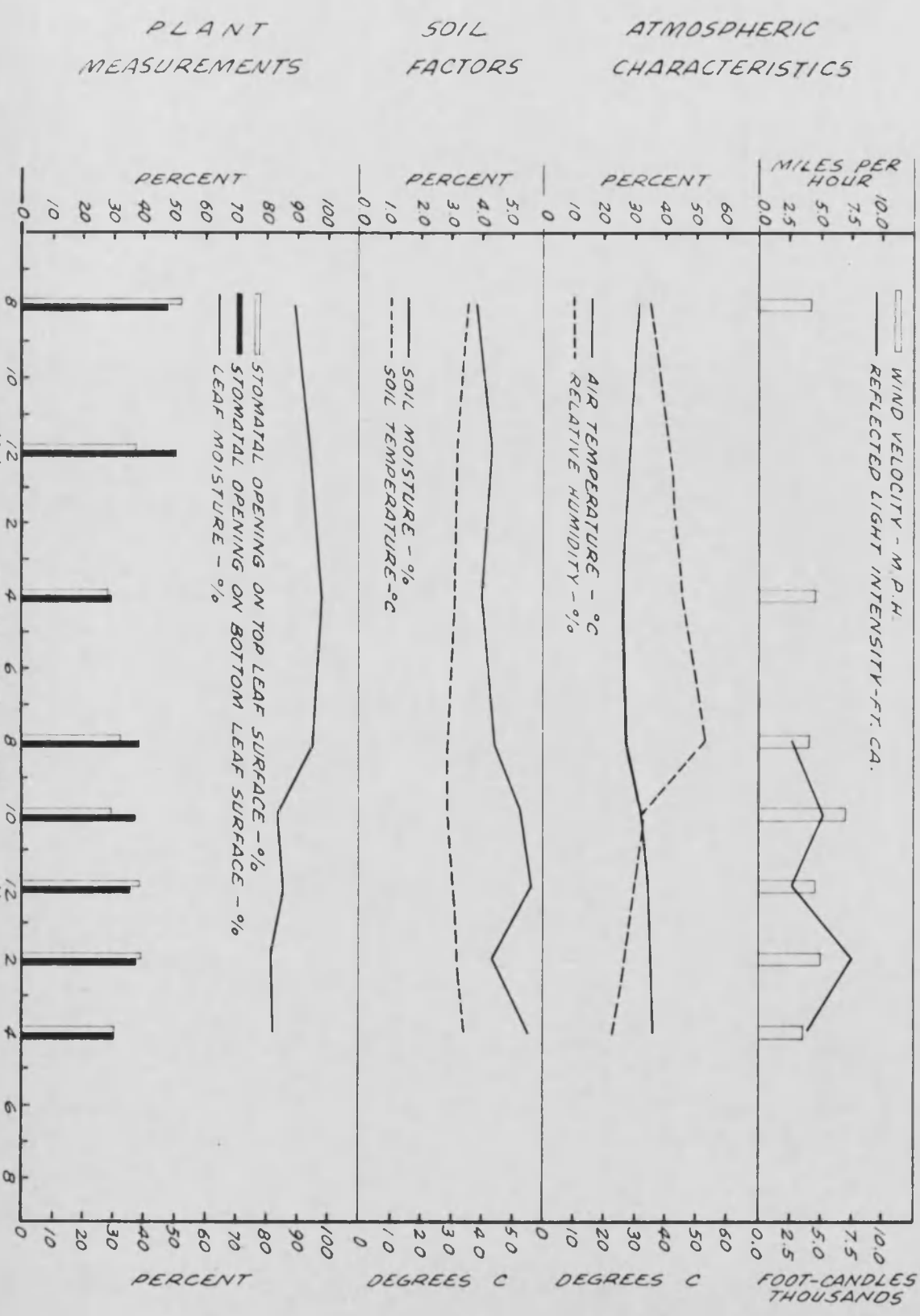

ATMOSPHERIC CHARACTERISTICS
SOIL FACTORS
PLANT MEASUREMENTS
MILES PER HOUR
WIND VELOCITY - M.P.H.
REFLECTED LIGHT INTENSITY-FT. CA.
FOOT-CANDLES THOUSANDS
PERCENT
AIR TEMPERATURE - °C
RELATIVE HUMIDITY - %
DEGREES C
PERCENT
SOIL MOISTURE - %
SOIL TEMPERATURE-°C
DEGREES C
PERCENT
STOMATAL OPENING ON TOP LEAF SURFACE - %
STOMATAL OPENING ON BOTTOM LEAF SURFACE - %
LEAF MOISTURE - %
PERCENT
TIME OF DAY
M.N.
N.

Figure 7. Results of growth-chamber studies showing the relationships of average temperature and relative humidity to average stomatal opening on top and bottom leaf surfaces of creosotebush at three temperature levels.

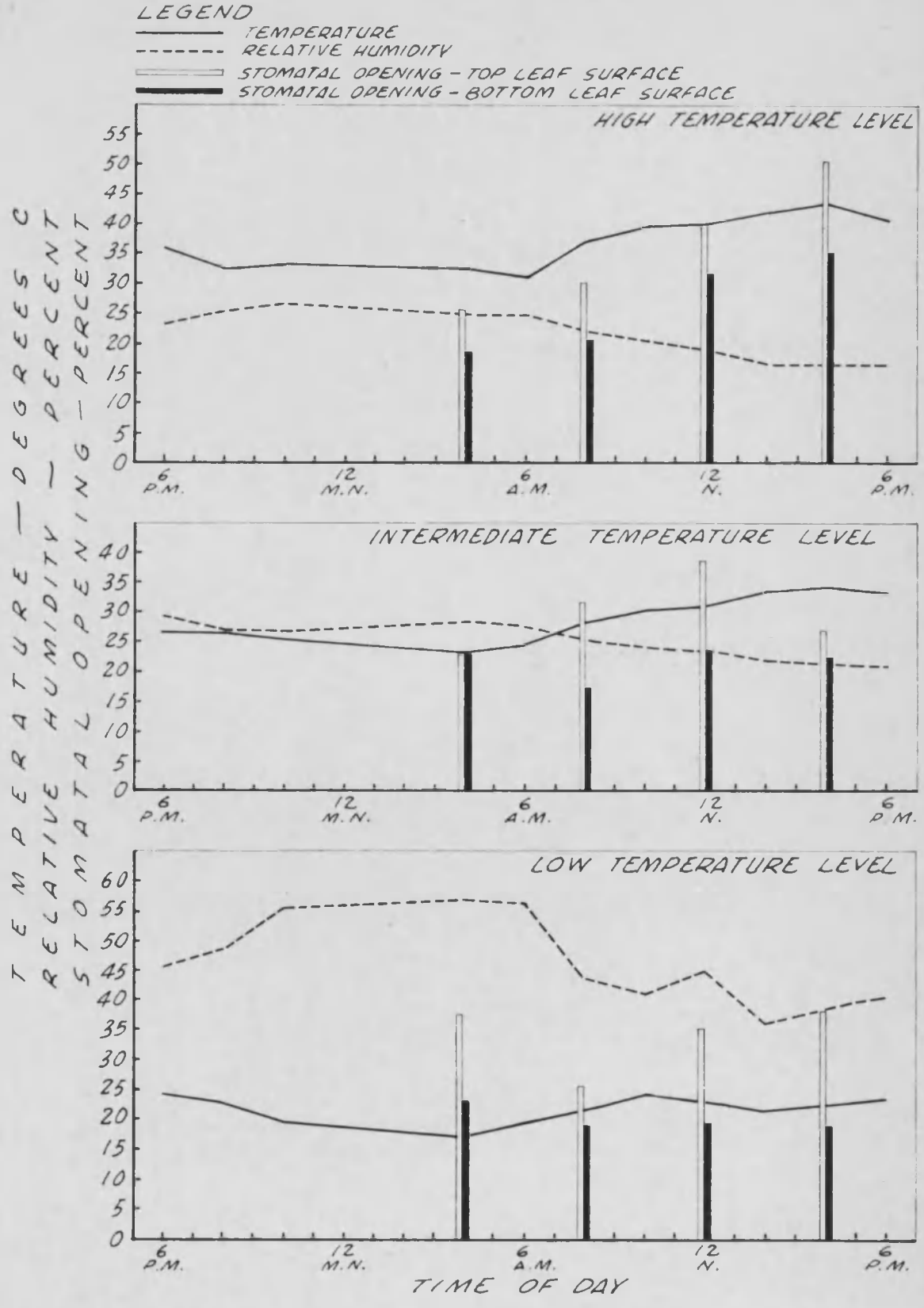

LEGEND
TEMPERATURE
RELATIVE HUMIDITY
STOMATAL OPENING - TOP LEAF SURFACE
STOMATAL OPENING - BOTTOM LEAF SURFACE
HIGH TEMPERATURE LEVEL
INTERMEDIATE TEMPERATURE LEVEL
LOW TEMPERATURE LEVEL
TEMPERATURE — DEGREES C
RELATIVE HUMIDITY — PERCENT
STOMATAL OPENING — PERCENT
6 P.M.
12 M.N.
6 A.M.
12 N.
6 P.M.
TIME OF DAY

Table 3. List of factors and interactions of factors studied in the field and their correlations with stomatal opening on each leaf surface and plant studied

Factors studied	Stomatal opening											
	Plant #1		Plant #2		Plant #3		Both surfaces			Three-plant ave.		
	Top	Bot.	Top	Bot.	Top	Bot.	#1	#2	#3	Top	Bot.	All
	Correlations											
Temperature	-.18	-.04	.21	.05	.14	.22	-.11	.11	.10	.07	.02	.04
(Temperature)2	-.17	-.02	.21	.03	.15	.20	-.09	.10	.11	.08	.02	.04
Relative humidity	-.06	.11	-.25	-.05	-.04	-.02	-.06	-.17	.05	-.19	.02	-.09
(Relative humidity)2	-.07	.16	-.23	-.07	-.03	-.05	-.06	-.17	.03	-.19	.02	-.09
Absolute humidity	-.23	.06	-.11	.07	.09	.25	-.18	-.04	.18	-.14	.05	-.05
(Absolute humidity)2	-.18	.08	-.08	.12	.09	.21	-.14	.01	.15	-.11	.05	-.03
Temperature x relative humidity	-.18	.07	-.19	.04	.01	.16	-.16	-.10	.12	-.20	.04	-.08
Temperature/relative humidity	-.07	-.01	.26	-.01	.10	.01	.00	.12	-.01	.13	.00	.06
Temperature x absolute humidity	-.23	.02	.03	.10	.14	.30	-.16	.04	.19	-.05	.04	-.01
Temperature/absolute humidity	.11	-.04	.26	.02	.00	-.18	.11	.17	-.15	.19	-.03	.08
Leaf moisture	-.23	-.25	-.31	-.01	-.12	-.23	-.31	-.11	-.16	-.31	-.19	-.27
Soil temperature	.46	.37	.45	.51	.36	.10	.50	.57	.21	.56	.42	.53
Soil moisture	.06	.01	.05	-.17	.09	.33	.02	-.03	.23	.08	.04	.07
Wind velocity	-.33	-.26	-.40	-.07	.03	.00	-.35	-.21	-.03	-.28	-.17	-.25
Light intensity	-.27	-.12	-.07	-.20	.07	.24	-.22	-.17	.13	-.08	-.11	-.12

Table 4. List of factors and interactions of factors studied in the growth chambers and their correlations with the stomatal opening of top and bottom leaf surfaces

Factors studied	Stomatal opening Three-plant average	
	Top	Bottom
	--- Correlations ---	
Temperature	.19	.37
$(\text{Temperature})^2$	.23	.41
Relative humidity	-.03	-.28
$(\text{Relative humidity})^2$	.02	-.23
Absolute humidity	.35	.28
$(\text{Absolute humidity})^2$	.35	.31
Temperature x relative humidity	.07	-.25
Temperature/relative humidity	.22	.42
Temperature x absolute humidity	.30	.41
Temperature/absolute humidity	-.02	.24

and epi-illumination studies indicated, however, that the cuticle was unbroken save for openings through which the guard cells protruded (Figures 9 and 10). Photographs of cross sections of creosotebush leaves supported the latter observation (Figure 11). These photographs show that the epidermis, with the exception of the protruding guard cells, is covered with a continuous, heavily-cutinized cuticle. Even though cross-sectional studies supported the latter observation, it should be noted that the gray splotches which occur in Figure 9 may be imperfections in the cuticle corresponding to the fragmentation lines appearing in Figure 8.

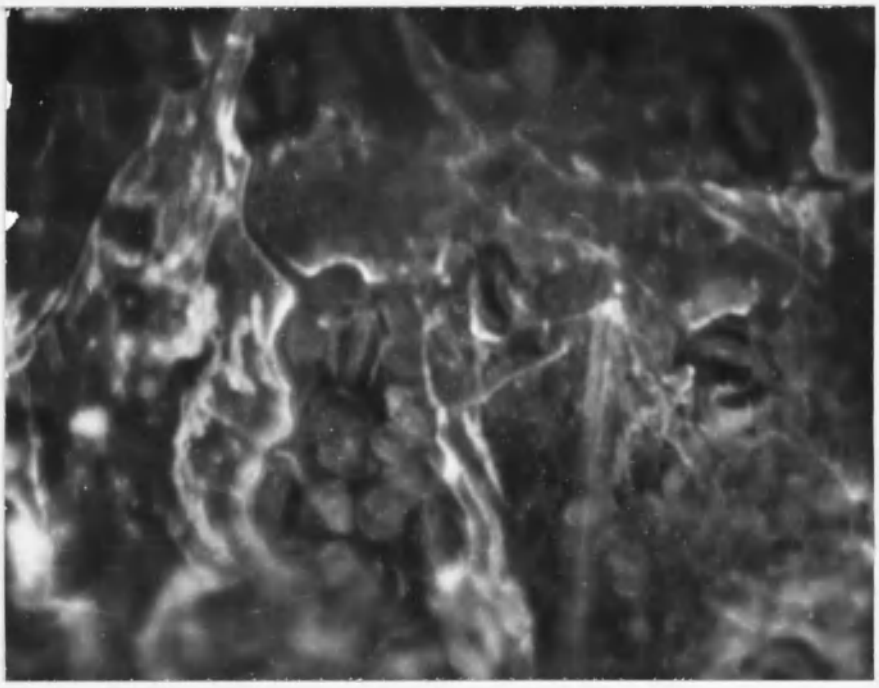


Figure 8. Fragmented appearance of the cuticle of a creosotebush leaf fluorescing under ultraviolet epi-illumination (200X).

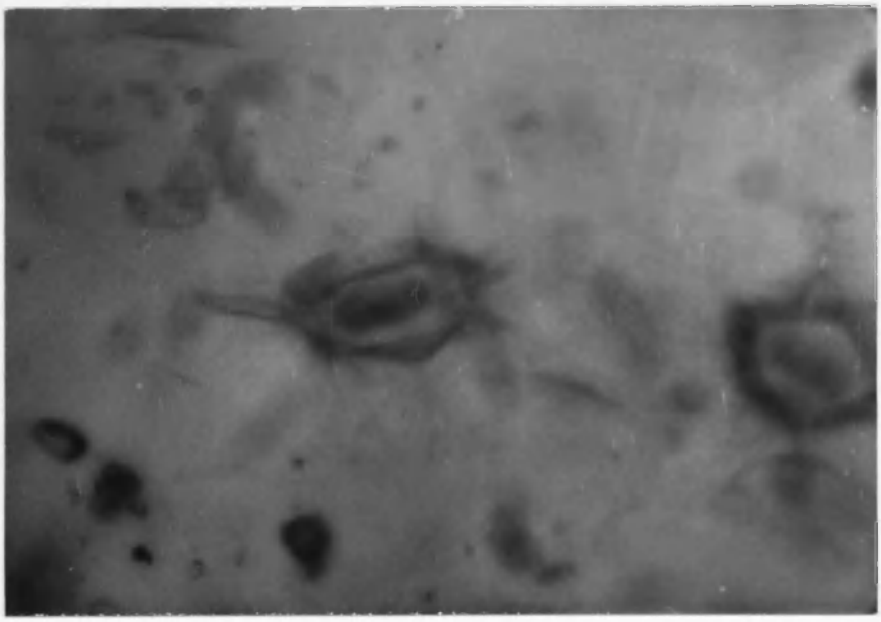


Figure 9. Continuous appearance of the cuticle of a creosotebush leaf photographed with transmitted infrared light (200X).

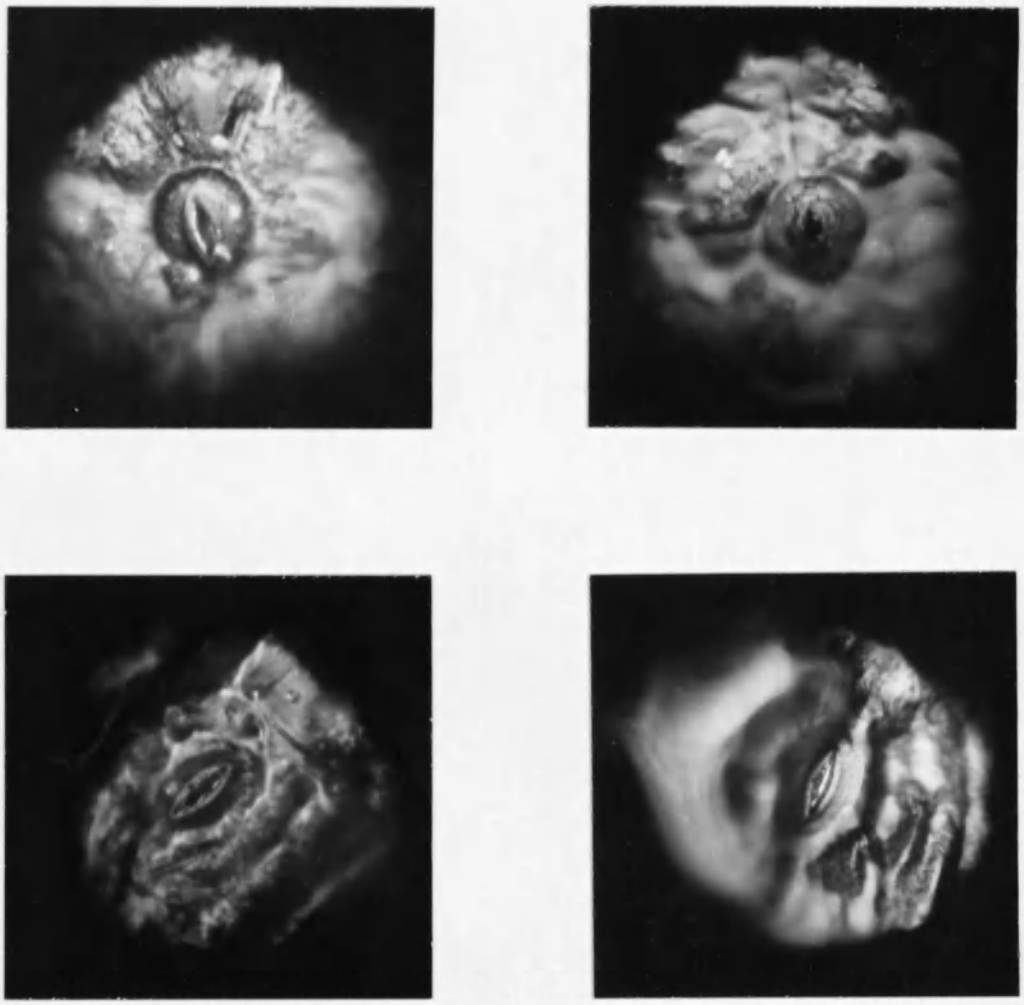


Figure 10. Creosotebush guard cells protruding through openings in the cuticle of living leaves (200X).

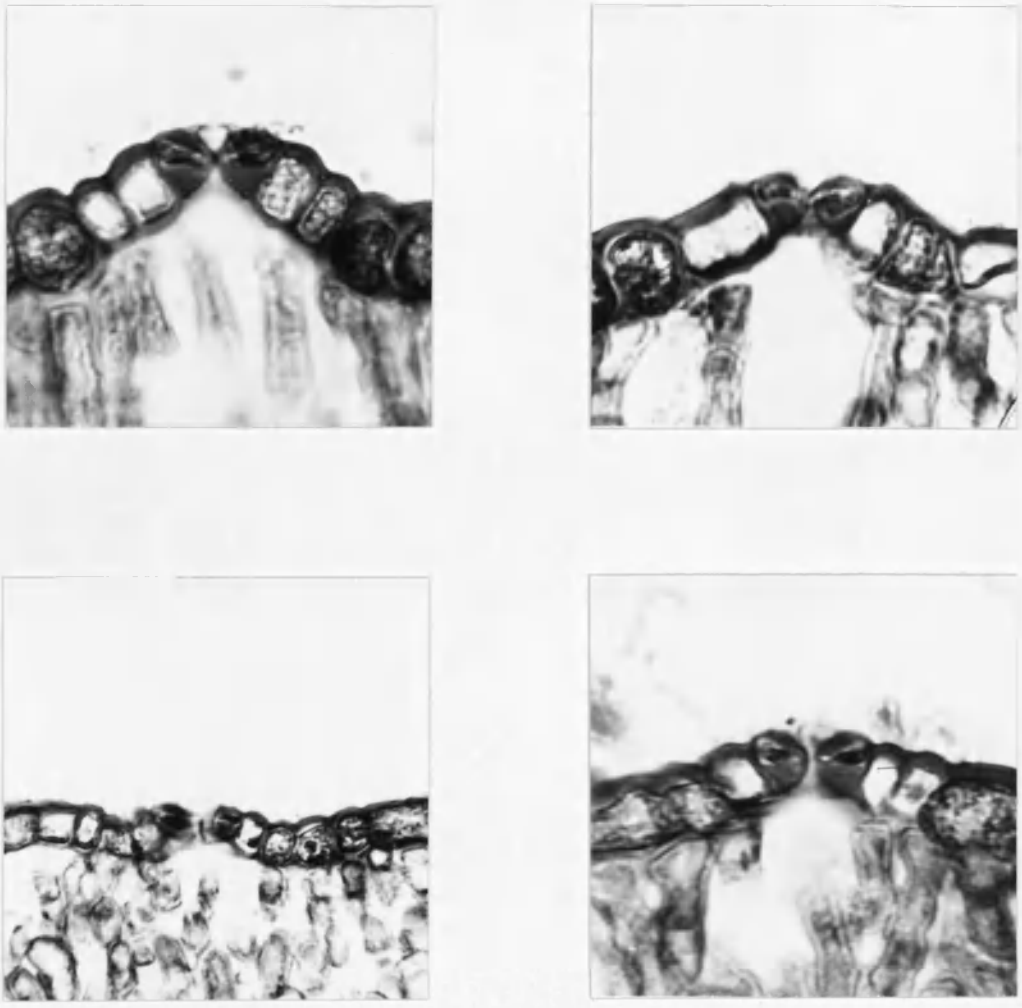


Figure 11. Guard cells protruding through openings in the heavy, continuous cuticle of mature creosotebush leaves (80X and 200X).

Dalton (1961) reported a chamber exterior to the stoma throat formed by cuticle extrusions on the guard cells. He called these extrusions "lips" or "horns". Photographs of cross sections of creosotebush stomata (Figure 12) confirmed Dalton's findings. Observation of living stomata under epi-illumination showed that the openings formed by the cuticle lips were irregular in outline and sometimes clogged with debris (Figure 13).

Further examination of cross sections of creosotebush leaves revealed that the guard cells were similar to the drawings of peach guard cells used by Esau (1953). Figure 14 shows that the guard cells have very thick cell walls and relatively small lumens when compared to the surrounding epidermal cells. Figure 15 shows the structural detail of the guard cell lumen. On a vertical cross section through both guard cells at the middle of the stomatal pore, the cell walls are the thickest and the lumen is tear shaped. On a similar section through the ends of both guard cells, the cell walls are thinner and the lumen is oval shaped. On a vertical plane running longitudinally through a single guard cell, the lumen appears to be shaped like a barbell.

A re-appraisal of the effects of 0.01 molar solutions of sodium azide and sodium bisulfite on stomatal opening indicated that the previous criterion of the shape of the guard cell walls outlining the stomatal pore (straight versus curved) could not validly be used to estimate the degree of stomatal opening. Apertures of stomata which had previously been thought to be open were found to be either open or closed depending on the development of the cuticle lips and the amount of debris

Figure 12. Cuticle extrusions (lips) on creosotebush guard cells (200X).

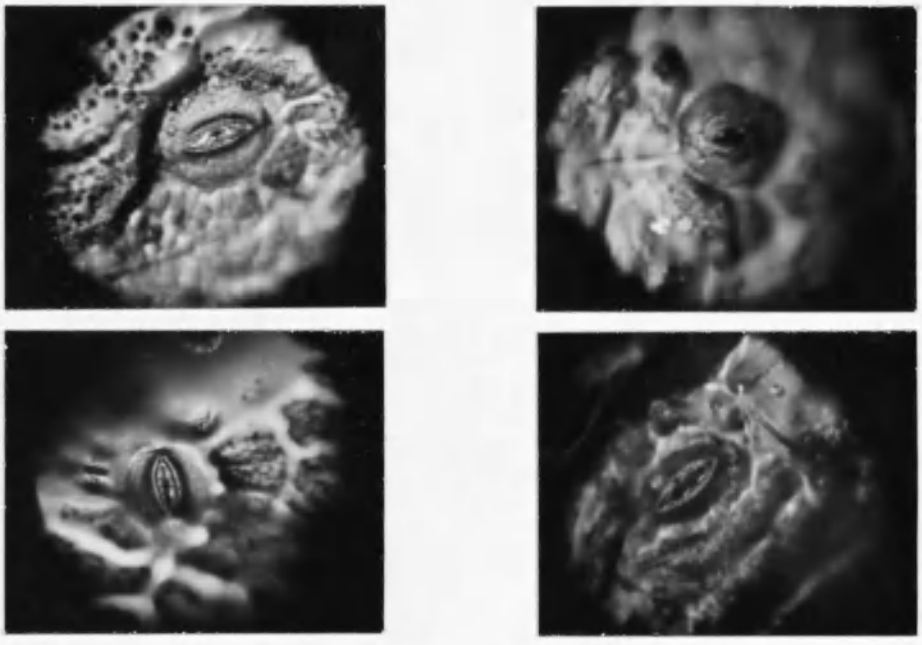


Figure 13. Irregular outline of aperture formed by the cuticle lips. Upper right hand photograph shows debris adhering to the cuticle lips (200X).

Figure 14. Thick cell walls and small lumens of creosotebush guard cells compared to the thinner cell walls and larger lumens of the surrounding epidermal cells (200X).

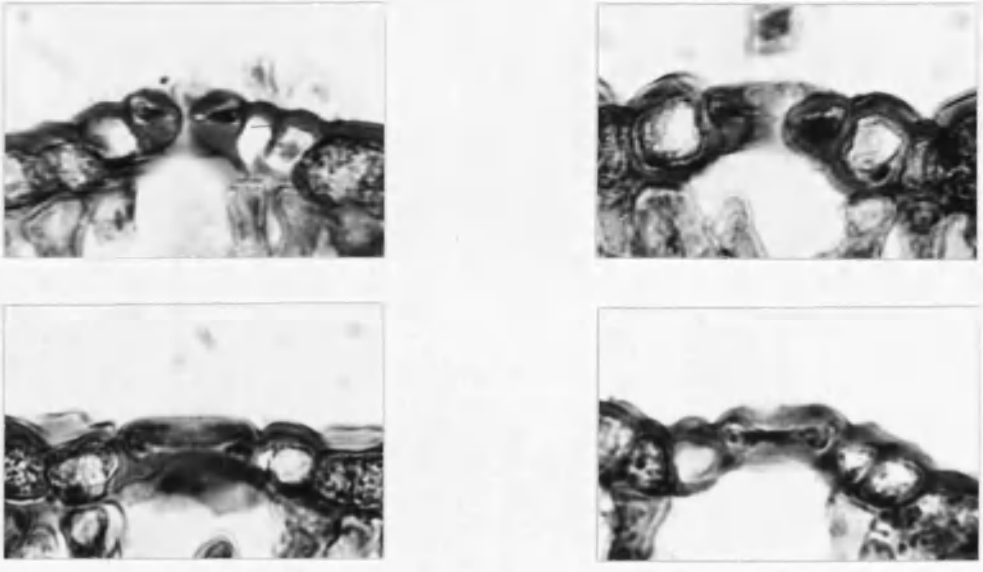


Figure 15. Lumen detail of mature creosotebush guard cells (200X). Upper photographs show cross sections of two guard cells through the center of the stomatal pore (left) and through the ends of both guard cells (right). Lower photographs show longitudinal cross sections of the lumen of a single guard cell.

clogging the aperture. For example, the top row of photographs in Figure 16 shows the guard cell shape originally estimated to be fully open, and the middle row, 40 to 60 percent open. All of these photographs, with the exception of the upper left photograph, show significant reduction in the stomatal aperture by the cuticle lips. The bottom row of photographs shows the guard cell shape originally thought to indicate fully closed stomata. The left-hand stoma is closed. The other two are still slightly open.

During the re-appraisal of the effects of chemicals on stomatal opening, the epidermal cells surrounding the guard cells were observed to be severely plasmolyzed by sodium bisulfite while the guard cells appeared to be only slightly affected and, in general, retained their ellipsoidal shape (Figure 17). This plasmolysis resulted in the greatest reduction in the size of the aperture formed by the cuticle lips seen throughout the entire study.

After the anatomical studies, the collodion imprints collected during the field and growth-chamber studies were re-evaluated. A sample of 154 stomatal imprints taken from five leaves during the field studies was examined. Stomatal opening could not be determined in 78% of the stomatal imprints. The low readability of the imprints was attributed primarily to the hardening of the collodion before it had formed a complete cast of the finer stomatal details. This difficulty might be overcome by increasing the percentage of solvent (alcohol or ether) in the collodion. The quality of the imprints was further reduced by the substantial amounts of debris adhering to the resinous leaves.

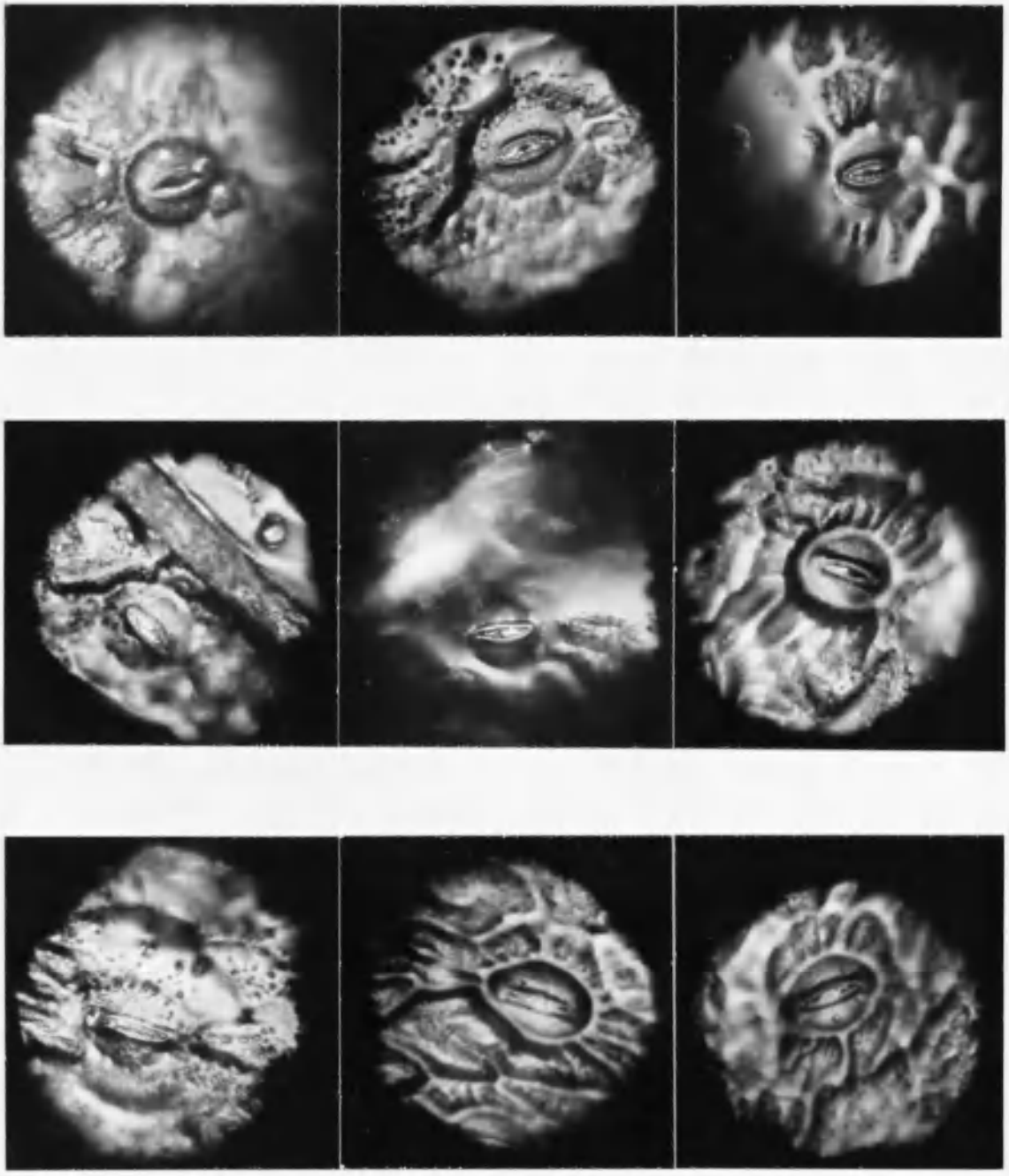


Figure 16. Variability of stomatal opening in creosotebush (200X). Top row of photographs shows guard cell shape originally thought to indicate maximum opening. Middle row shows guard cell shape originally thought to indicate an opening of 40 to 60%. The bottom row shows guard cell shape originally thought to indicate fully closed stomata.

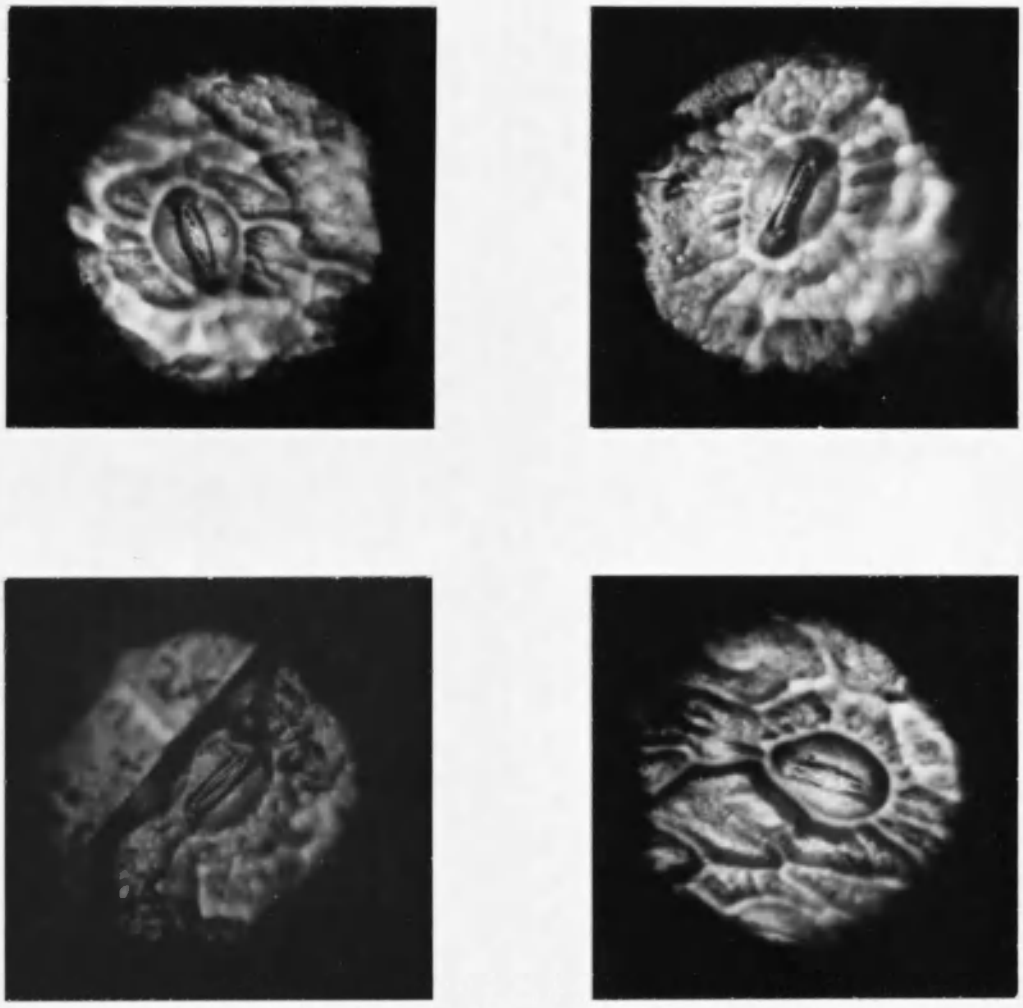


Figure 17. Effects of sodium bisulfite-induced plasmolysis on creosote-bush stomata (200X).

Discussion

The results of this and previous anatomical studies lead to three major conclusions: mature guard cells of creosotebush are generally incapable of producing stomatal movement solely by themselves; stomatal movement probably does not occur in creosotebush under normal field conditions; and, the effect of any stomatal movement that may occur is apparently reduced or nullified by the presence of the cuticle lips.

The relative lack of guard cell deformity in comparison to the severe puckering of the other epidermal cells during plasmolysis and the presence of very thick cell walls in the mature creosotebush guard cells suggest that mature creosotebush guard cells are too rigid to markedly vary the size of the stomatal aperture by themselves. This concurs with the conclusions of Florin (1931) regarding his observations of the stomata of conifers. He reports that the cell walls of conifer guard cells are very thick and are apparently more or less rigid. He suggests that the thin-walled subsidiary cells which support the guard cells may be involved in stomatal movement. This concept is further developed by Stålfelt (1929, 1956) and Ketellapper (1959) who indicate that stomatal movement may be effected by changes in the turgor of the epidermal cells surrounding the guard cells. Thus, plasmolysis of the epidermal tissue should affect stomatal opening.

The only change in the size of the aperture formed by the cuticle lips of creosotebush guard cells observed during the entire study was a closing which occurred during plasmolysis of creosotebush leaves with sodium bisulfite (Figure 17). Such plasmolysis could be recorded

by the collodion method. Careful examination of the collodion imprints obtained from more than 250 leaves during the field and growth-chamber studies revealed no epidermal plasmolysis -- even when the temperature was 42° C, the relative humidity 15% and the soil moisture 4.4% (Figure 5). Thus, stomatal movement apparently does not occur in creosotebush; but, it may occur under environmental conditions much more severe than those observed during the present study. Additional studies using an improved collodion technique (or another method for determining stomatal opening) are needed to fully determine the presence or absence of stomatal movement in creosotebush under field conditions.

Even if stomatal movement should occur in creosotebush, its effectiveness may be either reduced or eliminated by the presence of the cuticle lips on the guard cells. Dalton (1961) hypothesized that, as the stomatal pore closes, the aperture formed by the cuticle lips opens due to the rotation of the guard cells. Conversely, as the stomatal pore opens, the aperture formed by the cuticle lips closes. Debris between the cuticle lips could prevent their complete closure. Debris could also reduce the effective size of the aperture formed by the lips when they are open.

Lack of stomatal movement in creosotebush, or the ineffectiveness of any stomatal movement that does occur, is further supported by herbicide time-of-application studies. Skoss (1955), Leonard and Crafts (1956), Dybing and Currier (1959) and Pallas (1960) observed greater herbicide absorption when stomata were open than when they were closed. Loftfield (1921), Stålfelt (1929), Ashby (1932), Wilson (1948),

Daubenmire (1958), Dybing (1958) and Ketellapper (1959) reported a diurnal movement of stomata. Thus, it is reasonable to assume that increased herbicide effectiveness would occur during daylight hours. However, in studies reported by Schmutz (1963), the absorption and translocation of, and resultant creosotebush susceptibility to, 2,4,5-T applied at different times of the day showed no consistent pattern. Additional studies conducted by Schmutz in 1962 (Figure 18) and 1963 verified his earlier findings.[3]

Leaf-Moisture Studies

Analysis of the leaf-moisture data shown in Figures 5 and 6 revealed no significant correlations between creosotebush leaf moisture and the atmospheric and edaphic factors studied (Table 5). Despite the lack of statistical significance, leaf moisture in this study did vary with air temperature, relative humidity and light intensity in a manner similar to that reported by Böhning and Lusanandana (1952) and Bonner (1959). These researchers reported that leaf moisture varied inversely with light intensity. This they attributed to an increase in the transpiration rate caused by the elevation of the vapor pressure within the leaf as light energy was degraded to heat energy by the leaf tissues.

Böhning and Lusanandana further reported that leaf moisture varied inversely with air temperature and directly with relative humidity. Here they concluded that leaf moisture reduction was the result of an

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3. Personal communication from Dr. Ervin M. Schmutz, Department of Watershed Management, University of Arizona, Tucson. Unpublished data from the 1962 studies are used with the permission of Dr. Schmutz.

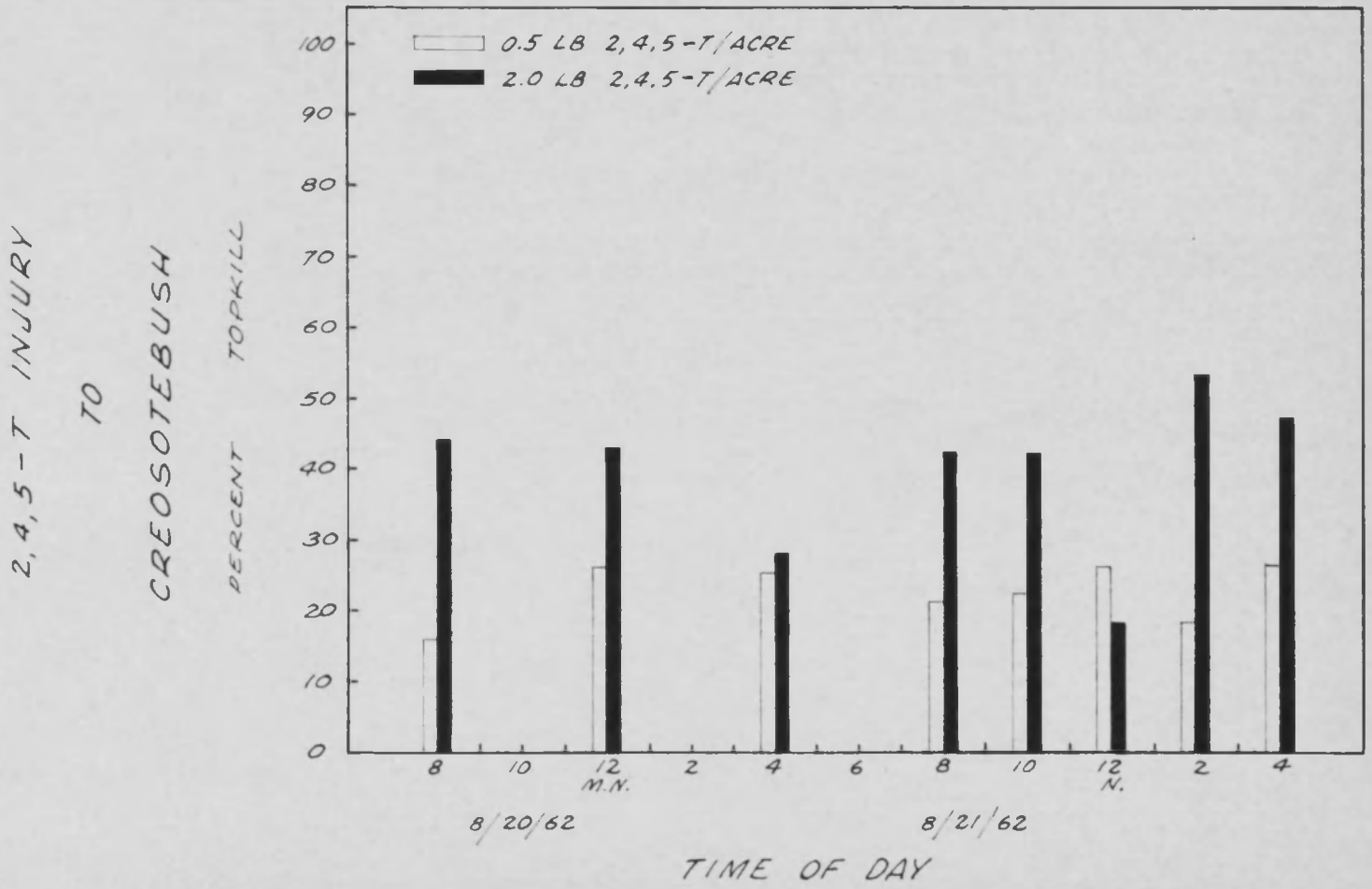

Figure 18. Relationship of time-of-day to the susceptibility of creosotebush to 2,4,5-T injury. Topkill data obtained 8/1/63.

Table 5. Correlation of leaf moisture with various atmospheric and edaphic factors

Factors studied	Leaf moisture: Individual plant average 1 n=32	2 n=29	3 n=32	Average of all plants All n=32
	Correlations			
Light intensity	-.30	-.30	-.30	-.30
Temperature	-.25	-.25	-.25	-.25
Relative humidity	.32	.36	.32	.32
Absolute humidity	.08	.12	.08	.08
Soil temperature	-.10	-.13	-.10	-.10
Soil moisture	-.18	-.16	-.18	-.18
Wind velocity	.06	.07	.06	.06

increase in the transpiration rate caused by a steepening of the atmospheric moisture deficit gradient as air temperature rose and relative humidity fell.

Herbicide Studies

Autoradiographs of the mounts of creosotebush branches treated with 2,4-D* during the growth-chamber studies indicated that the herbicide was translocated downward the full length of each branch (approximately 25 cm) in 3 hours regardless of light or temperature treatment. Autoradiographs of the branches treated at 12 noon and 4 p.m. indicated that the 2,4-D* applied earlier in the day was translocated downward to the crown of the plant and then 1 to 3 centimeters up the adjacent branches.

Further growth-chamber studies using better techniques and shorter translocation periods are needed to delineate the effects of length of photoperiod and temperature on herbicide translocation in creosotebush.

General Observations on Creosotebush Stomata

Ashby (1932) reported 24,000 stomata/cm^2 on the adaxial and 36,000 stomata/cm^2 on the abaxial leaf surfaces of creosotebush. In the present study no significant difference was found between the number of stomata on the top and bottom leaf surfaces. The average numbers of stomata obtained from examining 124 adaxial and 121 abaxial leaf surfaces from fifteen creosotebush plants were 22,000 stomata/cm^2 on the adaxial leaf surfaces and 21,000 stomata/cm^2 on the abaxial leaf surfaces (Table 6).

Table 6. Summary of stomatal counts on top and bottom leaf surfaces of fifteen creosotebush plants examined in field and growth-chamber studies

Leaf surface	No. of leaves examined	Total leaf area sampled - mm^2	No. of stomata	No. stomata per cm^2
Adaxial	124	12.4	2743	22,121
Abaxial	121	12.1	2548	21,058
Both	245	24.5	5291	21,596

GENERAL DISCUSSION AND CONCLUSIONS

Results of this study indicate that mature creosotebush guard cells are incapable of producing stomatal movement; that there may be little or no movement of mature creosotebush stomata in response to climatic and edaphic factors; and, that any stomatal movement which does occur is largely ineffective. Reasons for these conclusions:

1. The considerable thickness of the walls of the guard cells and their resultant resistance to plasmolysis largely precludes stomatal movement caused by changes in the turgor pressure of the guard cells.

2. Opening or closing of creosotebush stomata due to plasmolysis of adjacent epidermal cells was not observed in the field — even under conditions of severe stress. However, the possibility that changes in the turgor pressure of the epidermal cells may affect stomatal opening was shown by the plasmolytic closing of the aperture formed by the cuticle lips observed under artificial conditions.

3. This study confirmed the presence of the cuticle lips which were hypothesized by Dalton (1961) to reduce the effectiveness of stomatal opening.

4. Mature stomata were frequently clogged with debris, preventing their complete closure and nullifying their effectiveness when open.

5. If stomata are an important entryway for herbicides and if stomatal opening fluctuates diurnally, time-of-application studies

should show a corresponding herbicide response. No such response has been observed in herbicide studies involving creosotebush.

On the basis of this study, creosotebush stomata do not appear to have a significant effect on the effectiveness of herbicides: first, because stomatal effectiveness is low; and secondly, because the action of surfactant and oil carrier in the herbicide mixture facilitates the absorption of herbicides through the cuticle as well as the stomata (Crafts, 1961). The weak spots observed in the cuticle of creosotebush leaves may also be important in increasing the absorption of herbicides (Schiefferstein and Loomis, 1956). Thus, the effect of time-of-day, if any, would be through its influence on translocation within the plant and not by its effect on absorption through the stomata.

SUMMARY

Field and growth-chamber studies were initiated in 1962 to determine the effects of environmental factors on stomatal opening in creosotebush and their combined effect on herbicide activity.

The factors studied were soil temperature and moisture, light intensity, wind velocity, air temperature, relative humidity and time of herbicide application. Stomatal opening was determined from collodion imprints.

Analysis of the data obtained during these studies showed that results were erratic and that there were no significant correlations between stomatal opening and the factors studied. This led to the initiation of detailed anatomical studies, which revealed that the collodion technique used was inadequate to determine stomatal opening in creosotebush. This discovery invalidated most of the data obtained during the field and growth-chamber studies.

The anatomical studies revealed that creosotebush guard cells have very thick cell walls and confirmed the previous report of cuticle extrusions or "lips" on the guard cells. The aperture formed by these lips is irregular in outline and is frequently clogged with debris. Plasmolysis of adjacent epidermal cells induced by treatment with sodium bisulfite reduced the size of the exterior stomatal aperture but did not appear to affect the guard cells. No plasmolysis was observed under field conditions. These results led to the following conclusions:

1. Mature creosotebush guard cells are generally incapable of producing stomatal movement.

2. Little stomatal movement occurs in creosotebush under normal field conditions.

3. The effectiveness of stomatal opening is apparently nullified by the cuticle lips and the clogging of stomata by debris.

The absence of consistency in the results of the field herbicide studies substantiated these conclusions.

It was further concluded that the stomata of creosotebush do not affect herbicide activity and that herbicide absorption is influenced mainly through the action of the surfactants and oil carriers used.

The results of the leaf-moisture studies showed no significant correlation between leaf moisture and temperature, relative humidity and light intensity. However, leaf moisture did vary in the classical manner with these factors - that is, directly with relative humidity and inversely with air temperature and light intensity.

Studies of the number of stomata per unit area of leaf surface gave 21,000 and 22,000 stomata/cm^2 on the abaxial and adaxial leaf surfaces, respectively.

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