Time Responses and the Susceptibility of Roadside Plants to Growth Regulation

Willard L. Koukkari
in cooperation with
Minnesota Local Road Research Board
and Minnesota Department of Transportation

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TIME RESPONSES AND THE SUSCEPTIBILITY OF ROADSIDE PLANTS TO GROWTH REGULATION

Investigation No. 649

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ABSTRACT

Time responses, particularly daily oscillations, of seven species of plants were studied. Five of the species were weeds: Ambrosia artemisiifolia L., common ragweed; Ambrosia trifida L., giant ragweed; Cirsium arvense (L.) Scop., Canada thistle; Euphorbia esula L., leafy spurge; and Taraxacum officinale Weber, common dandelion. Two were desirable as roadside ground cover: Medicago sativa L., alfalfa, and Trifolium pratense L., red clover.

Methods were developed for germinating weed seeds, a process which is often difficult to accomplish in a laboratory. A chlorophyll assay that was selected and modified for the study should be valuable in monitoring the status of injury to a variety of roadside plants.

Variations in plant populations and the lack of good statistical evidence were important factors in not being able to designate any one time of day to be consistently better for controlling weeds by 2,4-dichlorophenoxyacetic acid (2,4-D) under controlled environmental conditions.

It is possible that changes which take place throughout the day in leaf orientation could be an important factor when considering procedures for controlling roadside weeds, such as sicklepod in southern states or velvetleaf in Minnesota.

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INTRODUCTION

Many species of plants are found along the sides of roads and highways. The management and maintenance of these stands of plants is, in most cases, the responsibility of the state or local highway agency. Some of the plants are very beneficial, while others are classified as noxious weeds. Methods for controlling the growth and development of the latter species by conventional chemical methods has not always proven to be satisfactory. Numerous factors may influence the efficacy of chemical agents on plants, including the internal timing mechanisms of the organism. In the summer of 1975 a research study, sponsored by the Minnesota Local Road Research Board, was initiated to study time responses of several species of roadside plants. The material in this document is the final report of this research project.

BACKGROUND

The main emphasis of this project was on the time responses of some roadside plants, particularly responses to a chemical agent. Strong support for initiating the project was based upon previous studies with plants (Table I) and with other organisms, including laboratory animals and humans (Table II). Especially germane to this project were the numerous published reports that the effects of herbicide injury are not always constant during the course of a single day (Table III). In other words, plants represented by a variety of species were sometimes injured more by a herbicide at one time of day than at another time of day. The list of herbicides, like the list of plant species, is relatively diverse (Table III). Included in Table III are four studies conducted in three different departments at the University of Minnesota. The results of these studies supported the premise that, at least with some plants and herbicides, a time-of-day response may be present.

Furthermore, it was evident that roadside maintenance personnel and farmers often experienced inconsistencies and difficultues in controlling the growth of weed species with chemical

growth regulators. The time of application appeared to be one of the variables which should be examined further. From a research point of view, understanding more about the temporal organization of roadside plants was important. Furthermore, the results of the study could have applications to other areas of knowledge. In addition to conserving energy by eliminating repeated applications of chemicals, knowing the best time to apply a growth regulator could eventually aid in reducing the amounts of chemical agents that are introduced into the roadside environment.

For a project that is in many ways new and unique, many basic problems had to be resolved and special techniques and procedures developed. Some of the techniques and procedures that were developed should be very helpful in any future projects conducted in this area.

SPECIES OF ROADSIDE PLANTS STUDIED

Approximately a dozen species of plants were originally considered for this project. After consultation and discussions with numerous individuals, including members of the Department of Transportation, seven species were selected. The plants included in this list were Ambrosia artemisiifolia L., common ragweed; Ambrosia trifida L., giant ragweed; Cirsium arvense (L.) Scop., Canada thistle; Euphorbia esula L., leafy spurge; Medicago sativa L., alfalfa; Taraxacum officinale Weber, common dandelion; and Trifolium pratense L., red clover. Five of the species are common weeds along the sides of Minnesota roads and highways, while two of the species, alfalfa and red clover, provide a ground cover for many roadside areas.

SEED GERMINATION

Introduction

Unlike commercially packaged vegetable seeds, seeds of "weed" species often require special treatment before they will germinate. In fact, seed dormancy has been reported to be one of the major problems in being able to have weed seeds grow when and where desired (Andersen, 1968). This study was no exception. Considerable time and effort was devoted during the first two years to obtaining seeds and developing a method for germinating them successfully.

Seed dormancy is a very common phenomenon. In some species of plants, dormancy may be part of an internal type of timing mechanism that controls seed germination (cf. Sweeney, 1974). An example may be found in certain seeds that will germinate after, but not before, they receive a specified duration (days) of cold temperature. In addition to specific temperature requirements, other agents or means of increasing seed germination may include light, scarification, removing chemical inhibitors, or adding chemical stimulators (e.g., hormones). However, before the problems of dormancy could be approached, it was necessary to obtain a source of seeds.

Source of Seeds

With the possible exception of some strains of dandelion, seeds of weed species are not available from commercial companies. Even for a specific species there are often many ecotypes, and plants from one region may be quite different from those that grow elsewhere. Therefore, with the exception of alfalfa and clover, stock plants from the Minneapolis-St. Paul metropolitan area were chosen for the study. The source of seeds and the location of the stock plants are presented in Table IV.

Harvest, Storage, and Percent Germination

Common and Giant Ragweed: Seeds from both species of Ambrosia were harvested and stored in the same manner. During the autumn, generally in October, whole branches bearing "mature" seeds in the leaf axils were placed in large plastic bags (ca. 830 cm x 100 cm). The bags were kept open to allow the plants to air dry (ca. 23 degrees C) for approximately two weeks. To separate the seeds or fruits from the rest of the plant, the bags were rolled and the contents "crushed." Seeds and accompanying debris were then removed from the bottom of each bag and placed in a "South Dakota Blower" (Burrows, Evanston, Illinois) to separate the seed from the chaff.

Freshly harvested ragweed seed will not germinate, and dormancy must be broken. During the first year of the project, published techniques which involved seed stratification (cf. Andersen, 1968; Willemsen, 1975) were employed but were found to be inadequate. A new procedure, similar to one used in another laboratory (personal communications, Dr. D.B. Dickinson, University of Illinois, Urbana) was tested the following year. The technique was found to be very successful for common ragweed. Generally, more than 80% of the seeds would germinate after they were removed from cold storage. The techniques for enhancing the germination of giant ragweed seeds were not perfected to the same degree as those for common ragweed. Often only approximately 4% of the giant ragweed seeds germinated. Because the duration of the project was limited and more than a year had been spent in developing a germination method for common ragweed seeds, a special study to investigate the cause of low germination rates in giant ragweed, or to develop better techniques, was not initiated.

Table I. Examples of daily oscillations in the sensitivity of plants to chemical agents.[1]

Agent	Response	Organism Affected	Reference
Water and ions	Inhibition of flowering	Duckweed (*Lemna*)	Halaban and Hillman, 1971
Sucrose	Changes in membrane potential	*Samanea*	Racusen and Galston, 1977
Herbicide	Inhibition of growth (elongation)	Cotton (*Gossypium*)	Gosselink and Standifer, 1967
	Changes in fresh weight	Beans (*Phaseolus*)	Campiranon and Koukkari, 1976
	Mortality	Velvetleaf (*Abutilon*)	Andersen and Koukkari, 1978
	Changes in plant weight	Velvetleaf (*Abutilon*)	Koukkari and Johnson, 1979
Foliar fertilizer	Changes in seed yield	Soybean (*Glycine max*)	Ham, 1977 (Preliminary report)

[1]The response of a plant to a chemical may change in relation to the time when the chemical is applied. Some chemicals affect the parameters of a rhythm. These are two separate factors. Included in the list of chemicals that affect rhythms are alcohol, theophylline, D_2O (Bünning and Baltes, 1962; Keller, 1960; Mayer, Gruner, and Strubel, 1975), valinomycin (Bünning and Moser, 1972; Sweeney, 1974), vanillic acid (Kiessig, Herz, and Sweeney, 1979) and lithium (Englemann, 1973).

Table II. Examples of oscillations in the sensitivity of organisms to chemical agents from medicine and animal research.[1]

Organism	Chemical Agent	Reference
Mice	Ethanol	Haus and Halberg, 1959
Rats	Pentobarbitol sodium	Pauly and Scheving, 1964
Cats	Atropine	Krieger and Krieger, 1967
Flies	Pyrethrum	Sullivan *et al.*, 1970
Humans	Histamine	Reinberg *et al.*, 1969

[1]More extensive lists are available, *e.g.*, Reinberg, 1973.

Table III. Studies in which the response of plants to herbicide applications was related to the time of day that the herbicide was applied. Modified from Koukkari (1980).

Plant	Herbicide	Reference
Abutilon theophrasti Medic., velvetleaf	bentazon[2] 2,4-D[1]	Doran and Andersen, 1976 Kraatz and Andersen, 1979
Acacia farnesiana (L.) Willd., huisache	picloram[8] 2,4,5-T[9]	Bovey, Haas, and Meyer, 1972
Agropyron repens (L.) Beauv., quackgrass	paraquat[11]	Putnam and Ries, 1968
Amaranthus sp., pigweed	chloroxuron[6]	Gossett and Rieck, 1970
Cassia obtusifolia L., sicklepod	2,4-D[1]	Kraatz and Andersen, 1979
Digitaria sp., crabgrass	chloroxuron[6]	Gossett and Rieck, 1970
Glycine max (L.) Merr., soybean	chloroxuron[6]	Black and Wilson, 1969
Gossypium hirsutum L., cotton	fluometuron[3] dicryl[4] EPTC[5]	Gosselink and Standifer, 1967
Nicotiana tabacum L., tobacco	MH[12], penar[13]	Seltmann and Peedin, 1972
Opuntia polycantha Haw., prickly pear	2,4,5-T[9]	Schuster, 1970
Phaseolus vulgaris L., bean	2,4-D[1]	Campiranon and Koukkari, 1976
Pisum sativum L., pea	MCPA[10]	Weaver and Nylund, 1963
Rosa bracteata Wendl., Macartney rose	picloram[8] 2,4,5-T[9]	Bovey, Haas, and Meyer, 1972
Sorghum halepense (L.) Pers., johnsongrass	dalapon[7]	Caulder and Fletcall, 1970
Xanthium pennsylvanicum Wallr., cocklebur	bentazon[2]	Doran and Andersen, 1976

[1](2,4-dichlorophenoxy)acetic acid

[2]3-isopropyl-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide

[3]1,1-dimethyl-3-(α,α,α-trifluoro-*m*-tolyl)urea

[4]3',4'-dichloro-2-methylacrylanilide

[5]*S*-ethyldipropylthiocarbamate

[6]3-[*p*-(*p*-chlorophenoxyl)phenyl] -1,1-dimethylurea

[7]2,2-dichloropropionic acid

[8]4-amino-3,5,6-trichloropicolinic acid

[9](2,4,5-trichlorophenoxy)acetic acid

[10] [(4-chloro-*o*-tolyl)oxy] acetic acid

[11]1,1'dimethyl-4,4'-bipyridinium salt

[12] 1,2-dihydro-3,6-pyridazinedione

[13]dimethyldodecylamine acetate

Table IV. Source of seeds.

Species	Location of Stock Plants	Comments
Ambrosia artemisiifolia L.	East of Biological Sciences Center, University of Minnesota, St. Paul	Stock plants were very prolific in areas where sod had been removed.
Ambrosia trifida L.	East of Biological Sciences Center, University of Minnesota, St. Paul	Stock plants were very prolific in areas where sod had been removed.
Cirsium arvense (L.) Scop.	Location unknown; seeds from unknown source planted in greenhouse.	No environmentally controlled experiments conducted with this species.
Euphorbia esula L.	Highway 100 and 70th Street, Edina, Minnesota	Area was under road construction. A different location selected the first year was mowed before seed could be collected.
Medicago sativa L.	Location not specified, seed from commercial seed companies and Department of Agronomy and Plant Genetics, University of Minnesota.	Seed provided by Dr. D. Barnes. Cultivars recommended by Department of Transportation.
Taraxacum officinale Weber.	Near Biological Sciences Center, University of Minnesota, St. Paul.	Seed available in many areas.
Trifolium pratense L.	Location not specified, seed from commercial seed companies and Department of Agronomy and Plant Genetics, University of Minnesota.	Seed provided by Dr. D. Barnes. Cultivars recommended by Department of Transportation.

To be able to germinate sufficient quantities of weed seed and to obtain uniform seedlings are frequently major problems in weed research. Having found or developed a good technique for germinating ragweed seeds was an important contribution derived from this project. Because of this achievement, the author's laboratory has received many inquiries on how to best germinate ragweed seeds.

The following procedure was selected and developed for germinating ragweed seed in the laboratory: Freshly harvested seeds were placed on top of a layer of two moist paper towels positioned on an aluminum foil-lined tray (35.5 cm x 46 cm). A layer of two moist paper towels was placed over the seeds and the top of the tray was covered with a plastic wrap (Saran Wrap). The tray containing the seeds was then stored at 3 degrees C for at least three months. The minimum number of days in cold storage necessary to break dormancy was not examined. According to other reports (Willemsen, 1975), 12 to 15 weeks' low temperature may be required to overcome the dormancy of common ragweed seeds.

Since approximately 80% of the common ragweed seeds would germinate, the seeds were planted directly in soil. However, because of low germination rates, the giant ragweed seeds from cold storage were first placed on top of moist filter paper over a 3- to 5-mm layer of damp vermiculite in a Petri dish. The dishes were maintained in the laboratory for approximately three days, after which the young seedlings were transplanted into soil.

Dandelion: Seeds of common dandelion were collected from May to October. In harvesting, the "fluff-ball" (mature achenes and pappi) was held with one hand over a 50-ml plastic bottle and the achenes (small fruits which contain the actual seeds) were separated from the pappi by rubbing the thumb of the other hand over the bottle's rim. The achenes were then transferred from the bottle to small paper envelopes (7.8 cm x 14 cm) and stored at 3 degrees C until used in an experiment. Although the germination rate

was generally near 45% (46% in an actual test), the seeds were first placed on moist filter paper as already described for giant ragweed. The seedlings were transplanted later into soil.

Leafy spurge: Fruits of Euphorbia, called capsules, were harvested during July and August. The capsules were picked by hand from mature plants and placed in 50-ml plastic bottles. The capsules of leafy spurge are trilocular with each locule containing one seed. The mature fruits are tan. While studying how to best separate seeds from the fruit, it was observed that when fruits were placed in a glass dish exposed to sunlight, some fruits would "explode" along a dehiscence line. Some of the seeds were observed to travel approximately 1.5 m. Based upon the results of these studies, a simple procedure was developed. The capsules were placed in a covered Petri dish and exposed to sunlight. Within two days, practically all the seeds were outside the fruit. A blower was used to separate the seed from the fruit walls. The seeds were then stored in paper envelopes at 3 degrees C until they were planted directly in soil. Generally, the germination rate was very good and comparable to that of common ragweed.

Alfalfa, Red Clover, and Canada Thistle: Seeds of these species were obtained from various sources, including the University of Minnesota Department of Agronomy and Plant Genetics, and no special techniques were developed for harvesting or storing them in this project. The seeds were planted directly in soil and the germination of both legume species was excellent. Except for one preliminary experiment, no environmentally controlled experiments with Canada Thistle were attempted. Results from the preliminary experiment indicated that a great deal of research on how to grow thistle plants, representative of those near roadsides, would have to be completed before thistles could be included in a project.

GENERAL MATERIALS AND METHODS

Plant Material and Environmental Conditions

Seeds, obtained from storage, were planted in 200-cc plastic cups containing soil. In the case of giant ragweed and common dandelion, the seeds were first germinated on filter paper and then transplanted. At the time of treatment, each cup contained a single plant that possessed two to six leaves (Figure 1). For most of the experiment, plants were grown at 25 degrees C in growth chambers (Figure 2) under a LD 15:9 regime, with the 15-hour light span (L) supplied by 16 fluorescent lamps (F72T12-CW-VHO, Sylvania), supplemented with 12 60-watt incandescent lamps (GE Code-60A21/TS Traffic Signal Type), and providing approximately 18,360 lux near plant height. Relative humidity (RH) was approximately 50% in the chambers. In one of the experiments in which the effects of low and high moisture levels were examined, the RH was either increased to 80% or reduced to near 27% three days before the treatment. Under the latter condition (27% RH), the plants were not watered for approximately 40 hours prior to the first application of 2,4-dichlorophenoxyacetic acid (2,4-D) or during the 24-hour treatment cycle. Otherwise, all plants were generally watered each day. In some of the earlier experiments, RH was not controlled and environmental conditions were similar to those reported elsewhere (Campiranon and Koukkari, 1976).

It should be emphasized that the scope of the project was very broad in that many species were included. However, more emphasis was placed on common ragweed. Approximately 17 experiments with this one species focused on time-of-day effects and another 31 experiments focused on special aspects relating to these effects. If one assumes that each experiment requires six weeks, then theoretically the 17 common ragweed experiments alone could account for more than two years of research time. For some of the special experiments (e.g., developing assay techniques) leaves from plants grown under natural, out-of-door conditions and leaves from older plants were used.

Herbicide Applications

The herbicide was applied to the leaves in a series of drops (Figure 3) with most of the drops over the midvein and a few drops on some of the other large veins. The applicator used was a micropipette (Drummond Microdispenser, the Drummond Scientific Co., Philadelphia, Pennsylvania).

A solution of 2,4-D (formulated as the alkanolamine salts--ethanol and isopropanol series of 2,4-dichlorophenoxyacetic acid, product of the Dow Chemical Company, Midland, Michigan) was prepared by diluting 10 to 75 µl of the commercial formulation (4 lbs 2,4-D acid equivalent per gallon) with distilled water to 10 ml. During the course of 24 hours, 2,4-D was applied every three or four hours to a new group of 16 to 20 plants arranged in a randomized block or Latin Square design. Five µl was applied on one to six leaves of each plant. The number of leaves treated depended upon the species and size of the seedlings, but was constant for any given experiment. Manipulations during darkness were conducted under dim green light (Campiranon and Koukkari, 1976).

Figure 1. Photograph of the six species of plants studied, at approximately the same size or stage of development as selected for most of the experiments. Left to right: common ragweed, giant ragweed, leafy spurge, common dandelion, red clover, and alfalfa.

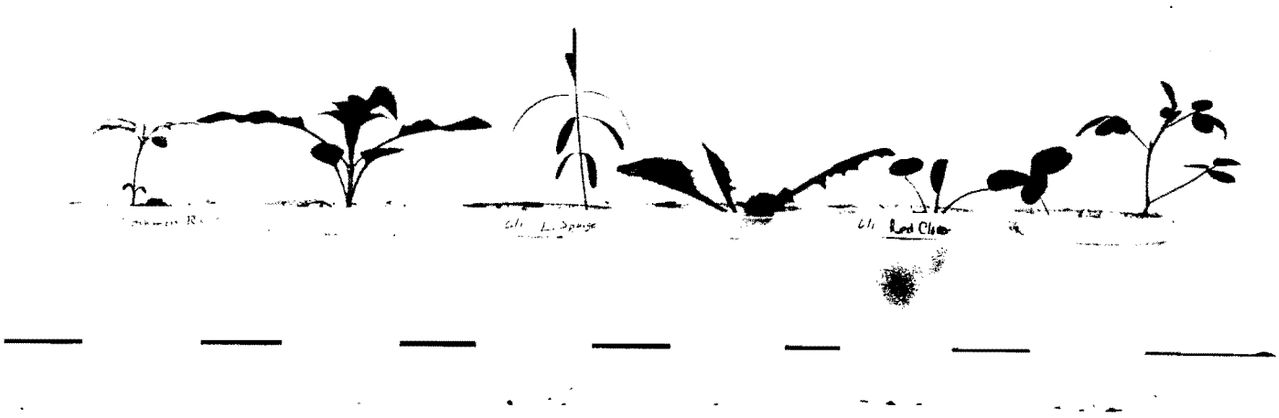


Figure 2. Young leafy spurge plants maintained in a growth chamber photographed about three days before treatment and prior to being thinned to one plant per cup.

Figure 3. Photograph illustrating the quantitative method for applying solutions of 2,4-D with a micropipet to precise locations on ragweed leaves.

Field Experiments

Two experiments were conducted under actual roadside conditions. One included an area that had an abundant population of dandelion; the other was an area of dense thistles.

The dandelion site was the median of an interstate highway. The experiment was arranged in randomized blocks. Five treatments (times of application) were included in each of three blocks. The areas treated were 15.2 m by 2.9 m. with 30.5 m separating each area. This arrangement accounted for the sprayer or boom (with a width of 2.9 m) and the space necessary for the equipment operator to prepare, adjust, and standardize the spray before reaching the area to be treated. The five treatment times were 0500, 0900, 1200, 1500 and 2100 hours. Plants were sprayed during early spring by Department of Transportation personnel according to their routine maintenance procedures and 2,4-D formulations.

Method of evaluating the effect of treatment consisted of counting the number of plants killed in two 25 cm x 1 m regions of each treated area. Eight days after treatment, the total plant population (live and dead plants) was counted in the areas bounded by a metal frame (25 cm x 1 m). Before the frame was removed, marker stakes were placed at opposite corners of the frame. One month later, the frame was replaced in the area between the stakes and the number of surviving plants were recorded.

The thistle site was located north of St. Paul and was arranged in a randomized block experimental design. The treated areas were 3.0 m x 2.4 m. As with the dandelion experiment, the spraying was conducted by personnel from the Department of Transportation according to their routine procedures.

Due to the terrain and plant population, the effects were rated visually by both Department of Transportation personnel and two members of the author's laboratory.

ASSAYS

Plant injury caused by the herbicide was evaluated approximately 14 days after application. Criteria selected for evaluating the extent of injury included visual injury rating, fresh weight, dry weight, chlorophyll, and protein levels.

Rating and weight

Treated plants were rated according to an injury index of 1 to 5 (with 5 being the most severely injured), based on visual observations. For fresh weight determinations, the plant was excised directly above the cotyledons and the fresh weight measured on either a digital electronic balance (Arbor 206, Arbor Laboratories Inc., Palo Alto, California) with a thermal printer (Datel Systems Inc., Mansfield, Massachusetts, model DPP07), or an analytical balance (Mettler H10, Mettler Instrument Corp., Princeton, New Jersey). In some experiments the plant material was placed in paper envelopes and air dried for approximately six to seven days, then weighed again.

Chlorophyll and Protein Assays

The chlorophyll assay was based on the procedure described by Knudson, Tibbetts, and Edwards (1977) but was modified to be more applicable for small quantities of tissue and large numbers of samples. Excised plant material (e.g., a cotyledon) was placed in a 12 mm x 75 mm tube containing 4 ml of 95% ethanol. The tube, hereafter referred to as the "extraction tube," was tightly capped and kept in darkness at approximately 23 degrees C for 24 hours. The liquid contents were decanted into a screw cap glass tube (20 mm x 150 mm). This glass tube served as the collection tube for all the chlorophyll extracted from a given sample of plant material. Two ml of 95% ethanol were added to the extraction tube, containing the cotyledon, with a syringe (B-D Cornwall Continuous Pipetting Outfit, Becton, Dickinson and Company, Rutherford, New Jersey) to rinse the plant material. The rinse solution was decanted into the collection tube. Four ml of 95% ethanol were added to the extraction tube. Both the collection tube and the extraction tube containing the plant material were placed in darkness for approximately 24 hours. The decanting and rinsing process was repeated and 4 ml of ethanol was again added to the extraction tube. After another approximate 24-hour span in darkness, the decanting and rinsing procedure was repeated. The combined 18-ml volume was mixed by gently swirling the tube. The absorbance (A) of the chlorophyll-ethanol extract was measured at 649 nm and 665 nm with a Beckman model 25 spectrophotometer (Beckman Instruments Inc., Palo Alto, California). The plant tissue from which the chlorophyll had been extracted was placed in a paper envelope (64 mm x 110 mm), air dried for one to two hours and then oven dried (70 degrees C) for three days. The dry weight of the plant tissue was measured on an analytical balance (Mettler H10). The amounts of chlorophyll a and chlorophyll b were determined by the equations (Knudson, Tibbetts, and Edwards, 1977):

$$\frac{\mu g \text{ Chl a}}{1 \text{ ml soln}} = (13.70)\ (A_{665\ nm}) - (5.76)(A_{649nm})$$

Equations Reference: Wintermans & Demots 1965 Biochim. Biophys. Acta 109: 448-453.

$$\frac{\mu g \text{ Chl b}}{1 \text{ ml soln}} = (25.80)(A_{649\ nm}) - (7.60)(A_{665nm})$$

The sum was then expressed as μg chlorophyll/mg dry weight.

The procedure described above includes a more dilute concentration of ethanol (95%) and much smaller volumes than reported elsewhere (Knudson, Tibbetts, and Edwards, 1977). Extracting with ethanol was preferred to the more classical acetone method (Arnon, 1949) because it is much easier and safer to work with ethanol in the laboratory. Furthermore, the extraction procedure appeared to be very successful. The modification of this assay and the demonstration of this analytical technique was an important contribution of the project and one that has already been adopted in other laboratories.

Several assay techniques were tried, including fluorescent procedures. The protein assay finally selected was that of Lowry et al. (1951). Procedures for preparing the material for assay from all three organs (leaf, stem, root) were similar. However, the roots were first washed once in tap water, then rinsed in distilled water and blotted between paper towels. The freshly harvested material was weighed, placed between paper towelling, covered with aluminum foil and frozen. Samples were thawed and the plant material placed in an extraction medium (1 gm per 9 ml of 0.0 M phosphate buffer, pH 7.0, 0.5 M NaCl) and homogenized for two minutes (Virtis homogenizer, The Virtis Company, Gardiner, New York). Generally 6 mM dithiothreitol was included in the extraction medium. Homogenates were centrifuged (e.g., 20,000 x g) for 15 minutes. Sometimes an additional 15 minutes was required to form a good pellet. The supernatant was collected and frozen until assayed. For the assays each sample was thawed, mixed and 0.45 ml transferred to a 12 mm x 75 mm tube. Proteins were precipitated with 50 μl of 50% trichloroacetic acid (5% TCA in final volume) and the samples were centrifuged at 7000 x g for 15 minutes. After removal of the supernatant, the pellet was washed and resuspended in 1 ml of 1% TCA and again centrifuged at 7000 x g for 15 minutes. The supernatant was again discarded and the protein pellet dissolved in 0.1 N NaOH and assayed by the method of Lowry et al. (1951).

STATISTICAL METHODS

Three methods were selected for the statistical analyses of the data: the analysis of variance and the f test, the Newman-Kuels test (cf. Snedecor and Cochran, 1967), and least squares fitting of cosine functions (Halberg, Tong, and Johnson, 1967). The latter cosinor method was utilized to determine the probability of cosine function periods (τ) of approximately 24-hour oscillations. These computerized procedures for analyzing oscillations were similar to those described in greater detail elsewhere (Koukkari, Halberg, and Gordon, 1973).

RESULTS AND DISCUSSION

General

The effects of 2,4-D on plants depend upon many factors, including the concentration and formulation (e.g., salt) of the chemical, weather (dew), stage of plant development, and species. This section of the report describes results from experiments in which the potential role of another factor, time of day, was studied. Stated more succinctly, the experiments were designed to address the matter of whether certain plants were more sensitive to 2,4-D at one time of day than they were at another time of day.

The morphological characteristics (shape or form) of the species selected for the project (Table IV) were distinctly different from each other. Because of morphological or anatomical features, and the effects of 2,4-D on the plant, several different assays for evaluating plant injury were utilized for this project. These characteristics or properties of both plant and herbicide account for the extended time and effort devoted to developing, testing, and selecting the best assay for each species. In most "weed control" studies, the entire plant is sprayed with a concentration of herbicide that will kill the plant. However, for the laboratory studies discussed in this section, exact amounts of 2,4-D were applied to precise locations on the leaf (Figure 3). Furthermore, the objective was not to completely destroy all tissues, but rather to monitor levels of injury produced by sublethal concentrations.

Results of several experiments with six species of roadside plants are presented in Tables VA and VB. Based on a rating scale of 1 to 5, with 1 representing no injury and 5 the most serious injury, pronounced differences were observed between treated and untreated plants. However, differences between the mean fresh weights of untreated and all of the treated groups were not always significant. Generally, the last group of plants treated was the heaviest. Such results can be predicted since the plants had more time to grow and develop before treatment.

Common Ragweed and Giant Ragweed

Results from one of the common ragweed experiments showed that plants treated at 1300 hours were the most severely injured (lowest FW).

Table V. Summary of representative experiments with: A. common ragweed (CR), giant ragweed (GR), and leafy spurge (LS); B. common dandelion (CD), red clover (RC), and alfalfa (A), illustrating the effects of 2,4-D when applied at various times as assayed by fresh weight (FW), fresh weight of roots (FWr), fresh weight of cotyledons (FWc), rating (R), chlorophyll level of cotyledons (Chl), protein levels of cotyledons (Pro), dry weight (DW), rating of cotyledons (Rc), rating of new growth (Rn), fresh weight of new growth (FWn), dry weight of new growth (DWn), and number of new leaves (Nnl). FW and DW expressed in mg; Chl in µg/mg DW; and Pro as µg/ml. Values are expressed as the mean ± standard error of the mean.

A. Common ragweed, giant ragweed and leafy spurge.

		Time of Treatment						
Exp.	Assay	0900	1300	1700	2100	0100	0500	Untreated
CR a	FW	969.45 ±40.90	773.80 ±52.36	981.40 ±76.13	908.70 ±59.90	1018.95 ±48.35	1082.65 ±47.29	1094.95 ±51.42
b	FW	263.0 ±49.8	271.6 ±59.8	320.1 ±63.5	336.6 ±63.9	465.1 ±75.3	555.5 ±78.0	1355.8 ±78.9
	FWr	53.8 ±13.8	81.6 ±26.5	95.1 ±19.7	164.2 ±58.8	229.2 ±75.3	337.7 ±88.1	2202.4 ±103.9
c	FWc	8.0 ±1.5	9.3 ±1.5	8.4 ±1.7	9.0 ±1.5	7.6 ±1.8	8.2 ±1.5	11.1 ±1.0
	R	3.35 ±0.18	3.10 ±0.18	3.20 ±0.17	3.10 ±0.12	3.20 ±0.21	2.85 ±0.17	1.00 ±0.00
	FW	547.6 ±51.9	544.2 ±52.1	524.0 ±54.8	634.2 ±57.8	605.9 ±63.0	663.0 ±52.8	805.4 ±71.6
	Chl	118.370 ±18.867	182.690 ±16.660	128.008 ±19.085	163.218 ±39.116	95.297 ±19.207	107.732 ±19.135	153.073 ±20.069
	Pro	55.40 ±3.75	53.36 ±5.24	54.22 ±4.75	59.57 ±5.20	58.22 ±6.00	44.32 ±1.51	82.46 ±3.17
GR	R	3.71 ±0.21	3.82 ±0.21	3.47 ±0.21	4.06 ±0.21	3.76 ±0.22	3.65 ±0.21	1.00 ±0.00
	FW	886.70 ±140.21	735.53 ±103.67	1218.35 ±188.94	876.35 ±146.68	982.06 ±167.40	1083.00 ±153.33	1960.88 ±98.55
	DW	163.82 ±13.37	166.47 ±12.56	207.47 ±21.91	180.18 ±16.31	189.12 ±19.71	181.59 ±15.79	470.88 ±26.16
LS	Rc	2.25 ±0.18	2.40 ±0.22	2.25 ±0.25	2.00 ±0.00	2.30 ±0.22	2.45 ±0.24	4.80 ±0.37
	R	3.05 ±0.11	3.35 ±0.11	3.10 ±0.07	3.05 ±0.09	3.10 ±0.07	3.10 ±0.07	1.00 ±0.00
	FW	168.90 ±10.15	150.60 ±12.52	182.50 ±11.94	172.90 ±11.52	174.70 ±12.60	205.20 ±13.99	497.60 ±31.22

B. Common dandelion, red clover, and alfalfa.

Exp.	Assay	Time of Treatment						
		0900	1300	1700	2100	0100	0500	Untreated
CD a	Rn	2.75 ±0.11	2.88 ±0.15	3.00 ±0.16	2.94 ±0.06	2.88 ±0.09	2.81 ±0.10	1.00 ±0.00
	FWn	1087.19 ±99.40	1047.31 ±119.86	898.50 ±93.78	1205.69 ±87.85	1286.88 ±142.16	1195.94 ±100.66	1399.00 ±135.90
	DWn	190.94 ±17.05	188.31 ±22.62	159.06 ±15.33	210.19 ±15.34	225.81 ±24.41	211.81 ±18.08	262.56 ±25.69
b	Rn	3.28 ±0.11	3.44 ±0.12	3.28 ±0.11	3.39 ±0.14	3.39 ±0.12	3.22 ±0.10	1.00 ±0.00
	Nnl	5.28 ±0.28	5.22 ±0.17	4.64 ±0.15	5.44 ±0.31	5.28 ±0.22	5.47 ±0.25	4.86 ±0.19
	FWn	1075.67 ±60.75	1021.22 ±55.41	1061.89 ±67.15	1258.56 ±127.16	1194.67 ±79.96	1294.56 ±100.05	1408.56 ±104.73
	DWn	151.17 ±8.38	143.61 ±8.06	154.22 ±9.36	190.56 ±19.38	171.78 ±11.97	201.33 ±14.88	253.44 ±14.01
RC	Rn	2.61 ±0.17	2.80 ±0.25	2.50 ±0.15	2.95 ±0.18	3.05 ±0.14	2.40 ±0.18	1.00 ±0.00
	Nnl	5.50 ±0.32	6.35 ±0.43	5.20 ±0.30	6.00 ±0.44	6.25 ±0.28	6.35 ±0.50	6.15 ±0.38
	FWn	1179.05 ±65.88	1220.20 ±61.61	1176.05 ±113.90	1291.30 ±78.21	1360.70 ±77.13	1612.75 ±75.99	1695.45 ±64.50
	DWn	149.85 ±8.26	150.15 ±7.85	151.80 ±14.83	171.20 ±11.79	177.30 ±10.04	221.05 ±13.59	285.80 ±10.64
A	Rn	3.67 ±0.18	3.33 ±0.16	3.61 ±0.16	3.28 ±0.11	3.22 ±0.15	2.89 ±0.11	1.00 ±0.00
	Nnl	1.44 ±0.12	1.72 ±0.16	1.61 ±0.16	2.11 ±0.23	2.39 ±0.35	3.39 ±0.50	19.94 ±1.14
	FWn	57.50 ±7.74	80.89 ±14.68	79.56 ±12.58	108.83 ±15.26	130.00 ±27.66	196.89 ±32.46	1159.78 ±19.94
	DWn	13.28 ±2.05	17.94 ±3.22	18.56 ±2.72	20.50 ±3.02	26.78 ±5.58	34.33 ±5.32	244.39 ±8.85

Statistical analysis of this data indicated that no significant differences were present among any of the other treatments, including the untreated plants. The fresh weights of the plants treated at 1300 hours were significantly less than all other groups except those treated at 2100 hours. A more appropriate statistical method for analyzing data such as this involves curve fitting (Halberg, Tong, and Johnson, 1967; Koukkari, Halberg, and Gordon, 1973). When

analyzed by the least squares cosinor method (see General Materials and Methods), a significant 24-hour oscillation with a probability (P value) of 0.01 was observed.

For the other ragweed experiments that were analyzed by the cosinor method, the P values for a 24-hour fit of the cosine function ranged from 0.17 to 0.24 and were not considered to be significant (e.g., Table VI).

There appears to be a relationship between the extent of herbicide injury to primary leaves and the senescence of cotyledons (Koukkari and Johnson, 1979). When the leaves of many species are injured, the cotyledons remain on the plant longer than they would if the plants had not been injured. The chlorophyll content of the cotyledons also follows the same trend: more injury, more chlorophyll. Even though the data were not statistically significant, the chlorophyll levels of the cotyledons from plants treated at 1300 hours in Experiment C, Table VA, were higher.

Common Dandelion

Although no statistically significant differences existed in fresh weight between groups of common dandelion plants treated at various times of day, only the 1700-hour (Exp. a) and 1300-hour (Exp. b) groups were significantly different from the untreated controls. Visual inspection of the rating results (Table VB) indicated a similar trend.

Results from a roadside experiment in which dandelion plants were sprayed by a Department of Transportation maintenance crew are presented in Table VII. Although the mean number of plants destroyed at 9:00 a.m. was higher than at any other time, no statistically significant differences between treatment times were observed.

Leafy Spurge

Experiments with leafy spurge were delayed because of a technical problem in first collecting the seeds, and later in obtaining sufficient numbers of uniform plants. Both problems have now been resolved, and even though the project was completed, the experiments with leafy spurge were continued in the laboratory. Current indications are that there are no significant statistical differences among treatment times.

Alfalfa and Red Clover

Alfalfa appeared to be very sensitive to 2,4-D. The alfalfa and clover plants were very young and grew quite rapidly. This was evident during the 24-hour span that they were subjected to the herbicide treatments. The later the time of treatment, the greater the weight of the plant material (Table VB). This linear relationship, which is due to rapid growth and development rather than any implied oscillation, may account for the general trends observed in this study. However, it is possible that with both species there could be daily oscillations in injury when plants are subjected to herbicidal sprays (described in the next section).

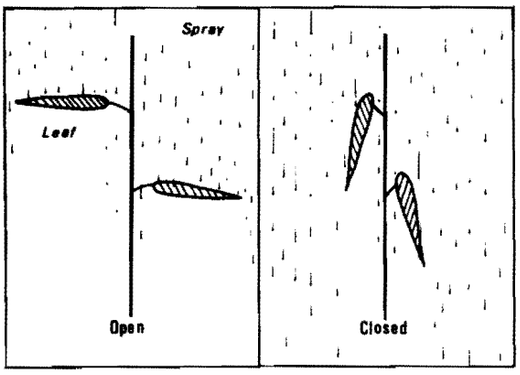
Orientation of Leaves

The orientation of many common weeds such as Abutilon theophrasti Medic., velvetleaf (Andersen and Koukkari, 1978); Amaranthus retroflexus L., redroot pigweed; Cassia obtusifolia L., sicklepod; Cassia occidentalis L., coffee senna; Chenopodium album L., common lambsquarters; Datura stramonium L., jimsonweed; Solanum nigrum L., black nightshade; and Xanthium pennsylvanicum Wallr., common cocklebur, changes dramatically throughout the day (Andersen and Koukkari, 1979). Results of experiments with velvetleaf have shown that the time of day when bentazon was applied influenced the effectiveness of the herbicide treatment (Doran and Andersen, 1976). It has been reported that there is a relationship among all three patterns, leaf orientation, spray retention, and the percent control of velvetleaf by bentazon (Andersen and Koukkari, 1978). More recently, significant time-of-day effects between leaf movement and 2,4-D efficacy have also been observed (Kraatz and Andersen, 1979). These results strongly suggest that although other factors may be involved (Koukkari and Johnson, 1979), rhythmic changes in leaf orientation responsible for changes in spray retention or interception could be a major cause of the time of day effect in the response of plants to herbicides.

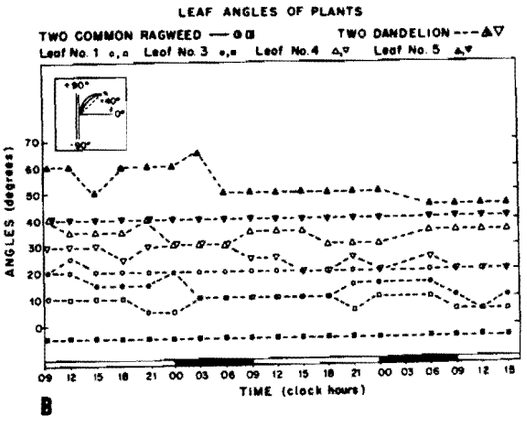
Since many plants display rhythmic leaf movements (Andersen and Koukkari, 1979; Bünning, 1973; Cumming and Wagner, 1968), and such movements could influence herbicidal efficacy (Andersen and Koukkari, 1978) in the manner illustrated in Figure 4A, leaf orientation of the six "roadside" species was also monitored. Very little, if any, daily change in the orientation of common ragweed and common dandelion leaves was observed (Figure 4B). Slight changes were observed for some leaves of giant ragweed and leafy spurge (Figure 4C). The most pronounced changes in leaf orientation displayed were of the leaflets of alfalfa and clover. Circadian oscillations of legume leaves, including those of clover (Scott and Gulline, 1972), are very common.

Whether or not changes in leaf orientation of roadside plants could affect the extent of control by 2,4-D, especially in legumes, awaits further study. In the case of velvetleaf, it has been recently reported that a slight, but

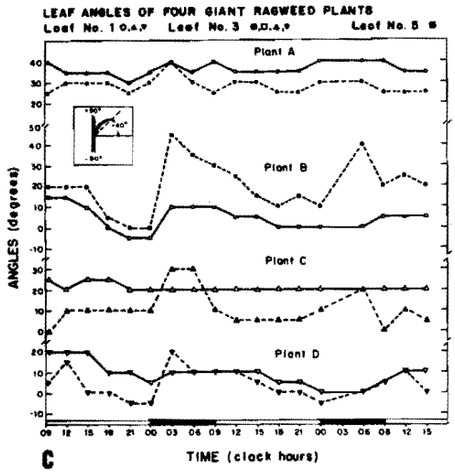
Figure 4. Leaf orientation: (A) diagram illustrating spray efficacy; and (B-F) of six species of roadside plants.



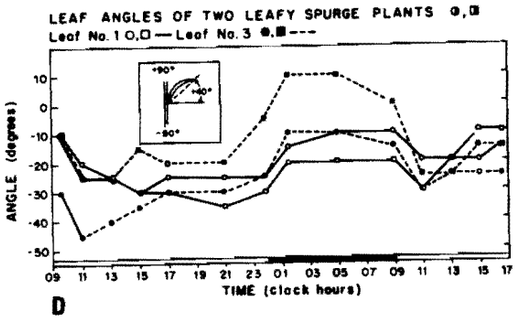
A



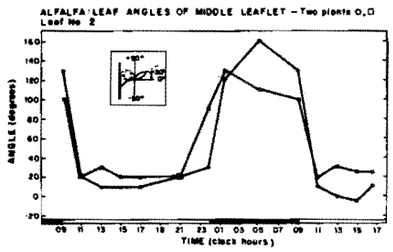
B

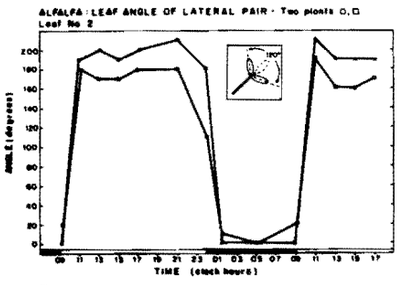


C

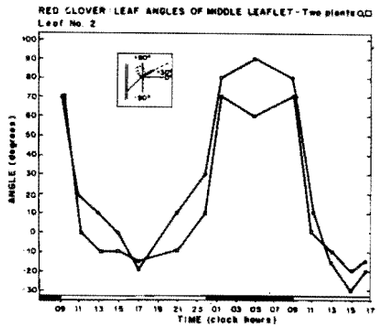


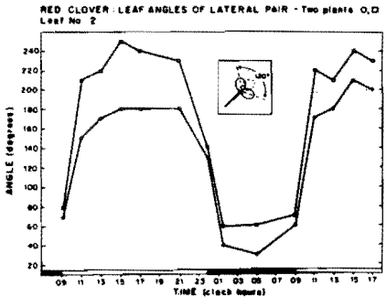
D





E





F

Table VI. Oscillation parameters in the sensitivity of young common ragweed plants to 2,4-D. Freshweight data analyses by the least squares method of cosine function periods.

	Period = 24 hrs.				Best period between 10-hour periods and 30-hour periods		
Exp.	P	Amplitude and standard deviation	Acrophase[1]	95% confidence limits	Period	P	Amplitude and standard deviation
a	.011	100.067 ±32.700	-43°	-6° to -8°	10 hours	.006	109.706 ±33.821
b	.174	60.101 ±31.872	-30°	0° to 0°	30 hours	.140	59.223 ±30.130

[1]Indicates crest time of cosine function for period of 24 hours, 1 hour = 15 degrees. Therefore, -30 degrees indicates 0200 hours.

Table VII. Mean percent ± the standard error of the mean of common dandelion plants destroyed by 2,4-D in a roadside experiment. Conditions as described under General Material and Methods.

	Time of Treatment				
	0500	0900	1200	1500	2100
% Kill	84.53 ±9.83	99.49 ±0.36	83.60 ±5.40	87.35 ±4.74	72.33 ±11.49

statistically significant, decrease in the percent control resulted from 2,4-D treatments in late evening, at night, and in early morning (Kraatz and Andersen, 1979). Results from experiments with sicklepod were similar, although the differences were more pronounced and suggest that changes in projected leaf area may be a factor in the efficacy of post-emergence herbicide treatments (Kraatz and Andersen, 1979).

FINDINGS AND CONCLUSIONS

1. Laboratory procedures were developed for germinating seeds of many roadside plants. Particularly important was the method devised for common ragweed. It was possible to increase the percent of seeds that germinated from less than 5 percent to approximately 80 percent.

2. A new chlorophyll assay was selected and modified for assaying large numbers of samples. The method (Knudson, Tibbitts, and Edwards, 1977) should be valuable in monitoring the status of roadside plants for a variety of projects.

3. Variations in plant populations and the lack of good statistical evidence were important factors in not being able to designate any one time of day to be consistently better for controlling weeds by 2,4-D under controlled environmental conditions.

4. Environmental factors, such as dew, temperature, wind, and stage of plant development, are perhaps of greater importance than time of day in influencing the amount of injury caused by 2,4-D.

5. It is possible that changes which take place throughout the day in leaf orientation could be an important factor when considering procedures for controlling roadside weeds, such as sicklepod in southern states, or velvetleaf in Minnesota.

6. Studies on the effects of chemical and mechanical agents in plants are being continued at the University of Minnesota. Results of these studies, especially those that may have implications to the management of roadside plants, will be published within the next two years.

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