KOSTEWICZ, Stephen Raymond, 1940-
THE EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID
ON THE ENDOGENOUS LEVELS OF GIBBERELLINS IN THE
ROOT-HYPOCOTYL TISSUES OF GERMINATING PHASEOLUS
VULGARIS SEEDLINGS.

The Ohio State University, Ph.D., 1970
Agriculture, plant culture

University Microfilms, A XEROX Company, Ann Arbor, Michigan
THE EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON THE ENDOGENOUS LEVELS
OF GIBBERELLINS IN THE ROOT-HYPOCOTYL TISSUES OF GERMINATING
PHASEOLUS VULGARIS SEEDLINGS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Stephen Raymond Kostewicz, B.S., M.S.

**********

The Ohio State University
1970

Approved by

E. K. Allan
Adviser
Department of Horticulture
ACKNOWLEDGMENTS

The author wishes to express his appreciation to Dr. E. K. Alban for his guidance and counsel while completing his course of study.

Appreciation is extended to Dr. D. W. Kretchman and Dr. P. C. Kozel for serving on the reading committee. He is especially appreciative of the guidance Dr. Kozel rendered in portions of this work.

Gratitude is extended to the Department of Horticulture for the graduate assistantship and for the use of its physical and financial facilities.

Special appreciation is given to my wife, Mary Beth, for her encouragement, work, and many sacrifices endured during the periods of graduate study.
VITA

June 13, 1940 . . . Born--Canton, Ohio

1963 . . . . . B.S. in Biology, Kent State University, Kent, Ohio

1963-1965 . . . . Teaching Assistant, Department of Horticulture, The Ohio State University, Columbus, Ohio

1965 . . . . . M.S., The Ohio State University, Columbus, Ohio

1965-1969 . . . . Teaching Associate, Department of Horticulture, The Ohio State University, Columbus, Ohio

1969-1970 . . . . Research Associate, Department of Horticulture, The Ohio State University, Columbus, Ohio
# CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENT</td>
<td>ii</td>
</tr>
<tr>
<td>VITA</td>
<td>iii</td>
</tr>
<tr>
<td>TABLES</td>
<td>v</td>
</tr>
<tr>
<td>FIGURES</td>
<td>vi</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>3</td>
</tr>
<tr>
<td>The response of plants to the herbicide 2,4-D</td>
<td>3</td>
</tr>
<tr>
<td>The theoretical modes of action of the growth regulating herbicides</td>
<td>9</td>
</tr>
<tr>
<td>The germination process</td>
<td>11</td>
</tr>
<tr>
<td>Germination and plant hormones</td>
<td>14</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>17</td>
</tr>
<tr>
<td>Treatment and harvest</td>
<td>17</td>
</tr>
<tr>
<td>Extraction of gibberellins</td>
<td>18</td>
</tr>
<tr>
<td>Fractionation of the gibberellins</td>
<td>20</td>
</tr>
<tr>
<td>Bioassay and thin layer techniques</td>
<td>24</td>
</tr>
<tr>
<td>Safeguards</td>
<td>30</td>
</tr>
<tr>
<td>PRESENTATION OF RESULTS</td>
<td>31</td>
</tr>
<tr>
<td>The control treatment</td>
<td>32</td>
</tr>
<tr>
<td>2,4-D treatment</td>
<td>36</td>
</tr>
<tr>
<td>Gibberellin treatments</td>
<td>36</td>
</tr>
<tr>
<td>2,4-D plus gibberellin treatments</td>
<td>39</td>
</tr>
<tr>
<td>DISCUSSION OF RESULTS</td>
<td>52</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>57</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>58</td>
</tr>
<tr>
<td>Table</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
</tr>
<tr>
<td>1</td>
<td>The step-wise elution pattern for gibberellins $A_{1-9}$ from silica gel partition columns</td>
</tr>
<tr>
<td>2</td>
<td>Physical data of the harvested root-hypocotyl tissues of <em>Phaseolus vulgaris</em> seeds germinated in various solutions at the time of harvest</td>
</tr>
</tbody>
</table>
### FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td>2</td>
<td>23</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
</tr>
<tr>
<td>4</td>
<td>33</td>
</tr>
<tr>
<td>5</td>
<td>37</td>
</tr>
<tr>
<td>6</td>
<td>40</td>
</tr>
<tr>
<td>7</td>
<td>42</td>
</tr>
<tr>
<td>8</td>
<td>45</td>
</tr>
</tbody>
</table>

1. Extraction and fractionation procedure for the separation of gibberellins from the treated root-hypocotyl tissue of germinating *Phaseolus vulgaris* seeds.
2. Elution gradient curve for the ethyl acetate-n-hexane solvent system used to fractionate gibberellins A₁₋₉ from silica gel partition columns.
3. The emission spectrum of Sylvania warm white "Lifeline" fluorescent tubes as measured at a 30 cm distance.
4. Gibberellin activity in the fractionated extract of root-hypocotyl tissue of germinating *Phaseolus vulgaris* seedlings in the control treatment as measured by the lettuce hypocotyl bioassay.
5. Gibberellin activity in the fractionated extract of root-hypocotyl tissue of germinating *Phaseolus vulgaris* seedlings in a 1 ppm 2,4-D solution as measured by the lettuce hypocotyl bioassay.
6. Gibberellin activity in the fractionated extract of root-hypocotyl tissue of germinating *Phaseolus vulgaris* seedlings in a 1 ppm gibberellin A₇ solution as measured by the lettuce hypocotyl bioassay.
7. Gibberellin activity in the fractionated extract of root-hypocotyl tissue of germinating *Phaseolus vulgaris* seedlings in a 5 ppm gibberellin A₇ solution as measured by the lettuce hypocotyl bioassay.
8. Gibberellin activity in the fractionated extract of root-hypocotyl tissue of germinating *Phaseolus vulgaris* seedlings in a 1 ppm 2,4-D plus 1 ppm gibberellin A₇ solution as measured by the lettuce hypocotyl bioassay.
FIGURES (continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Gibberellin activity in the fractionated extract of root-hypocotyl tissue of germinating <em>Phaseolus vulgaris</em> seedlings in a 1 ppm 2,4-D plus 5 ppm gibberellin A₇ (5-day) solution as measured by the lettuce hypocotyl bioassay</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td></td>
<td>47</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>49</td>
</tr>
</tbody>
</table>
INTRODUCTION

Plants treated with the herbicide 2,4-D (2,4-dichlorophenoxy-acetic acid) have demonstrated tissue proliferations of aerial stem and hypocotyl regions. This morphological response has become an advanced "symptom" of plants treated with phenoxy herbicides. The literature shows that much work has been done to determine which tissues have proliferated or are affected by 2,4-D treatment and tended to be a popular avenue of early 2,4-D research. Work along this line has stimulated several researchers to offer the explanation that the proliferation is due to a stimulation of a reversion to a meristematic condition of certain "mature" tissues resulting in a proliferation of tissues.

Our preliminary investigations had shown that snap bean (Phaseolus vulgaris L.) cv. Contender seeds germinated in varied concentrations of 2,4-D resulted in swollen root-hypocotyls of the seedlings due to a proliferation of tissues. The hypocotyls continued to swell in diameter quite outstandingly while the root tips became discolored and appeared to be non-functional. If the seedlings were kept in the 2,4-D solutions, they died without the appearance of the epicotyls.

It was observed that concurrent application of gibberellin A7 to the treatment solution of 2,4-D resulted in a degree of prevention of the swelling. In place of a swollen hypocotyl a moderately elongated root-hypocotyl tissue appeared.
Gibberellins have been shown to be active in the elongation mechanism of hypocotyl and stem tissues of "older" seedlings for various species of plants. Relatively little work has been reported dealing with the gibberellins of developing embryos during germination or more specifically the emerging root-hypocotyl portion of the seedling.

It has been postulated that gibberellins may trigger the synthesis of hydrolytic and proteolytic enzymes in the cotyledons. It has been shown that the level of gibberellins in cotyledonary tissue usually rises during germination and that de novo synthesis occurs in these tissues. It has been postulated that the roots are sites of synthesis of gibberellins also, thus it would be expected that the root-hypocotyl tissues of emerging seedlings would contain gibberellins either from de novo synthesis or translocation from the cotyledons. It would also be reasonable to suspect that the tissues of the germinating seedlings would be high in gibberellins because of the rapid elongation of these tissues during germination and development. The action of the exogenous gibberelin in moderately preventing the 2,4-D induced swelling indicated that gibberellins may be involved in the process.

The objectives of the research thus were to determine the status of the endogenous gibberellins of the root-hypocotyl tissues of germinating seeds of *Phaseolus vulgaris* and to determine the effect of 2,4-D upon such status. The effect of applied gibberellins both with and without the concurrent application of 2,4-D upon endogenous gibberellin levels in the emerging root-hypocotyls of the seedlings was also investigated.
The response of plants to the herbicide 2,4-D

1. Formative effects

2,4-D has been shown to exhibit growth regulating properties and has been classified as a growth regulating herbicide (5). Most growth regulating herbicides are used at relatively low rates compared to other herbicides and their effects upon susceptible plants are extremely conspicuous. Most growth regulators exert certain characteristic responses upon plants when used in high quantities.

Smith (74) found that as little as 0.01 to 0.25 pounds per acre of the phenoxy herbicides caused moderate to severe morphological effects on soybeans. The effects were more than sufficient to reduce the yield significantly.

Beans were shown to exhibit pronounced formative defects after being planted in a muck soil which had been treated one week earlier with 100 parts per million of 2,4-D (25). Seeds germinated in as low as 1 ppm solution of 2,4-D were shown to exhibit similar effects. At 10 ppm the seedlings were "checked" and swelling occurred in the hypocotyl.

Beal (8) reported that hypocotyl activity which resulted in swelling or in root formation in that tissue was a common response of bean plants to 2,4-D and 2,4,5-T. The greatest activity occurred in the ray and phloem parenchyma tissue with less in the cambium.
Swanson (77) concluded that meristematic tissues and those capable of reverting to a meristematic condition were most readily affected by 2,4-D. He found that the derivatives of such tissues remained meristematic for considerable periods, and, if differentiation did occur, it was never in an orderly fashion.

Allard, De Rose, and Swanson (1) found that the general effect of 2,4-D was to retard the rate of germination and decrease the percent germination of 22 cereal and broadleaf crops they tested. They found that with dicots 1 ppm or greater caused a swelling of the hypocotyl and root regions when germinated in that solution. Cereals were found not to exhibit this swelling, but did exhibit coleoptile curvatures and gall-like growths on the root tips. Applying high rates of 2,4-D (3.12 mg/plant) to kidney bean plants resulted in the inhibition of normal root growth. There was increased stunting and distortion of the hypocotyl region with marked hypertrophy of the adventitious root system.

It was shown that relatively low rates (0.04 to 3.3 ppm) of 2,4-D consistently resulted in the swelling of the lower hypocotyl and parts of roots of Red Kidney bean when grown in solution culture (78).

Eames (21) concluded that all the tissues between the cortex and primary xylem were involved in the proliferation and destruction of hypocotyls in young bean plants treated with 2,4-D. The first divisions were periclinal followed by anticlinal and transverse divisions. A thick sheath of proliferated tissue resulted including parenchyma cells which disrupted phloem function. He stated that
destruction of phloem was a contributing factor in the phytotoxic action of 2,4-D.

Tissue abnormalities were reported in colonial bentgrass (Agrostis tenuis) after applying 2,4-D, 2,4,5-TP, and 4(2,4-DB) at rates from 1 to 3 pounds per acre. The abnormalities involved proliferations of the pericycle, hypertrophic cortical cells, and massed lateral root and root hair formations (14). It was concluded that the difference in susceptibility of monocot versus dicot could be the result of fewer cambial layers in the stems of monocots.

The monocot nutsedge (Cyperus rotundus L.), and the dicot bean (Phaseolus vulgaris), were shown to exhibit the same internal modification of the vascular system in response to butyl 2,4-dichlorophenoxyacetate (20). The monocot differed in that it had a basal leaf meristem versus the apical and marginal leaf meristem of the dicot. The leaves of the monocot were affected in transverse segment only but the entire blade was affected in the dicot. The leaves in both became narrower but were more distinctly noticeable in the dicot as being irregular.

2. Biochemical and physiological effects

The physiological responses most often associated with 2,4-D according to Penner and Ashton (58) are:

1. Change in respiration rate
2. Nastic responses
3. Profusion of adventitious roots
4. Cell and tissue proliferation
5. Induction of parthenocarpy
6. Depression of IAA translocation in the stem
The responses Penner and Ashton listed are often listed as the responses characteristic of growth regulating substances in general. The activity of 2,4-D in the *Avena* coleoptile elongation and curvature tests support the classification of the compound as a growth regulator.

The literature is voluminous with regard to reporting the contents of plant species following the application of 2,4-D (9, 19, 46, 58, 63, 68, 73, 75, 84, 87, 88). The major responses or effects of 2,4-D on the metabolic contents of plant species are:

1. Initially enhanced soluble sugar content at the expense of reserve carbohydrates.
2. Reduction in storage lipids.
3. Increase in total N in proliferating tissues.
4. Higher nitrate levels in some plants.
5. Increased amino acid and protein content in proliferating tissues.
6. Stimulated ethylene production in plants.
7. Decreased mineral content of leaves.
8. Increased levels of nucleic acids.

Humphreys and Dugger (29) found that 2,4-D increased respiration rate of etiolated pea seedlings. They found that there was an increase in the amount of glucose catabolized via the pentose shunt pathway.

Black and Humphreys (10) showed that 10⁻³ M 2,4-D resulted in an *in vitro* increase in utilization of ribose-5-phosphate with increased formation of heptulose and hexose. These data supported the observation that 2,4-D affected glucose catabolism in etiolated corn plants by increasing the pentose phosphate shunt pathway.

An increase in the utilization of carbohydrates for the production of protein was noted after 2,4-D application (64). It was thought that the increase in protein content itself was responsible for proliferation of the stems.
Bourke et al. (11) however concluded that the action of 2,4-D and 2,4,5-T was not singularly due to disruption of glucose catabolism since other non-herbicidal compounds could cause similar effects.

Wort and Cowie (87) found that 5 and 500 ppm of 2,4-D stimulated an increase in the activities of phosphorylase, beta-amylase, catalase, and peroxidase in aerial portions of wheat plants. The activities reached a maximum by the first or second day after treatment.

Rebstock, Hamner, and Sell (65) stated that the most striking response to 2,4-D was an increase in the level of nucleic acids in treated stem tissues. It was suggested that 2,4-D regulated the growth of mitochondria from soybeans by affecting the nucleotide metabolism (39). The phosphorylation reactions of isolated mitochondria could be uncoupled with ribonuclease.

Baxter (7) showed that mitochondria isolated from swollen 2,4-D treated hypocotyls of soybean were equally efficient as those from controls in terms of respiratory and phosphorylative mechanisms. However, there was a more rapid incorporation of amino acids into mitochondrial protein in treated hypocotyls. 2,4-D also appeared to stimulate the incorporation of nucleotide triphosphates into RNA in the mitochondria. This high molecular weight RNA species was found only in the mitochondria in both treated and untreated particles.

O'Brien et al. (53) showed that 2,4-D pretreatments of soybeans with 1000 micrograms per milliliter doubled the activity of RNA polymerase extracted 12 hours later. The RNA products of isolated polymerases of 2,4-D and control hypocotyls showed different elution
profiles when separated. The RNA produced supported amino acid incorporation in cell-free systems. Actinomycin-D did not have a preferential inhibition of RNA synthesis by 2,4-D induced chromatin. They concluded that production of RNA polymerase may be an initial step of 2,4-D action.

Fisher (22) found that in germinating corn the highest activity of RNA polymerase occurred in the nucleoli of cells immediately behind the "quiescent center" itself and decreased distally from the center. It was found that 50 percent of the transfer-RNA was lost from the embryo of germinating wheat seeds during the first 10-15 hours of germination (81). A rapid resynthesis occurred so that at 20 hours the level was near normal. They also found that there were two kinds of RNase enzymes present in the system. They suggested a relationship between these RNases and t-RNA with M-RNA in regard to translational control of protein synthesis.

To test the hypothesis that in resistant species high levels of bound ribonuclease occur and prevent excess RNA synthesis, a survey of levels of endogenous ribonuclease in several plant species was made (12). It was found that resistance to 2,4-D was correlated with ribonuclease level. In more critical analysis the results were inconclusive but the expected general trends were there.

Shannon et al. (70) showed that ribonuclease increased with age in corn mesocotyl tissue. Low levels of 2,4-D accelerated growth and ribonuclease activity but high levels inhibited both. Time course studies showed that ribonuclease activity continued to increase after growth rates declined. They showed that protein synthesis, however, was needed for ribonuclease activity to continue.
The theoretical modes of action of the growth regulating herbicides

The mode(s) of action of growth regulating herbicides has not been clearly elicited as have the modes of action for other classes of herbicides. Indeed, many theories of action have been proposed. The most recent and most well regarded theories are those that deal with the effect of the herbicides upon the RNA-DNA-protein synthesis mechanism and those theories that deal with the disruption of the plant hormone interaction.

1. RNA-DNA-protein synthesis mode of action theory

This theory holds that protein synthesis is the regulating process that determines whether growth will occur or not. It is known that certain inhibitors will interfere with the normal sequence of nucleic acid synthesis and reduce growth of excised tissues. It has been found that certain substances like 2,4-D affect the synthesis of nucleic acids, either stimulating or inhibiting depending upon concentration.

West et al. (85) found that 400 ppm of 2,4-D sprayed on cucumber hypocotyl and mesocotyl and corn mesocotyl resulted in an increase in fresh weight and RNA levels.

Key and Hanson (38) found that 2,4-D treated soybean seedlings and excised hypocotyl sections had elevated RNA contents and maximum content existed just prior to the initiation of cell proliferation. The protein-RNA ratio increased markedly up to 48 hours after treatment after which there was a decrease.

It was shown that 2,4-D caused the RNA level in subcellular particles of soybean hypocotyl to double within 48 hours after
treatment (18). The authors concluded that the herbicidal action of 2,4-D lies in a renewal of nuclear activity in the tissue, leading to a synthesis of RNA and protein and to tissue proliferation, and that the cytochemical basis of auxin herbicide action lies in a reversion to a meristematic metabolism.

Key and Shannon (41) reported that in fully elongated cells, auxin induced a 25-30 percent increase in RNA, primarily ribosomal. 2,4-D and IAA produced similar effects. Actinomycin-D prevented RNA synthesis as measured by $^{14}C$ incorporation.

Key (36) showed that RNA and protein synthesis are essential for cell elongation to proceed at the normal rate. He showed further that the 2,4-D stimulation of the rate of cell elongation in soybean hypocotyl requires active RNA synthesis and in turn protein synthesis.

Key et al. (40) reported that 2,4-D treatment blocked nucleic acid and protein synthesis in the apical zone of soybean hypocotyl, with the resumption of synthesis occurring before the recovery of growth. In other areas of the hypocotyl the rate of RNA synthesis increased prior to DNA and protein synthesis. They report that there was a preferential synthesis of normal r-RNA in response to 2,4-D. They concluded that the results were consistent with the view that herbicidal levels of 2,4-D cause a renewal of RNA and protein synthesis leading to massive tissue proliferation, disorganized growth, and finally death of the plant.

Key (35) found that in excised corn mesocotyl tissue low concentrations of 2,4-D enhanced the metabolic breakdown of RNA whereas high concentrations inhibited the catabolism of RNA. They suggested
that the role of auxin in the system was not to alter synthesis of RNA, but rather to alter some process(es) related to the degradative metabolism of RNA.

2. Hormone interaction theory

The most recent view holds that there is an interaction involving the endogenous growth promoters and inhibitors to the extent that at any given time the response of the plant to a stimulus will depend upon the dynamics of the hormone status. It is also postulated that at different stages of the life cycle the most influential hormone will be different.

Overbeek (55) postulated that the difference in activity between 2,4-D and the other non-herbicidal auxins was accounted for on the basis of undissociated molecules. He concluded that 2,4-D was more active since undissociated molecules would result in a considerably greater auxin activity at all concentrations and an increasingly greater relative activity with increasing auxin concentration. The mode of action was suggested as being an accumulation of metabolites such as the inhibitor coumarin.

It was suggested that 2,4-D may act by increasing the amount of unsaturated lactones such as coumarin, scopoletin, and methyl umbelliferone which are active in interfering in the phosphorus metabolism of the plant (51).

The germination process

The process of germination has remained somewhat aloof of the probing tools of research. The exact nature of the "triggering" mechanism has yet to be elucidated with any certainty. There are
certain responses which are associated with the initiation of the germination process (79). They are: imbibition, increase in temperature, increase in respiration, and ultimately emergence of the radicle through the seed coat, which is the first "visual" indication that germination is occurring. The primary mechanism by which germination is stimulated lies somewhere between initial imbibition and radicle emergence.

Simon and Meany (71) reported that in germinating Phaseolus seeds the initial hydration of the seed was independent of temperature between 15 and 35°C. There was a lag period that followed which was highly temperature-dependent in terms of its length, inversely related with temperature. The rate of fresh weight increase after the lag phase was likewise dependent on temperature. The abrupt commencement of dry weight increase in the root-shoot axis was paralleled by an equally sudden rise in the total N content of the axis. They showed that the root-shoot axis had 0.8 mg soluble sugar and each cotyledon had 43.3 mg (½ stachyose ½ sucrose) which they stated was sufficient to support the germinating seed for 60 hours. They maintained that hydrolysis of starch for the commencement of growth was not needed, thus the regulatory mechanism probably did not depend upon percent hydration alone.

A decline in the rate of appearance of N and soluble compounds in germinating corn seeds after 72 hours was postulated as being the result of a steady state condition between synthesis and mobilization of reserves in the tissues (30). However, it was pointed out that certain compounds such as the nucleic acids are not contained in
storage areas of seeds and an increase in their levels must represent a \textit{de novo} synthesis.

Marre et al. (50) showed that there was a \textit{de novo} synthesis of all RNA species in the endosperm of germinating castor bean seed. They reported a doubling of the RNA content within the first 24 hours. The increase in RNA was followed by an increase in ribosomes.

Ingle and Hageman (31) found that in germinating corn, as contrasted to their results with barley, the level of endosperm RNA remained practically constant over the germination period. They found that the activity of RNases increased 340-fold over the germination period in the axis. They showed that little nucleotide or nucleic acid was stored in the corn grain thus the increase in RNA and DNA must have been due to \textit{de novo} synthesis.

Holdgate and Goodwin (26) found that RNA exhibited an initial decline then an increase in the endosperm of germinating rye (\textit{Secale cereale}) seeds prior to the initiation of cell division. No explanation of the decrease could be given. They found that DNA increased by \textit{de novo} synthesis in the radicle and the plumule.

Anderson and Fowden (2) followed the change in s-RNA synthetase activities in germinating \textit{Phaseolus vulgaris} seed. They found that in the plumule and radicle the absolute activity of the synthetases increased exponentially during germination.

It is known that proteolytic and hydrolytic enzymes are synthesized in the germinating seeds of plants. There must, then, be a relationship between the DNA-RNA-protein synthesis process within germination. It is not difficult to rationalize the relationship
within the framework of the process we know. The mystery revolves
around the actual triggering mechanism that regulates the process.

Germination and plant hormones

Most recently, certain of the plant growth hormones have been
found to regulate certain enzymatic reactions occurring in germinating
seeds and isolated seed parts. It is felt in some academic circles
that germination may involve interaction of certain growth promoting
and growth inhibiting substances. Galston and Davies state that
hormones do not act alone but in an interrelated manner (24). They
state that the action of hormones seems to be related to the mechanism
of enzyme production through the DNA-mRNA-tRNA system.

The gibberellins have been shown to exert positive effects
upon germinating seeds. It is known that gibberellic acid can sub­
stitute for red light conditions on certain plant species which need
light to stimulate germination.

Gibberellic acid has also been shown to exert a pronounced
effect upon certain enzymatic reactions of germinating seeds. Kaufman
et al. (34) showed that gibberellic acid was necessary for invertase
activity in Avena internodes. They reported that the increase in
activity was about five-fold when gibberellic acid was added to the
media.

Varner and Johri found that gibberellin enhanced the synthesis
of alpha-amylase, protease, and ribonuclease in isolated aleurone
layers of barley (80). They found that gibberellin was required con­
tinuously for the de novo synthesis to occur and that RNA synthesis
was required.
Wain (81) described the function of gibberellins in plants to be that of interaction with auxins to promote cell extension growth and in certain seeds to promote hydrolytic and proteolytic enzymes.

Penner (57) showed that several herbicides inhibited the gibberellin stimulated production of amylase in embryo-free barley and squash seeds. This type of action, on germinating seeds, was postulated as being the reason seedlings are more susceptible to herbicides than mature plants.

Phinney et al. (59) found gibberellins present in the immature seeds of higher plants including *Phaseolus vulgaris*. The gibberellins A$_1$, A$_2$, A$_5$, A$_6$, and A$_8$ were shown to be present in the immature seeds of *Phaseolus vulgaris* (48, 86). These gibberellins have been reported to occur in *Phaseolus multiflorus* also but recently MacMillan and Pryce (48) have shown that others are present, notably A$_{17}$, A$_{19}$, A$_{20}$, G-alpha, G-beta, and phaseic acid.

These works have been done utilizing immature seeds of beans. It has been shown that the content of gibberellin-like substances extractable in ethyl acetate disappears as seeds mature and dry (66). Chemically neutral gibberellin-like substances appear to increase and a water soluble gibberellin-glucoside has been found.

Sembdner et al. (69) showed that in *Phaseolus coccineus* gibberellin sigma was a glucopyranoside of GA$_8$. They found that the glucoside increases in content of seeds as it matures and in dry seed it is the only GA substance detected. The function of the glucoside was not proven but it was suggested that it may be a storage mode.
Andrews (3) found three gibberellin-like substances in *Avena fatua* L., one of which was present in both dormant and mature seeds at an equal level. The other two were lower in the mature seeds. He found that the dormant seeds contained a water soluble inhibitor of germination and postulated that it blocked synthesis of gibberellin.

Gibberellins and a water soluble inhibitor may function as the regulating agents of seed dormancy-germination. It has been proposed that water may wash out the germination inhibitor and hydrolyze the seed so that gibberellin can take over. The gibberellin would then regulate the production of hydrolytic and proteolytic enzymes (54). The production of IAA becomes necessary then to promote elongation in seedling growth. It was related that there is a sequence of action of the plant hormones in which they interact to produce growth (24).
MATERIALS AND METHODS

Treatment and harvest

Two layers of absorbent cotton were placed in the bottom of covered plastic boxes (10" x 14") which had been light-proofed with flat black enamel paint on the outside surfaces. The cotton was then saturated with one of the following substances at the concentration indicated: 1 ppm 2,4-D; 1 ppm GA (gibberellic acid A-7); 5 ppm GA; 1 ppm 2,4-D + 1 ppm GA; 1 ppm 2,4-D + 5 ppm GA; and a control.

Two hundred seventy-five seeds of Phaseolus vulgaris L., cv. Contender II, were selected for uniformity in size and quality and surface sterilized in sodium hypochlorite. A one percent solution was utilized and the seeds were left in the solution with gentle agitation for three minutes. The seeds were then washed with running water for five minutes followed by a quick rinse in single distilled water. The seeds were then immediately placed in the treatment boxes and the boxes placed in a germination area at 25°C. They remained undisturbed for 5 to 8 days, depending upon the treatment, until harvested.

The seedlings were removed from the boxes and rinsed quickly in double pyrex glass distilled water. The seedlings were selected for uniform size in an attempt to maintain a similarity in physiological age.
The root and hypocotyl were harvested from the seedling by cutting it at the point of emergence from the seed coat. Care was exercised to exclude pieces of the seed coat from the harvested tissue to prevent contamination from possible inhibitors in the seed coat. The severed root-hypocotyl tissue was then placed in cold 80 percent methanol for extraction. The extraction was allowed to progress for two weeks at -20°C.

**Extraction of gibberellins**

The alcohol extract containing the gibberellins was removed from the freezer and reduced to the aqueous phase on a Buchi Rota-vapor at 40°C. The aqueous phase was then handled according to a method reported by Kozel and Tukey (44). Sodium bicarbonate was added to the aqueous phase to bring the pH to 8.2. The sample was then extracted three times with equal volumes of methylene chloride to remove any inhibitors which may have been present. The methylene chloride fractions were discarded. The aqueous phase was then brought to pH 3.2 with 3N hydrochloric acid. The extract was again extracted three times with equal volumes of methylene chloride. The aqueous phase was discarded. The methylene chloride fractions were combined and reduced in volume on the Rotavapor to about 10-20 milliliters. Two milliliters of n-decane were added and the methylene chloride evaporated off. The gibberellins thus were contained in the n-decane. The procedure is outlined in Figure 1.

A 10-microgram quantity of gibberellic acid placed in 80 percent methanol was extracted to determine the efficiency of the procedure. All the extracts destined to be discarded were saved and
Aqueous extract
  ↓
add sodium bicarbonate to raise pH to 8.2
Aqueous extract at pH 8.2
  ↓
3 extractions with methylene chloride (CH₃Cl)

Aqueous extract
  ↓
acidify to pH 3.0 with HCl
Aqueous extract at pH 3.0
  ↓
3 extractions with methylene chloride

methylene chloride fractions (discard)

methylene chloride fractions
  ↓
Reduce in volume
20 ml methylene chloride fraction
  ↓
add 2 ml n-decane
CH₃Cl evaporated off
2 ml n-decane containing gibberellins
  ↓
n-decane placed on silica gel column
  ↓
eluted with 0-100% ethyl acetate in hexane
20 ml fractions
  ↓
Lettuce hypocotyl bioassay
  →
Thin layer chromatography

Fig. 1. Extraction and fractionation procedure for the separation of gibberellins from the treated root-hypocotyl tissue of germinating Phaseolus vulgaris seeds.
reduced in volume. The volumes were then spotted on silica gel-G thin layer chromatography plates and developed for a 10-cm distance in a benzene:n-butanol:acetic acid (85:15:5) solution. The plates were then air-dried and sprayed with an ethanolic sulphuric acid detection spray. The plates were then placed in a 100°C oven for 20 minutes, then viewed beneath ultra-violet light. In this system gibberellins fluoresce beneath the ultra-violet light and can be readily located. Comparison of the \(R_f\) values for fluorescing spots of spotted known gibberellins with any suspected fluorescing areas enabled gibberellin presence to be confirmed. In this work no gibberellin presence could be detected in any of the discard fractions of the extraction method. This indicated that the gibberellins present in the samples were extracted efficiently without loss in the extraction process. The lower limit of detection of this system is 0.1 microgram per spot. A negative reaction to the detection spray meant less than 0.1 microgram was present which on this basis allows the assumption of 98 percent efficiency.

**Fractionation of the gibberellins**

A silica gel column was used to separate the gibberellins in the extract. The method utilized was that described by Powell (62) and modified slightly by Kozel and Tukey (44).

The silica gel was sized by initially suspending one pound of 100-200 mesh silica gel in two liters of double pyrex glass distilled water. The suspension was agitated vigorously for 20 seconds, then allowed to settle for 45 minutes. The supernatant liquid was poured off quickly. Two liters of water were added to the remaining slurry
and agitated again. The settling time was reduced to 30 minutes at which time the supernatant liquid was removed as before. The procedure of adding water and agitating was repeated and the following settling times utilized: 20 min, 10 min, 10 min, 5 min, 5 min, 2 min, and 2 min with a discarding of the supernatants at each interval. The silica gel remaining after the conclusion of the sizing operation was that utilized in the experiment. The silica gel was then washed with 10 N HCl and rinsed with double pyrex glass distilled water until the effluent was neutral. The silica gel was then dried to a constant weight at 100°C. This sizing procedure was carried out to insure the elution pattern stability of columns prepared from the silica gel. Columns prepared from unsized lots of silica gel can vary in the elution pattern and flow times due to fluctuation in the content of different sized particles comprising the column. The sizing procedure resulted in a final usable weight of 257 grams remaining from an initial 454 grams of the silica gel.

Silica gel columns were then prepared according to the following procedure. Eight grams of silica gel were hydrated with 5 ml of 0.5 M formic acid to charge the active exchange sites of the gel. The mixture was shaken until a free flowing powder was obtained. The silica gel was then slurried with n-hexane saturated with 0.5 M formic acid and pipetted into glass columns. The columns were 11 mm inside diameter and had a stopcock on one end. The silica gel was packed with air pressure to a height of 10 cm in the column. One liter of n-hexane saturated with formic acid was allowed to flush the column before use.
The n-decane samples containing the gibberellins were pipetted onto the tops of the silica gel columns and eluted off of the columns with increasing concentrations listed in Table 1. The table also shows the fractions expected to contain gibberellins $A_1$-$A_9$ according to Powell (62). Figure 2 is a graphical representation of the elution gradient utilized as given in Table 1.

**TABLE 1. The step-wise elution pattern for gibberellins $A_1$-$A_9$ from silica gel partition columns**

<table>
<thead>
<tr>
<th>Fraction Number</th>
<th>Percent Ethyl-acetate in n-hexane</th>
<th>Gibberellin Eluted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>$A_9$</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>$A_4$</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>$A_4,7$</td>
</tr>
<tr>
<td>9</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>19</td>
<td>$A_5$</td>
</tr>
<tr>
<td>12</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>24</td>
<td>$A_6$</td>
</tr>
<tr>
<td>14</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>42</td>
<td>$A_3,1$</td>
</tr>
<tr>
<td>18</td>
<td>44</td>
<td>$A_3,1$</td>
</tr>
<tr>
<td>19</td>
<td>46</td>
<td>$A_2$</td>
</tr>
<tr>
<td>20</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>62</td>
<td>$A_8$</td>
</tr>
<tr>
<td>24</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 2. Elution gradient curve for the ethyl acetate-n-hexane solvent system used to fractionate gibberellins $A_{1-9}$ from silica gel partition columns.
An attempt was made to automate the system by the use of a fraction collecting device. The sensing unit which determined when to change test tubes beneath the column was a multi-mode unit. It was found that drop counting and volumetric siphon methods of collecting the fractions were not usable. The n-hexane and ethyl acetate solvents used were highly different with regard to density and surface tension causing a constant change in density and surface tension as the percentage of the ethyl acetate in n-hexane increased. Thus it was found that the fraction size collected by these methods continuously changed as a function of time lapsed.

The final collection method utilized was a manual pipetting of 20 ml of the various concentrations onto the column. The fraction was allowed to elute the column and was collected in a test tube. The procedure was repeated for each concentration. The elapsed time for one complete elution of the column was six hours.

Bioassay and thin layer techniques

Gibberellin activity was determined by a modified lettuce hypocotyl bioassay of Frankland and Waring (23). The modification used was described by Kozel and Tukey (44). The hypocotyls of germinating lettuce seeds, variety Grand Rapids, are sensitive to gibberellins. The hypocotyls will elongate in proportion to the amount of gibberellin present. The response of the hypocotyls has been shown to follow a straight line between the concentrations of 0.001 and 10 micrograms per milliliter of gibberellic acid. A series of standard concentrations ranging in concentration from 0.001 to 100 micrograms
per milliliter was used in each bioassay as a means of correlating the amount of activity present in the unknowns with these known quantities. Control samples were also included and the results of the bioassay are reported as net hypocotyl elongation of the unknown and standard samples over the control hypocotyls.

Frankland and Waring in the original description of the system did not elaborate in detail on several of the factors essential to the system. Therefore several preliminary experiments were run in an attempt to standardize and add reliability to the system utilized in this work. One of the factors not described in detail by the original workers was the light source under which the lettuce seedlings were subjected during the test period. Initially in this work a mixture of cool white and "Gro-Lux" fluorescent tubes were used in holders which had V-shaped reflectors for each tube. Because of the closeness of the tubes to the germinating seeds an uneven distribution of the light resulted. This resulted in differences in elongation of the hypocotyls attributable to location beneath the light bank. It was found that the use of a flat reflector helped but that the use of only warm white tubes (Sylvania "Lifeline" Warm White) in combination with a flat reflector gave the best results.

The effect of several surfactants and types of filter paper on the bioassay system was investigated. Surfactants in general have in the past been rather indiscriminantly used in biological systems without the effect of the surfactant itself on the system being investigated. The surfactants tried in this work were: Tween 20, Tween 21, Tween 60, Tween 80, and X-77. Each was tried at the
concentrations 0.01, 0.1, and 0.5 percent. Six different filter papers were used: Whatman numbers 1, 3, 41, and 42 and Schleicher and Schuell numbers 540 and 589. The papers were used to determine whether any differences could be noted due to pretreatments of the papers in their manufacture or the surface textures and finishes of the papers.

It was determined that the type of filter paper did not affect the response of the lettuce hypocotyls; however, it was noticed that the smooth, hard-surfaced papers (S & S 595) enabled the seedlings to be removed more easily which facilitated the handling of large numbers of hypocotyls.

It was found that the surfactant X-77 was inhibitory to the hypocotyls at all levels used. The other surfactants showed relatively little inhibition in the range of 0.01 to 0.1 percent. The surfactant Tween 80 at 0.08 percent was used in subsequent work because it had been described with this system previously and was satisfactory in this work.

It was also noted from preliminary work that the source of distilled water had an important effect on the response of the lettuce hypocotyl. Initially the double distilled water used in the work was obtained in 10-quart plastic "handi-tap" containers. It was found that the results would vary when the source of water would switch from container to container. The use of pyrex double distilled water obtained in glass carboys eliminated this variability. The variability was thought to be possibly the result of contaminants in the plastic containers remaining from the manufacturing process.
Utilizing these data the lettuce hypocotyl bioassays were run according to the following method. Filter paper discs (S & S 595) were placed on the bottom of 18 mm diameter x 60 mm long glass vials. Three-tenths of a milliliter of the sample solutions was pipetted onto the filter papers of each of three vials per sample. Three-tenths of a milliliter of controls and standard solutions of .001, .01, .1, 1, and 10 micrograms per milliliter concentrations of GA-3 were also utilized in every series of the bioassays. Ten lettuce seeds were placed in each vial and the vials were then placed in covered plastic boxes the sides of which were lined with aluminum foil and the bottom with paper towels. The paper towels were saturated with water and a glass plate was placed on top of the plastic box. The moist towels and glass top helped to maintain a saturated atmosphere in the vials. The boxes were then placed under fluorescent lights in a growth chamber at 25°C. The aluminum foil lining helped to eliminate low light spots in the box. The vials remained undisturbed for 7 days. The fluorescent lights utilized were Sylvania warm white tubes having the emission spectrum graphically represented in Figure 3. The lights were 30 cm from the seeds and at 3,150 uW/cm² intensity.

The vials were removed at the end of 7 days and the tallest five seedlings from each vial used. The seedlings were dipped in a 0.5 percent crystal violet solution to stain the root tissue but not the hypocotyl. The hypocotyl was then measured to the nearest tenth of a millimeter with a pair of dial calipers.

Initially only control treatments were run to determine if any differences within the plastic boxes or beneath the light bank
Fig. 3. The emission spectrum of Sylvania warm white "Lifeline" fluorescent tubes as measured at a 30-cm distance.
could be detected. Initial differences were attributable to the light
bank and solvent contamination and were corrected. Subsequent bio-
assays were repeatable and when internal gibberellin standards were
used the net hypocotyl elongation of these with the control treatments
were significant statistically. In general, it was assumed that any
net hypocotyl elongation value which was less than the 0.001 micro-
gram per ml internal standard of GA₃ net elongation value was insig-
nificant in terms of its relevance to the test.

Fractions exhibiting gibberellin activity were analyzed by
thin layer chromatography to confirm gibberellin presence. Three ml
of the sample fraction were acidified to pH 3.2 with 3N HCl. The
sample was then extracted with three volumes of methylene chloride to
obtain the gibberellins. The methylene chloride fractions were com-
bined and evaporated on a Buchler evaporator to dryness. The fractions
were then resuspended in a drop of methylene chloride and applied to
thin layer plates.

The thin layer technique of MacMillan and Suter (49) was
utilized which involved the use of thin layer plates of Silica Gel G.
The gel was 25 microns thick on glass plates. The developing solution
utilized was benzene:n-butanol:acetic acid (80:15:5). The plates were
developed to a distance of 10 cm which required approximately one hour.
The plates were sprayed with a detection spray of ethanol and concen-
trated sulfuric acid (95:5) and allowed to react at 120°C for 20
minutes. The plates were then viewed beneath ultraviolet light to
detect gibberellin fluorescence. Comparison of Rf values with known
gibberellins spotted on the plates and comparison with published
values (56), allowed confirmation of gibberellin presence.
Safeguards

All glassware utilized in the procedures was methodically cleaned before use. The glassware was first "chemically cleaned" by the use of a dichromate-sulfuric acid cleaning solution. After rinsing with water the glassware was then washed in a laboratory detergent and rinsed liberally with single distilled water. All items then were rinsed with pyrex double distilled water at least three times. The glassware was then placed in a 550°F oven for five hours to decompose any remaining organic contaminants or residues.

Solvents utilized in the extraction and fractionation procedures were all of at least reagent grade initially. The methanol and methylene chloride solvents were obtained in "spectro" grade and were used as they came. The n-hexane and ethyl acetate were obtained in reagent grade and were double distilled in an all pyrex glass distillation apparatus before use.
RESULTS

The seedlings were harvested as outlined in the previous section. A descriptive representation of the physical measurements of the treatments at the time of harvest is given in Table 2. The data indicate that the number of seedlings harvested from one treatment to another was highly variable. The control treatment yielded the greatest number of harvested seedlings as expected, 242 of 275.

TABLE 2. Physical data of the harvested root-hypocotyl tissues of Phaseolus vulgaris seeds germinated in various solutions at the time of harvest

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days to Harvest</th>
<th>Number Harvested</th>
<th>Weight of Tissue (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>242</td>
<td>71.2</td>
</tr>
<tr>
<td>1 ppm 2,4-D</td>
<td>8</td>
<td>329\textsuperscript{a}</td>
<td>119.6</td>
</tr>
<tr>
<td>1 ppm GA\textsubscript{3}</td>
<td>5</td>
<td>199</td>
<td>44.1</td>
</tr>
<tr>
<td>5 ppm GA\textsubscript{7}</td>
<td>5</td>
<td>242</td>
<td>45.2</td>
</tr>
<tr>
<td>1 ppm GA\textsubscript{3}</td>
<td>5</td>
<td>\textsuperscript{b}</td>
<td>-</td>
</tr>
<tr>
<td>1 ppm 2,4-D plus 1 ppm GA\textsubscript{3}</td>
<td>5</td>
<td>\textsuperscript{b}</td>
<td>-</td>
</tr>
<tr>
<td>1 ppm 2,4-D plus 1 ppm GA\textsubscript{7}</td>
<td>5</td>
<td>133</td>
<td>15.4</td>
</tr>
<tr>
<td>1 ppm 2,4-D plus 5 ppm GA\textsubscript{7}</td>
<td>5</td>
<td>109</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>181</td>
<td>53.6</td>
</tr>
</tbody>
</table>

\textsuperscript{a}500 seeds initially instead of 275.
\textsuperscript{b}Seedlings not large enough to harvest.
The treatments which contained \( \text{GA}_3 \) were not harvested because the seedlings did not reach the minimum harvestable size. The minimal size selected was a 2- to 4-centimeter length of the emerged root-hypocotyl. This size was selected to insure a sufficient amount of material to yield detectable quantities of gibberellins in the samples. All the treatments involving \( \text{GA}_3 \) failed to attain the minimal size at the end of a 10-day period and at that time the seeds were discarded.

**The control treatment**

The seeds were allowed to germinate for five days at which time 242 of 275 were harvested. The harvested root-hypocotyl tissue weighed 71.2 grams fresh weight prior to extraction.

Following liquid-liquid extraction and silica gel column fractionation the samples were submitted to a lettuce hypocotyl bioassay. The results of the bioassay are represented in Figure 4. The results are illustrated in the form of a histogram showing the net hypocotyl elongation of the eluted fractions over the control fraction in each instance. The histogram gives the net hypocotyl elongation in mm on the vertical scale. The length of the lettuce hypocotyls of the controls were subtracted from each fraction individually to yield the net elongation value. In all bioassays conducted the average hypocotyl length of the controls was 3.9 to 4.0 mm. In general a net difference of one mm represents a statistically significant increase in elongation. The horizontal axis represents the fraction numbers eluted off of the silica gel column and correspond to the numbers of Table 1. The internal standards of various concentrations of \( \text{GA}_3 \) used in every bioassay are also given on the horizontal
Fig. 4. Gibberellin activity in the fractionated extract of root-hypocotyl tissue of germinating *Phaseolus vulgaris* seedlings in the control treatment as measured by the lettuce hypocotyl bioassay.
ELUTION PATTERN OF KNOWN GIBBERELLINS
axis at the extreme right of the figures. They represent the net hypocotyl elongation a known concentration of GA$_3$ will yield. Below the horizontal axis is illustrated the fractions at which the gibberellins A$_1$-9 are known to elute according to Powell (62).

Figure 4 shows that the preponderance of gibberellin activity in the control occurred in the fractions where GA$_1$ and GA$_3$ are known to elute. A lesser amount of activity appeared to be present in the fraction. The GA$_{1-3}$ fraction contained an activity of between 0.001 and 0.01 micrograms per milliliter equivalent GA$_3$ activity. The GA$_8$ fraction yielded an activity of less than 0.001 micrograms per ml GA$_3$ equivalent. Brian and Lowe (12) have shown GA$_8$ to be typically inactive in any bioassay which could account for its low activity in this application.

Thin layer chromatography of the fractions showing possible activity in the lettuce hypocotyl bioassay were conducted. The fractions were prepared as detailed in the methods section and spotted on the silica gel-G plates, developed in the benzene:n-butanol:acetic acid (80:15:5) solvent, and sprayed with the detection reagent. $R_f$ values of 0.55 and 0.58 were obtained for fluorescing spots from fractions 18 and 19 respectively, of the control treatment. The GA$_3$ spotted concurrently on the plate had a $R_f$ value of 0.58. Thus, the presence of GA$_3$ was confirmed in the control sample. The confirmation of GA$_8$ could not be made by thin layer chromatography.
2,4-D treatment

The seeds in 1 ppm 2,4-D were cultured for eight days before harvest. Of 500 seeds initially sown, 329 seedlings were harvested. All the harvested root-hypocotyl tissue harvested was at least 2 cm long. The 329 excised root-hypocotyls had a fresh weight of 119.6 grams.

After extraction and fractionation of the sample a lettuce hypocotyl bioassay was conducted. The results of the bioassay are given in Figure 5. The histogram shows that a minimal amount of activity occurred but slight peaks did occur in the fractions representative of some of the gibberellins. These peaks, however, are considerably below the lowest standard GA$_3$ concentration utilized.

The fractions which corresponded to GA$_{1-3}$ exhibited fluorescence and R$_f$ values of 0.58 and 0.55 when analyzed by thin layer chromatography. GA$_3$ spotted concurrently with the fractions gave a R$_f$ value of 0.54. GA$_3$ was confirmed as being present in the sample but present in a quantity less than 0.001 micrograms/ml equivalents of GA$_3$ as the bioassay indicated.

Gibberellin treatments

At the end of five days 199 seedlings of the 1 ppm GA$_7$ treatment were harvested which yielded 44.1 grams of root-hypocotyl tissue. The 5 ppm GA$_7$ treatment yielded 242 seedlings and 45.2 grams of root-hypocotyl tissue. The GA$_3$ treatment did not yield any harvestable tissue at the end of 10 days.
Fig. 5. Gibberellin activity in the fractionated extract of root-hypocotyl tissue of germinating *Phaseolus vulgaris* seedlings in a 1 ppm 2,4-D solution as measured by the lettuce hypocotyl bioassay.
The GA$_3$ treated seeds germinated and the root-hypocotyls attained a length of 1-2 centimeters but remained at that length and were discarded after 10 days.

The harvested GA$_7$ treatments were extracted and fractionated then placed in a lettuce hypocotyl bioassay. The results of the bioassays are represented in Figures 6 and 7. The results revealed that a great deal of activity was present. Utilizing the thin layer techniques to confirm the gibberellin presence, it was shown that GA$_3$ was present in both the 1 and 5 ppm GA$_7$ treatments. The $R_f$ values were variable due to trailing of the spots but are concluded as valid. The 1 ppm GA$_7$ treatment yielded $R_f$ values of 0.44 for the fractions attributed to GA$_{1-3}$ while the internal standard of GA$_3$ gave a $R_f$ of 0.38 to 0.42. The 5 ppm GA$_7$ treatment GA$_{1-3}$ fractions gave $R_f$ values of 0.38 while the internal standard of GA$_3$ had a $R_f$ of 0.41 to 0.56.

In the 1 ppm GA$_7$ treatment four fractions exhibited activity in the bioassay. Two exhibited 0.01 to 0.1 micrograms/ml GA$_3$ equivalent activity and two exhibited 0.001 to 0.01 micrograms/ml GA$_3$ equivalent activity.

The 5 ppm GA$_7$ treatment had six fractions which gave activity in the GA$_{1-3}$ fractions. Three fractions had activity equal to one microgram/ml GA$_3$ activity and three fractions exhibited between 0.1 and 1 microgram/ml GA$_3$ equivalent activity.

2,4-D plus gibberellin treatments

The treatment 1 ppm 2,4-D plus 1 ppm GA$_7$ was harvested at the end of five days at which time 133 seedlings were harvested. The root-hypocotyl tissue harvested had a fresh weight of 15.4 grams.
Fig. 6. Gibberellin activity in the fractionated extract of root-hypocotyl tissue of germinating Phaseolus vulgaris seedlings in a 1 ppm gibberellin A₇ solution as measured by the lettuce hypocotyl bioassay.
ELUTION PATTERN OF KNOWN GIBBERELLINS
Fig. 7. Gibberellin activity in the fractionated extract of root-hypocotyl tissue of germinating Phaseolus vulgaris seedlings in a 5 ppm gibberellin A₇ solution as measured by the lettuce hypocotyl bioassay.
ELUTION PATTERN OF KNOWN GIBBERELLINS
The treatment 1 ppm 2,4-D plus 5 ppm GA\textsubscript{7} had two harvest dates. A 5-day harvest of 109 seedlings yielded a root-hypocotyl weight of 10.3 grams. An 8-day harvest sample of 181 seedlings yielded a 53.6-gram root-hypocotyl tissue weight.

The treatment 1 ppm 2,4-D plus 1 ppm GA\textsubscript{3} was not harvested because the seeds germinated but the root-hypocotyl failed to attain a length greater than 1-2 centimeters after 10 days.

The harvested samples were extracted and fractionated prior to lettuce hypocotyl bioassay. The results of the lettuce bioassay of the 1 ppm 2,4-D plus 1 ppm GA\textsubscript{7} fractionated sample are shown in Figure 8. The activity was shown to be in four fractions. Two fractions exhibited .01 to .1 micrograms per ml GA\textsubscript{3} equivalent activity and two fractions showed .001 to .01 microgram per ml GA\textsubscript{3} equivalent activity. Thin layer chromatography confirmed the presence of GA\textsubscript{3} in fraction 18 while no fluorescence was evident in the other fractions. No explanation for this result can be given.

The treatment 1 ppm 2,4-D plus 5 ppm GA\textsubscript{7} (5-day sample) after extraction and fractionation was followed by a lettuce hypocotyl bioassay of the sample and yielded the results as given in Figure 9. The histogram shows that the 5-day sample exhibited three fractions where activity was due to the GA\textsubscript{1-3} eluted. Two of the fractions exhibited activity to the extent of 0.01 and 0.1 micrograms/ml GA\textsubscript{3} equivalents and one fraction between 0.001 and 0.01 micrograms/ml activity.

The treatment 1 ppm 2,4-D plus 5 ppm GA\textsubscript{7} (8-day sample) was handled in a similar manner and the results are given in Figure 10.
Fig. 8. Gibberellin activity in the fractionated extract of root-hypocotyl tissue of germinating *Phaseolus vulgaris* seedlings in a 1 ppm 2,4-D plus 1 ppm gibberellin $A_7$ solution as measured by the lettuce hypocotyl bioassay.
NET HYPOCYOTEL ELONGATION (mm)

<table>
<thead>
<tr>
<th>Fraction Number</th>
<th>5</th>
<th>11</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>.001</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>4-7</td>
<td>5</td>
<td>6</td>
<td>1-3</td>
<td>2</td>
<td>8</td>
</tr>
</tbody>
</table>

ELUTION PATTERN OF KNOWN GIBBERELLINS

.1 μg/ml GA₃
Fig. 9. Gibberellin activity in the fractionated extract of root-hypocotyl tissue of germinating *Phaseolus vulgaris* seedlings in a 1 ppm 2,4-D plus 5 ppm gibberellin A₇ (5-day) solution as measured by the lettuce hypocotyl bioassay.
NET HYPOCOTYL ELONGATION (mm)

ELUTION PATTERN OF KNOWN GIBBERELLINS

FRACTION NUMBER

0.001
0.01
0.1
1.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
45.0
50.0
55.0
60.0
65.0
70.0
75.0
80.0
85.0
90.0
95.0
100.0

Net hypocotyl elongation ranges from -0.5 to 4 mm. The elution pattern of known gibberellins is shown with fraction numbers 1-3, 2, 8, 10, 15, 20, 25, 5, and 9. Specific concentrations are marked along the Y-axis.
Fig. 10. Gibberellin activity in the fractionated extract of root-hypocotyl tissue of germinating *Phaseolus vulgaris* seedlings in a 1 ppm 2,4-D plus 5 ppm gibberellin A$_7$ (8-day) solution as measured by the lettuce hypocotyl bioassay.
ELUTION PATTERN OF KNOWN GIBBERELLINS
The histogram shows very clearly two areas of activity in the fractions. These corresponded to $GA_{1-3}$ and $GA_8$. Three fractions were in the $GA_{1-3}$ area and two fractions in the $GA_8$ area. Two fractions in the $GA_{1-3}$ area exhibited about 0.001 micrograms/ml activity and one between 0.001 and 0.01 micrograms/ml $GA_3$ equivalent activity. The fractions of the $GA_8$ area approached 0.001 micrograms/ml $GA_3$ equivalent activity.

Thin layer chromatography failed to show any activity in these samples.
DISCUSSION OF RESULTS

The data show that the endogenous gibberellins of the root-hypocotyl tissue as measured in the described system were found to be GA_1 and/or GA_3 and possibly GA_8. Gibberellin A_8 is not highly active in bioassay systems but showed a slight activity in some samples. Thin layer chromatography confirmed the presence of GA_1 and/or GA_3 but no confirmation could be made of GA_8. Several workers have reported gibberellins A_1, A_3, A_5, A_6, and A_8 to be present in Phaseolus vulgaris tissue (49, 86). Most of their work was done on immature seeds of the species or with leaf tissues. Detectable amounts of gibberellins become less as the seed matures. The finding that certain of the gibberellins can be associated with sugar molecules, such as the glucopyranoside of GA_8, and increase in content during seed maturation, has been interpreted as indicating the possibility that they may serve as storage forms in the seeds (69).

The function of gibberellins in germination has not been fully elucidated but it is known that they play a dominant role in initiating and maintaining the synthesis of certain hydrolytic and proteolytic enzymes. In isolated aleurone layers of barley, Varner and Johri (80) found that gibberellin enhanced the synthesis of alpha-amylase, protease, and ribonuclease. They found that gibberellin was required continuously for the de novo synthesis to occur and that RNA synthesis was required. During germination there is a great increase in RNA and
DNA in the young tissues. This is expected due to the necessary synthesis of essential enzymes and proteins for growth and development of the seedling.

It was reported that Radley (80) has shown that in barley the embryo can be a source of synthesized gibberellins during germination in addition to that synthesized in the aleurone layers. Others have postulated that the root tip is a site of synthesis of gibberellins (55, 15). Thus if the root tip is an active center for the production of gibberellins in germinating seedlings, any inhibition of the normal function would reduce the synthesis of gibberellin.

The endogenous level of gibberellins in the root-hypocotyl tissue of the seedlings could be the result of synthesis by the root tip or result from translocation of the gibberellins from the cotyledons.

The addition of 2,4-D to the germination medium resulted in a decreased level of gibberellin in the root-hypocotyl tissue. 2,4-D has been characterized as causing tissue proliferations in many species. It also has been shown by Key and Shannon (41) and Chrispeels and Hanson (18) that in low concentrations 2,4-D and other auxin-type growth regulators result in increased DNA and RNA levels in treated tissue. In a recent review Key (37) stated that work to date has shown that, generally, intact plants respond to high levels of the auxin-type growth regulators by (a) inhibition of growth, (b) inhibition of nucleic acid synthesis in normal growing points, and (c) the more mature stem tissues are "activated" relative to nucleic acid synthesis and massive cell proliferation.
If the 2,4-D treatment resulted in an inhibition of growth of the root tip in our work in terms of interruption of normal function, then the synthesis of gibberellins in the root tip would also be assumed to be stopped. The 2,4-D histogram for gibberellin activity shows that indeed the activity was drastically reduced by the treatment. The results, however, do not exclude the possibility that the synthesis of gibberellins or the conversion from storage forms in the cotyledons was also inhibited. The possibility exists that the reduced activity might be accounted for in terms of reduced movement from the cotyledons.

The addition of gibberellins to the germinating seeds both with and without 2,4-D attempted to determine if inhibition by 2,4-D could be circumvented. If gibberellin could prevent the inhibition then a positive association of 2,4-D action and gibberellin synthesis could be hinted. Preliminary research indicated that a moderate reversal was possible; however, lower non-herbicidal rates of 2,4-D were utilized in those studies.

An examination of the bioassay histograms reveals an interesting aspect of the experiment. The GA\textsubscript{7} added resulted in an increase in level of activity exhibited in the GA\textsubscript{1-3} fractions of the samples in all instances. No activity was detectable in the fractions where GA\textsubscript{7} are known to elute. Thin layer chromatographic analysis of the active fractions confirmed that activity was due to a gibberellin that had the same R\textsubscript{f} value of concurrently spotted GA\textsubscript{3}. 
Wright (89) reported that Verbiscar was able to demonstrate the conversion of $^{14}$C labelled GA$_4$ and GA$_7$ into $^{14}$C-GA$_3$ in cultures of the fungus Gibberella fujikori.

Jones (33) reported that in pea tissue a conversion of added GA$_5$ to GA$_1$ occurred. He described the conversion as a mechanism by which the addition of an (-OH) group to GA$_5$ converts it to GA$_1$. He reported that several her workers had reported GA$_5$ to be a pre­ursor of other gibbe...ins.

The data presented in this experiment are circumstantial but it appears that the conversion of GA$_7$, not an endogenous Phaseolus vulgaris gibberellin, to GA$_1$ or GA$_3$ was made. The conversion of GA$_7$ to GA$_3$ involves an addition of an (-OH) group at the 7 position on the gibbane skeleton. This is the same type of reaction that is involved in the conversion of GA$_5$ to GA$_1$ reported in other species.

The 2,4-D treatments with the concurrent application of gibberellins yielded levels of activity in the tissues that approached the levels of the samples which had been treated with gibberellins alone. At first exposure that data appear to indicate that the amount was independent of the 2,4-D application. However, the amount of tissue extracted in each sample must be considered. The treatments involving gibberellins alone yielded harvested tissue weights that were relatively equal. The data show the expected trend that the greater the treatment concentration the greater the amount present in the tissue. Utilizing estimates for the activities of the fractions and correcting for dilution factors, the results show that the one ppm GA$_7$ treatment approached 1.1 micrograms per 44.1 grams of tissue. A
good approximation cannot be given for the 5 ppm GA\textsubscript{7} treatment due to the loss of the internal standards for that treatment. However, it can be noted that more activity than indicated for the one ppm treatment is evident. The treatments involving 2,4-D were approximated in a similar manner and corrected for weight differences and estimated. The yield was 3.3 micrograms per 44 g tissue for the 1 ppm 2,4-D plus 1 ppm GA\textsubscript{7} treatment. The yield was estimated at 4.2 micrograms per 44 g tissue for the 1 ppm 2,4-D plus 5 ppm GA\textsubscript{7} treatment. It appears that applied GA\textsubscript{7} did result in a considerable increase in the level of GA\textsubscript{3} in the tissues and indicated a conversion. When 2,4-D was added concurrently with the GA\textsubscript{7} it is noted that the amount present in the tissues of the treatments is much less than when GA\textsubscript{7} was added alone. The amount is still more than when 2,4-D was used alone. This result indicates that although GA\textsubscript{7} may be converted to GA\textsubscript{3} in these tissues the application of 2,4-D might limit the amount that is converted.
SUMMARY

The endogenous gibberellin status of the emerging root-hypocotyl of germinating *Phaseolus vulgaris* seedlings was investigated and the gibberellins found were GA₁ and/or GA₃ and possibly GA₈. The work has shown that the preponderance of gibberellin activity in these tissues was due to the GA₁₃ fraction of the extracted fractions.

The herbicidal rate of 2,4-D (1 ppm) resulted in a reduction in the levels of gibberellins in the root-hypocotyl tissue when used in the germination solution.

Applied GA₇ was utilized both with and without 2,4-D to determine the effect upon endogenous gibberellin levels. It was shown that endogenous levels could be increased substantially by the GA₇ treatments. The 2,4-D plus GA₇ treatments showed that endogenous levels were increased over the 2,4-D alone treatment but the levels were lower than when the GA₇ was used alone.

It was noted that the applied GA₇ resulted in an increase in activity of the GA₁₃ fractions of the sample. Investigation showed that no activity was detectable in the fractions where GA₇ would normally elute and that the preponderance of activity was due to the GA₁₃ fraction. It is suggested that the GA₇ could have been converted to GA₃ in the tissues.
1. Allard, R. W., H. R. DeRose, and C. P. Swanson. 1946. Some
effects of plant growth-regulators on seed germination and

sRNA synthetases of Phaseolus vulgaris as related to germi-
nation. Plant Physiol. 44:60-68.

B:7.

several herbicides on proteolytic activity of squash seedlings.

5. Audus, L. J. 1964. The Physiology and Biochemistry of Herbi-

activation of 2,4-dichlorophenoxyacetic acid and cessation of

7. Baxter, R. 1967. The effect of 2,4-dichlorophenoxyacetic acid
upon the metabolism and composition of soybean hypocotyl
mitochondria. Dissertation Abstracts 28:4887-B.

8. Beal, J. M. 1945. Histological reactions of bean plants to
certain of the substituted phenoxy compounds. Botanical

1963. Comparative effects of 2,4-dichlorophenoxyacetic acid
on nitrate metabolism in corn and cucumber. Plant Physiology
38:675-679.

10. Black, C. C. and T. E. Humphreys. 1962. Effects of 2,4-dichloro-
phenoxyacetic acid on enzymes of glycolysis and pentose


34. Kaufman, P. B., N. Ghosheh, and H. Ikuma. 1968. Promotion of
growth and invertase activity by gibberellic acid in develop­
ing avena internodes. Plant Physiol. 43:29-34.

35. Key, J. L. 1963. Studies on 2,4-D induced changes in
ribonucleic acid metabolism in excised corn mesocotyl tissue.

36. ______. 1964. Ribonucleic acid and protein synthesis as
essential processes for cell elongation. Plant Physiol. 39:
365-370.


38. Key, J. L. and J. B. Hanson. 1961. Some effects of 2,4-dichloro­
phenoxyacetic acid on soluble nucleotides and nucleic acid of

39. Key, J. L., J. B. Hanson, and R. F. Bils. 1960. Effect of 2,4-
dichlorophenoxyacetic acid application on activity and com­
oposition of mitochondria from soybeans. Plant Physiology 35:
177-184.

Relation of 2,4-D induced growth aberrations to changes in
nucleic acid metabolism in soybean seedlings. Botanical
Gazette 127:87-94.

41. Key, J. L. and J. C. Shannon. 1964. Enhancement by auxin of
ribonucleic acid synthesis in excised soybean hypocotyl tissue.
Plant Physiology 39:360-364.

Gradient elution column chromatography systems for the
separation and identification of gibberellins. Analytical
Biochemistry 12:113-118.

and Food Chemistry 17:1294-1297.

44. Kozel, P. C. and H. B. Tukey, Jr. 1968. Loss of gibberellins by
leaching from stems and foliage of Chrysanthemum morifolium

investigations of the effect of tordon and 2,4-D on leaf and
root tissue. Down to Earth 23:21-23.

46. Luecke, R. W., C. L. Hammer, and H. M. Sell. 1949. Effect of
2,4-dichlorophenoxyacetic acid on the content of thiamine,
riboflavin, nicotinic acid, pantothenic acid and carotene in stems and leaves of red kidney bean plants. Plant Physiology 24:546-548.


