Epidermal cell/stoma ratio, stomatal area, and 2,4-D C14 penetration of Canada thistle ecotypes as related to 2,4-D response
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A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE Agronomy
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Abstract:
Several possible morphological explanations for the differential response of 10 Canada thistle ecotypes to treatment with 2,4-D were investigated. The distribution of stomata and stomatal area of the upper leaf surface were studied in relation to the penetration rate of 2,4-D C^14 and response to treatment with 2,4-D. The distribution of stomata was studied in terms of the epidermal cell per stoma (e/s) ratio.

Significant differences were found among the 10 Canada thistle ecotypes in E/S, ratio and stomatal area. Some ecotypes followed a set pattern for every location while others showed no relationship from one location to the next, or from one year to the next.

Carbon 14 labeled 2,4-D penetrated into the upper surface of Canada thistle leaves at a more rapid rate when treatment was made in the light than when treatment was made in the dark. The rate at which 2,4-D C^14 penetrated into the upper surface of leaf sections varied significants Iy among the 10 ecotypes. This difference, however, was questioned because of the large amount of variation found between replications of a given ecotype. Abnormalities of the epidermal layers, even when slight, caused a drastic increase in the penetration rate of the 2,4-D C^14 into the leaf sections.

No correlation.was found between the E/S ratio or the stomatal area and the response of the 10 Canada thistle ecotypes to 2,4-D treatment. Likewise, there was no correlation found between the stomatal area and the rate that 2,4-D C^14 penetrated into leaf sections from the 10 ecotypes or the stomatal area and the response of the 10 ecotypes to 2,4-D treatment.

Stomata do not appear to be the major portals for the entry of 2,4-D into the leaves of Canada thistle. Furthermore, the rate at which 2,4-D penetrates into the leaves of the various Canada thistle ecotypes does not appear to be the factor that is responsible for the differences in response to treatment with 2,4-D found among the 10 ecotypes.

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of

MASTER OF SCIENCE

Agronomy

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June, 1968

ACKNOWLEDGMENT

This study was conducted as part of the duties of the author as an Agricultural Research Technician with the Crops Research Division USDA, ARS. The author wishes to acknowledge the Agricultural Research Service for the permission to use this work as a thesis for a Master of Science degree. Special thanks is due J.M. Hodgson, the author's supervisor and chairman of the thesis committee, for advice and counsel.

Appreciation is expressed to John Henry of the ARS, Entomology Research Division for the use of special photomicroscopy equipment and assistance in dark room procedures.

The author also wishes to thank Dr. A.E. Carleton, Dr. S.R. Chapman, and Dr. I.K. Mills for serving as members of the graduate committee.

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ABSTRACT

Several possible morphological explanations for the differential response of 10 Canada thistle ecotypes to treatment with 2,4-D were investigated. The distribution of stomata and stomatal area of the upper leaf surface were studied in relation to the penetration rate of 2,4-D C^{14} and response to treatment with 2,4-D. The distribution of stomata was studied in terms of the epidermal cell per stoma (E/S) ratio.

Significant differences were found among the 10 Canada thistle ecotypes in E/S ratio and stomatal area. Some ecotypes followed a set pattern for every location while others showed no relationship from one location to the next or from one year to the next.

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INTRODUCTION

Canada thistle (Cirsium arvense (L.) Scop.) is one of the most widely distributed perennial weeds which causes extensive agricultural losses in the Northern Hemisphere. Methods of control have varied in effectiveness under the wide range of conditions under which this weed thrives. Response to treatment with 2,4-dichlorophenoxy acetic acid (2,4-D) has been especially variable. Hodgson (21,22) reported a group of 10 ecotypes in which he found a marked variation in morphological and phenological characteristics. These ecotypes also varied in their response to treatment with 2,4-D.

In this experiment the variable response of Canada thistle to treatment with 2,4-D was studied in terms of the variation in numbers and area of stomata on the leaves of the 10 ecotypes studied earlier by Hodgson (21,22). My initial objective was to determine if there were significant variations in the number or area of stomata on the upper surface of leaves of Canada thistle. Next the relationship of this characteristic to penetration of 2,4-D C^{14} and response of Canada thistle ecotypes to treatment with 2,4-D were studied.

REVIEW OF LITERATURE

Stomatal Penetration

Foliar applied herbicides must penetrate the outer protective layers of the plant before action against the plant metabolism can occur. The nature of the outer layers of the plant and the formulation of the herbicide applied are two of the factors determining the rate at which penetration takes place.

Stomata have been proposed as important sites of rapid entry of foliar herbicides (3,6,25,32,35,38). Others (8,19,23,34,37,39) found no evidence that foliar applied herbicides penetrated more rapidly into species of plants or areas of a plant that have a greater number of stomata than species of plants or areas of a plant that have fewer numbers of stomata. Fogg (12) found no evidence that penetration of 3,5-dinitro-o-cresol into the leaf took place through open stomata, as no evidence of penetration through open stomata could be seen when the epidermis was stripped from the leaf and observed under the microscope. Rice (30) reported that more 2,4-D was absorbed by bean leaves when treated in the dark than when treated in the light. He also reported no significant difference between the amount of 2,4-D absorbed at 100 versus 900 foot candles light intensity, when the air temperatures were the same. He therefore stated that stomata were not a factor in 2,4-D entry in these cases. Crafts (5) stated that regardless of the role of stomata in the uptake of spray solutions, stomatal opening cannot be depended upon unless special care is taken to determine the extent of opening at the time of spray application. Adams (1) presented a theoretical model, based on the principles of mathematics and physics,

that implies that a solution with the surface tension of water could not penetrate an orfice the size of the average open stomatal pore. Holley (24) stated that, since the difference in penetration rate between various plant species was so small in relation to the difference in the dose of 2,4-D necessary to inhibit growth, penetration differences between species can only be a minor part in governing the response of the species to 2,4-D treatment. Williams et al. (43) found that species that were sensitive to foliar applied 2,4-D were also sensitive to like doses of 2,4-D applied in a root absorbed nutrient solution. Resistant species were resistant whether application was foliar or root applied. Thus, a physical barrier to the penetration of the herbicide could not be the dominant factor in determining the response of the species to 2,4-D treatment. It was reported by Whitworth and Muzik (42) that the differences in external morphological characters of stomata numbers and degree of pubescences found among selected clones of field bindweed had no relationship to the selective action of 2,4-D. They further reported that the rate of entry of 2,4-D into the plant and its translocation inside the plant were not related to the selective action of 2,4-D.

It has been shown that surfactants lower the surface tension of an aqueous herbicide solution and thus penetration through the open stomata is enhanced (2,6,7,9).

The entry of foliar applied nutrients into leaves has been studied to determine whether or not penetration of spray solutions is more rapid into leaf areas with higher concentrations of stomata. Fisher (11) and others (38,40), found no evidence that stomata are major portals for

entry of foliar applied nitrogen into various plants. Conversely, Boynton et al. (3,4) stated that there is an increasing body of circumstancial evidence to indicate that stomata are frequently the most important paths of entry of nutrients in solution, even though the cuticle or uncutinized guard cells may also be important pathways over a long period of time.

Some researchers (17,18,26,41) contended that penetration is wholly cuticular, but not enough evidence was found to exclude the possibility that penetration through stomata may also have taken place. Pallas (27) stated that a rather large increase in the penetration rate of 2,4-D was evident when the stomata were open. He also contended that the higher penetration rate observed, with conditions of high humidity at the time of application, was partially the result of an increase in stomatal penetration. Jyrung (25) found the rate of Rubidium uptake by bean leaves to be highly correlated with stomatal frequency. Many workers (3,4,25,34,35,38) reported that foliar applied substances penetrated the lower surface of leaves faster than the upper surface. Since more stomata were found on the lower leaf surface than on the upper surface they concluded that entry was via stomata. Another reason for the rapid penetration into the lower surface of leaves could have been the thinner cuticle on that surface.

Prasad et al. (29), conducting tests on sections of the hypostomatous leaves of Zebrina, found that stomata played an important role in the entry of dalapon. They stated that, if the stomata were open and the surface which contains them was wet, stomata were an important portal for

entry of spray solutions into leaves.

An attempt was made by Currier and Dybing (6,7) to locate, at the cellular level, the route of penetration of a fluorescent dye solution. When excised leaves were emersed in the dye solution for two minutes with the stomata closed due to darkness, no penetration was evident. If the stomata were open during either a two-minute or a five-minute emersion, they found that the substomatal chambers and guard cells contained the fluorescent dye solution. When leaves were left submerged in the dye solution for 30 minutes, penetration was evident into the epidermal cells indicating that cuticular penetration took place, but at a reduced rate compared to open stomatal penetration.

Sargent and Blackman (32) used C^{14} labeled 2,4-D to show that penetration into leaf sections of Phaseolus was correlated with stomatal concentration even though the application of the 2,4-D material is made when the stomates are closed. They proposed that penetration takes place through the less cutinized stomatal guard cells and/or subsidiary cells rather than through the open stomates.

Franke (13,14,15), working in Germany, found direct evidence that penetration took place through the stomatal guard cells and subsidiary cells, by way of ectodesmata, which he reported to be concentrated in these areas. Using microautoradiographs, Franke was able to show that C^{14} labeled sucrose and proteins penetrated the leaves of Spinacea sp. and Viola sp. by way of ectodesmata.

A later study by Sargent and Blackman (33) showed evidence that at least one step regulating the rate at which C^{14} labeled 2,4-D penetrated

leaf sections of Phaseolus sp. was controlled by the metabolism of the plant. The stomata on the leaf disks opened at 500 foot candles light intensity. However, at both 500 and 800 foot candles the rate of penetration of the C^{14} labeled 2,4-D into the leaf was not significantly higher than when treatment was made in the dark. When the treatment was made at light intensities above 1000 foot candles, a very marked increase in penetration rate was observed. This increase was termed a metabolic surge. With further tests they showed that low temperatures and pre-treatment with ultraviolet light halted the surge in penetration rate. This supported the theory that the surge was metabolic in nature. Removing leaf sections from the light into darkness during the period of metabolic surge resulted in an immediate drop in penetration rate to the constant rate observed when leaf sections were treated with 2,4-D at light intensity near 500 foot candles or at low temperatures.

Stomatal Counting

The number of stomata per unit area and size of stomata are reported to vary greatly within species and within varieties grown at different locations (10). Stomatal number was reported to vary more than stomatal size. Many workers (10,16,20,28,31) reported that stomatal number varied from one area to another on the leaf surface. There was a higher concentration of stomata near the tip and margins of the leaf than on other areas of the leaf. Environmental factors were found to effect the concentration of stomata per unit area of leaf. Penfound (28) observed that light intensity was more important in its effect on the number of stomata per unit area than soil moisture. He contended that more sunlight

initiated the formation of additional stomata; whereas, soil moisture affected the number of stomata per unit area by increasing the size of all the epidermal cells. Hirano (20) reported that species and varieties of citrus showed differences in unit area density of stomata which appear related to their place of origin. He gave as an example that most species of citrus indigenous to the tropics had more stomata per square millimeter than species from outside the tropics.

Salisbury (31) reported that the proportion of stomata to ordinary epidermal cells was fairly constant regardless of environmental conditions. Stalfelt (36) proposed calculating the number of epidermal cells per stoma ratio as an index of stomatal concentration. He pointed out that this ratio eliminated differences in numbers of stomata per unit area caused by variation in epidermal cell size.

One of the easiest methods for counting stomata was reported by Gustafson (18). "Duco" cement was applied to the leaf in a very thin layer. When dry, the thin layer of cement was peeled off the leaf and mounted on a microscope slide for counting of the stomatal impressions.

Methods and Materials

The 10 Canada thistle ecotypes studied were originally collected by Hodgson (22). He designated the ecotypes with letters corresponding to their place of origin. The collection was made in 1956 and the ecotypes were maintained through successive plantings of sections of horizontal roots. For a description of the 10 ecotypes see Appendix Table I.

Field Plantings

The field data for this study were collected from a set of Canada thistle plots that had been established in 1965 to study the variations in response of the 10 Canada thistle ecotypes to treatment with three herbicides. The field plantings were in 8 ft. by 8 ft. plots at three locations; Bozeman and Huntley, Montana and Newdale, Idaho. One of the herbicides to be used in the original experiment was 2,4-D so that these data were also utilized in this study. Sample leaves were taken from the check plots for each ecotype for the various tests.

Each plot contained a permanent reference stake which was used to locate its center. Establishment and vigor of the thistles was measured by counting the shoots found growing within a thirty inch diameter circular frame with the reference stake at its center.

Treatment with 2,4-D

In 1966, when a few plants at each location had reached the early bloom stage, 2,4-D was applied at a rate of one and one-half pounds acid equivalent per acre to all ecotypes. Response to treatment was measured in 1967 by comparing the number of shoots present in the 30 inch circular area of each treated plot with a similar count of an untreated check

plot of the same ecotype.

Stomatal Counting

Fully mature leaves were collected from each check plot in 1966 and 1967 for measurement of the concentration of stomata on the upper leaf surface. In 1966, the leaves were selected from the 16th to the 18th node or insertion above the surface of the ground. Leaf samples were collected from the 14th thru 16th insertion in 1967.

Collected leaves were immediately coated with a very thin layer of "Duco" cement at the widest portion of the leaf blade. Sometimes this initial coating was removed to clean the leaf surface and a second coat of cement was applied. When the layer of cement had dried completely, it was carefully stripped from the leaf, trimmed and mounted leaf side up on a clean microscope slide. A cover slip was mounted on top of the leaf impression with additional cement at the corners. The slide was placed on a warm hot plate (150 F) with pressure applied to the cover slip to flatten out the leaf impression. Since "Duco" cement melts easily, over heating the slides at this point was avoided to preserve the leaf impressions.

Uniform and clear areas on the slides were located through the microscope and photographed with a 35 mm camera. The leaf surface was magnified 156 times in the finished $3\frac{1}{4}$" x $4\frac{1}{2}$" photomicrographs. Two subsample photomicrographs were made from each slide.

The number of stomata and number of epidermal cells on each photomicrograph were counted. The ratio of epidermal cells to stoma (E/S ratio) was then computed for each photomicrograph.

Stomatal Area

Photomicrographs showing uniform stomatal opening were selected for determination of stomatal area. Photomicrographs of each of four replications of each ecotype were enlarged further to a total of 2420 magnifications of the original leaf. Three stomatal images were cut from each of the enlarged photomicrographs and weighed. The weight per unit area of the photographic paper was measured and the weight of the stomata outline was converted to area. The area of each stoma and its surrounding epidermal cells was computed by using the epidermal cell per stoma ratio. This figure was applied to a square centimeter of leaf area and the area involved in stomata was computed for each ecotype.

Leaf Insertion

Three ecotypes, represented by 2 plants each were grown in the greenhouse during the winter of 1966. Impressions were made from the 9th, 16th, and 25th leaf of each plant to ascertain what effect, if any, leaf insertion had on the epidermal cell per stoma ratio. Since field data was obtained from the 14th through the 18th leaf, it was necessary to know what differences might occur because leaves came from different insertions on the leaf.

Position On the Leaf

Variation of E/S ratios occurring in different places on the leaves was studied to determine the necessity for sampling identical areas in the study.

Eleven leaves were selected from various greenhouse-grown thistle plants for this study. All leaves were from the same leaf insertion.

Impressions were taken at the base, near the center and near the tip of each leaf and the E/S ratios were determined.

Penetration of 2,4-D C^{14} in Darkness and Light

The rate of penetration of 2,4-D C^{14} into the upper surface of Canada thistle leaf sections under light and dark conditions was studied.

Five leaves were selected from greenhouse grown plants of the ecotype LW. Two sections of 15 mm diameter were punched from each leaf; one to be treated in the dark, the other in the light. The two sets of samples were prepared and treated the same except that the dark treatment was made in a germination dish that had been completely covered with black tape to exclude all light, while the light treatment was made in a clear plastic germination dish. Each section was carefully inspected for visible damage to the leaf surface. Any small leaf scratches at the location of the 2,4-D application were reported to have a profound effect on the resultant penetration rate (32). A 7 x 7 mm O.D. glass "Raschig ring" was attached to each section with lanolin. The leaf sections were then placed on water saturated blotter pads in the two germination dishes. The lids were placed on the germination dishes and the dishes incubated in a growth chamber for 1 hour at 21 C with a light intensity near 2000 foot candles. A 10 microliter droplet of the 2,4-D solution (0.1 microcurie of C^{14} in 1.8 micrograms of 2,4-D acid as the Na salt) was placed in each ring. The solution was carefully run down the side of the ring so that no air was trapped between the solution and the leaf surface.

Immediately following treatment, the lids of the germination dishes were replaced and the dishes were put back into the growth chamber for

four hours. The glass rings were then carefully removed from each leaf section with the excess 2,4-D solution held in the ring by capillarity. The leaf sections were then throughly rinsed with a water jet from a wash bottle. A cotton swab, moistened with benzene, was used to remove the remaining lanolin from the surface of the leaf section. The sections were rinsed once more and blotted dry. The leaf sections were mounted in copper planchets, dried, and counted in a gas flow planchet counter.

Penetration of 2,4-D C^{14} in Field Grown Leaves

In this study, uniform leaves of each ecotype were collected from the check plots of the field planting. The leaves were, as near as possible, the same age and from the same insertion. The samples were prepared and the treatment was made as described above except that the C^{14} was detected with a liquid scintillation detector. Each leaf section was placed in a liquid scintillation vial with 1 ml. of methanol. After one hour, 12.5 ml. of a fluor solution, containing 4 gm. of PPO (2,5 Diphenyloxazole) and 100 mg. POPOP (1,4-bis-2-(5-Phenyloxazolyl)-Benzene) per liter of toluene, was added to each vial. A green color due to extracted chlorophyll was present in each vial. Check data indicated that vials containing leaf sections and the green color gave a calculated dpm (disintegrations per minute) equal to 85% of the same treatment without the leaf section and green color. It was assumed that all samples were quenched near the same rate by the presence of the leaf section and the extracted chlorophyll. For final analysis the dpm data, calculated for all samples, were compared disregarding the presence of the green color and leaf sections.

Penetration of 2,4-D C^{14} in Greenhouse Grown Leaves

The final study was run as a check on field data. Ecotypes G4, AI, G1, FM, PW, and YM were selected for this study because of their relative response to 2,4-D treatment and total area involved in stomata. Ecotypes G4, AI, and G1 were among the most resistant in response to 2,4-D. FM, PW, and YM were among the most susceptable. Similarly FM, AI, and PW had the greatest stomatal area while YM, G4, and G1 had less stomatal area per unit of leaf area.

Five leaves of each ecotype were selected for treatment. Following the previously described procedures, sections were punched from each leaf and treated for four hours with C^{14} labeled 2,4-D.

A slightly smaller dose, 0.08 microcuries of C^{14} in 10 microliter, of the 2,4-D C^{14} solution was applied. One modification of the previously described procedure was made. One milliliter of benzene was used to extract the 2,4-D C^{14} instead of 1 milliliter of methanol. With this modification the counting liquid did not become green from extracted chlorophylls. One milliliter of benzene quenches more than one milliliter of methanol, but, in the presence of green plant material, the counting efficiency is higher with benzene. Exact cross references could not be made between samples that were extracted with methanol and samples that were extracted with benzene.

RESULTS AND DISCUSSION

The distribution of stomata on the upper leaf surface of ten Canada thistle ecotypes was studied in relation to the penetration rate of 2,4-D C^{14} and response to treatment with 2,4-D. The results reported show the development of the stomatal sampling procedure, the ecotype effect on the E/S ratio, and the rate of penetration of C^{14} labeled 2,4-D into leaf sections of the various Canada thistle ecotypes.

A. Effects of Leaf Insertion On E/S Ratio

No significant difference of E/S ratio was found among leaf samples collected from the 25th, 16th, or 9th insertion above the soil surface (Table I). There was, however, a trend towards a higher E/S ratio on the 9th leaf which, in this case, was one of the last rosette leaves formed early in the development of the plants. These data indicate that in younger leaves where more epidermal cells per unit area were present there was a corresponding increase in the number of stomata per unit area. Leaf samples of the field-grown plants were obtained from the 14th to 18th insertion which was well above the rosette leaves, thus avoiding the lower leaves where greater variation was indicated to be present. It was assumed that plants grown in the greenhouse were similar to field grown plants in respect to the effect of leaf insertion on the E/S ratio.

B. Effect of Sample Position on the E/S Ratio

When greenhouse grown leaves of the various ecotypes were sampled at the base, near the center, and at the tip, no significant difference in E/S ratio was found. Means of 13.90 for the base, 14.08 for the center, and 15.29 for the tip were found when the 11 samples were averaged. These values were not significantly different at the five per cent level

of probability although there was a trend for more stomata per given number of epidermal cells toward the base of the leaves. These data indicate that the sampling technique used is reliable. All field samples were collected at or near the center of the leaf to the side of the midrib on the upper surface.

TABLE I. Effect of insertion level on the epidermal cell per stoma ratio of greenhouse grown Canada thistle leaves.

Ecotypes	Insertion Level 25	16	9	Total	Avg[1/]
LW	11.09	13.09	15.81	39.99	13.20
AI	11.77	12.50	15.56	39.83	13.14
G4	13.70	12.70	13.69	40.09	13.23
Total	36.56	38.29	45.06		
Average[1/]	12.07	12.64	14.87		

[1/] means not significantly different at the 5% level of probability. (F test)

C. Effect of Ecotype and Location on the E/S Ratio

Stomata on leaf surfaces of the ten Canada thistle ecotypes were determined by Hodgson[1/] in 1964. Stomatal counts per square centimeter were found to vary significantly because of age of leaf, side of leaf (more on the under side) and position on the leaf (more near the tip). Statistical analysis of the data gathered in 1966 by the author showed no differences in numbers of stomata or numbers of epidermal cells among the ten ecotypes. However, when the E/S ratio was determined, significant differences among ecotypes were detected (Table II). The E/S ratio was uniform and not significantly influenced by leaf age or size.

The Canada thistle ecotypes are listed in descending order of E/S ratio for the years 1966 and 1967 (Table II). Some ecotypes seem to follow a pattern for every location while others show no relationship from one location to the next or from one year to the next. In addition to the significant differences among ecotypes, significant interactions of ecotypes x location, ecotypes x years, years x locations, and ecotypes x locations x years were found. Environment apparently affected the E/S ratio of the upper surface of Canada thistle leaves. The ten ecotypes varied considerably because of environment or location. The E/S ratio was greater at Huntley than at Bozeman and Newdale and greater in 1967 than in 1966.

1/ J.M. Hodgson, "1964 Annual Report, Weed Control Investigations." The Crops Research Division, ARS, USDA, in cooperation with Montana Agri. Exp. Stat. and Region 6, Bureau of Reclamation, USDI.

TABLE II. Ratio of epidermal cells per stoma of 10 Canada thistle ecotypes for three locations in two years.

1967				1966			
Location	Ecotype	E/S Ratio		Location	Ecotype	E/S Ratio	
Huntley	G3	13.08	a[1]	Huntley	G4	12.16	a
Huntley	LW	12.63	ab	Huntley	FM	12.13	a
Bozeman	G3	11.96	abc	*[2] Huntley	G3	11.64	
Bozeman	FI	11.96	abc	Huntley	AI	11.62	ab
Huntley	G4	11.38	abcd	Bozeman	G4	11.46	abc
Huntley	FI	11.36	abcd	* Huntley	G2	10.69	
Huntley	G1	11.32	abcde	Huntley	PW	10.57	bcd
Newdale	G3	11.30	abcde	* Bozeman	G3	10.40	
Bozeman	PW	11.26	abcde	* Newdale	G3	10.33	
Bozeman	YM	11.22	abcde	Newdale	G4	10.17	cde
Bozeman	G1	11.22	abcde	Huntley	G1	9.91	def
Huntley	G2	11.21	abcde	Newdale	FM	9.96	def
Newdale	FI	10.82	bcdef	Huntley	YM	9.73	defg
Huntley	FM	10.59	bcdefg	Bozeman	FM	9.66	defg
Huntley	YM	10.58	bcdefg	Newdale	G1	9.48	defgh
Bozeman	G4	10.30	cdefg	Newdale	PW	9.47	defgh
Huntley	AI	10.26	cdefg	* Newdale	G2	9.47	
Bozeman	LW	10.25	cdefg	* Bozeman	FI	9.31	
Newdale	G4	10.09	cdefg	Bozeman	AI	8.96	efgh
Newdale	YM	9.95	cdefg	Bozeman	PW	8.94	efgh
Bozeman	G2	9.90	cdefg	* Newdale	FI	8.78	
Newdale	G1	9.76	defg	Newdale	AI	8.76	efgh
Newdale	PW	9.72	defg	Newdale	YN	8.75	efgh
Newdale	FM	9.68	defg	Huntley	LW	8.68	fgh
Huntley	PW	9.52	defg	Bozeman	YM	8.49	fgh
Newdale	G2	9.36	defg	Bozeman	LW	8.30	gh
Bozeman	AI	9.32	defg	Bozeman	G1	8.29	gh
Bozeman	FM	9.14	efg	Newdale	LW	8.17	h
Newdale	LW	8.67	fg	* Bozeman	G2	7.90	
Newdale	AI	8.65	g	Huntley	FI	Missing	

[1] Values with the same letter are not significantly different at the 5% level of probability.

[2] These samples were not included in the original analysis of variance test because of missing data. They are placed in their numerical order here for comparative purposes.

Both years and locations were significantly different. In 1966, the field plots were infested by Buckmoth larvae and many of the lower leaves were completely devoured. Consequently, 1966 sample leaves were taken from slightly higher insertions than those taken in 1967. The change in insertion should have had little influence on the E/S ratio, but the removal of the lower leaves by the larvae could have had an effect on the subsequent growth of the higher leaves. This factor could have had some effect on the differences in the E/S ratio found between the years 1966 and 1967.

D. Effect of Ecotype on Stomatal Area

Photomicrographs from the four replications were enlarged and the average area of a single stoma was calculated for each ecotype. This value was applied to the E/S ratio data for 1967 at Bozeman and a stomatal area was computed for each field replicate. The average size of stomata did not vary significantly among ecotypes. However, the total area involved in stomata, that is the area of the stomatal pore and its corresponding guard cells, per square centimeter of upper leaf surface varied significantly (Table III). The area of stomata was calculated using an average value for the size of epidermal cells, which in the 1966 Bozeman data did not vary significantly in size.

These values for the area of stomata (Table III) can also be expressed as the percentage (2.92 mm^2 equals 2.92% of 1 cm^2) of the total upper leaf surface area that is involved in stomata. The difference between the high ecotype FM with 2.92 mm^2 of stomatal area and the low ecotype of G1 with 1.88 mm^2 of stomatal area, was 1.04 mm^2 of stomatal

area. When considering the leaf area, 1.04 mm^2 per 1 cm^2 or 1.04% of the total leaf area would be a very small portion to consider. If, however, only the stomatal area is considered, the upper surface of G1 leaves had 35.6% less stomatal area than the upper surface of FM leaves. If stomata were the major portals for the entry of 2,4-D into leaf surfaces, this much difference could be very significant. However, if stomata were only minor portals, the small percentage of stomata and the even smaller difference between ecotypes would be completely overshadowed by the high rate of non-stomatal penetration.

TABLE III. The area involved in stomata on 1 square centimeter of the upper leaf surface of 10 Canada thistle ecotypes at Bozeman, Montana in 1967.

Ecotype	Stomatal area $mm^2\ cm^{-2}$ leaf area
FM	2.92 a[1/]
G2	2.76 a
AI	2.49 ab
PW	2.23 bc
LW	2.16 bc
G3	2.16 bc
YM	2.09 bc
FI	2.08 bc
G4	2.06 bc
G1	1.88 c

1/ Values with the same letter are not significantly different at the 1% level of probability.

E. Penetration of 2,4-D C^{14} in Light or Dark

Carbon 14 labeled 2,4-D penetrated into leaf sections treated in the dark at a slower rate than into leaf sections treated in the light. Leaf sections treated in the dark averaged 70 CPM (counts per minute) while those treated in the light averaged 146. Whether this difference is due to stomata being closed in the dark and open in the light or due to some metabolic process that occurs in the light but does not occur in the dark is not known. The means given above are for a single, five-replication experiment only. Various other experiments were run in which more variation was found. The leaf sections were carefully selected and fairly uniform data was obtained giving the above result. Any type of defect or injury at the point of application caused a tremendous increase (up to about 100 times) in the amount of C^{14} detected in the leaf. The planchet counter did not count the disintegrations of the C^{14} very efficiently. No correction was made for count efficiency or sample geometry as it was felt that the samples were very near the same in this respect.

F. Penetration of 2,4-D C^{14} into Field Grown or Greenhouse Grown Leaves

Penetration of 2,4-D C^{14} was significantly different among ecotypes when leaves were grown in the field or in the greenhouse in 1967 (Table IV.). However, the coefficient of variability for the field data was quite high indicating that penetration of 2,4-D under field conditions was extremely variable and more dependent upon method of herbicide application or the physical condition of the leaf epidermal layers than upon ecotype differences.

TABLE IV. DPM of C^{14} labeled 2,4-D that penetrated into leaf sections of field and greenhouse grown Canada thistle, Bozeman, Montana, 1967.

Ecotype	DPM [1] Field Grown	Greenhouse Grown
G4	1,295 a[2]	500 b
FM	1,094 ab	415 b
G3	902 ab	
YM	866 ab	654 a
LW	634 ab	
AI	504 ab	417 b
G1	504 ab	431 b
PW	501 ab	482 b
FI	339 b	
G2	321 b	

[1] Disintegrations per minute

[2] Numbers with different letters are significantly different at the 5% level of probability.

Mechanical damage to the leaf appeared to the major cause of this variation. Therefore, it appears that poor penetration caused by impervious epidermal layers could be overcome by scratching or otherwise disrupting the leaf surface prior to treatment.

The same type variation, but to a lesser extent, was experienced with leaves grown in the greenhouse. A sample would occasionally turn up with a reading 10 to 30 times greater than the usual range. On most occasions, these samples could be picked out before the readings were taken. These samples usually had visible damage or the treatment solution was completely absorbed. Such samples were discarded.

G. Relation of E/S Ratio and 2,4-D C^{14} Penetration to Response to 2,4-D

The data collected in 1967 for the response of the ten ecotypes to treatment with 2,4-D in 1966 were not used in this analysis as many of the plots became contaminated by mixing of ecotypes and the data was quite erratic. Response of these ecotypes to 2,4-D treatment was studied previously by Hodgson [2/] and the ranking of ecotypes found in that study is used here for comparisons.

The four year average percent survival, the stomatal area for 1967, and the relative penetration rates of the ten Canada thistle ecotypes are listed in descending order, (Table V).

The correlation coefficients for the three possible relationships between the above three characters are also given. There was no

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2/ J.M. Hodgson, "Annual Reports, Weed Control Investigations." The Crops Research Division, ARS, USDA, in cooperation with Montana Agri. Exp. Stat. and Region 6, Bureau of Reclamation, USDI, 1960 thru 1963.

significant correlation between the four year average percent survival and either the stomatal area or the 2,4-D C^{14} penetration rate. There was also no significant correlation between the rate of penetration of 2,4-D C^{14} and the stomatal area.

There seems to be very little evidence that the variation in response of the ten Canada thistle ecotypes to 2,4-D treatment was related to either the number of stomata present on the upper surface of the leaf or the rate at which 2,4-D penetrated into this surface.

Stomata did not appear to be the major portals for the entry of 2,4-D into the upper surface of Canada thistle leaves. Furthermore, the rate at which 2,4-D penetrated into the upper surface of Canada thistle leaves did not appear to be the limiting factor in determining the response of a given ecotype to treatment with 2,4-D.