NITROGEN NUTRITION AND METABOLISM AS IT AFFECTS TRANSLOCATION PATTERNS OF AUXINS

By

JEAN ALICE PITTMAN WINTERS

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Thesis Approved:

Thesis Adviser

Dean of the Graduate College

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ABBREVIATIONS

ATPase	Adenosine triphosphatase
ATP	Adenosine triphosphate
2,4-D	2,4-dichlorophenoxyacetic acid
DPM	Disintegrations per minute
DCCD	N,N-dicyclohexylcarbodiimide
IAA	Indole-3-acetic acid
NPA	Sodium alanap (N-1-naphtylphthalamic acid)
OAA	Oxaloacetate
PEP	Phosphoenolpyruvate
RNA	Ribonucleic acid
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
2,3,5-TIBA	2,3,5-triiodobenzoic acid

CHAPTER I

INTRODUCTION

The auxin indole-3-acetic acid is one of the most widely studied of the plant hormones, perhaps because of the hormone's characteristic role in plant growth and development. The coordination of growth processes occurs, in part, by the control of the transport of auxins as well as other plant hormones. A site of synthesis of auxin is believed to be in the young shoots and developing leaves. To be effective as a growth promoting hormone, auxin must be transported from the shoot to the area of cell elongation where the auxin accumulates in the proper concentrations.

Goldsmith (1977), reported on two types of transport for exogenously applied auxins. In the first type called polar auxin transport, auxins moved at a relatively slow rate from the young leaves at the apex towards the roots. Morris et al. (1969) showed that IAA moved at a velocity of 11 mm/hr when applied in a 5-µl droplet, containing 0.25 µ ci of ^{14}C IAA, to the undamaged apical bud of an intact twelve to fourteen day old Pisum sativum L. CV Meteor seedling. Hollis and Tepper (1971) reported a similar velocity for the translocation of IAA in two year old

white ash (Fraxinus americanus L.) saplings. This translocation is auxin-specific as experiments have shown that other labeled compounds such as sugars, cytokinins, purine bases, and amino acids are not transported in this manner. Polar auxin transport has other distinctive characteristics: it can be blocked by the compound 2,3,5-TIBA (Morris et al., 1974) and transport does not seem to occur via the phloem since aphids (Acyrthosiphon pisum) feeding on the stem below the site of application of label do not become radioactive (Morris and Kadir, 1972).

The other type of auxin translocation has different characteristics. This type of translocation occurs when the auxin is exogenously applied to a mature leaf or stem. It has a transport velocity of 10-24 cm/hr. Movement is acropetal as well as basipetal (Goldsmith et al., 1974). Lepp and Peel (1971), applied labeled IAA or labeled sugars to an abrasion made in a segment of willow (Salix viminalis L.) stem and collected honeydew from aphids feeding below the source. Since honeydew contained both labeled sugars and labeled IAA, Lepp and Peel (1971) concluded that the IAA must be transported via phloem. Since intact plants were not used in this experiment the validity of this conclusion can be questioned. Eschirch (1968) fed Vicia faba L. seedlings with 0.005 molar IAA through the first primary leaf and was able to collect labeled IAA in the honeydew of aphids feeding on the stem. Microautoradiography indicates that labeled auxins are concentrated in the phloem of the midrib

and petiole of the fed leaf (Goldsmith et al., 1974). Perhaps most distinctive of this form of transport is that application of 2,3,5-TIBA does not block auxin translocation (Goldsmith et al., 1974).

The modern theory of polar auxin transport called the chemiosmotic polar diffusion theory (Goldsmith, 1977), was independently suggested by Rubery and Sheldrake (1974) and Raven (1975). This model suggests that cells expend ATP to maintain a pH gradient so that the pH is lower outside the cell than in the cytoplasm. Because of this pH gradient, IAA molecules are less dissociated in the acid environment exterior to the cell, are fat soluble, and are able to passively diffuse into cells. Once the molecule is within the cytoplasm it encounters a higher pH which induces the molecule to ionize and become fat insoluble. It is thus trapped within the cell because of the impermeability of this form of the molecule in the fats of the plasma membrane. Polar transport occurs if it is assumed that the basal end of the cell has a plasmalemma that is either more permeable or has more auxin anion carriers than the apical end of the cell.

Recently research interests have concentrated on factors involved in auxin uptake and efflux. Auxin uptake is now thought to occur by the cooperation of two mechanisms; the diffusion of undissociated auxin through the membrane, or by a saturable, specific, proton/IAA anion symport located on the plasma membrane (Rubery 1978, and

Hertel et al., 1983). Polar transport is thought to be due to a specific anion exporter located at the basal end of cells as suggested by Sheldrake (1974). Jacobs et al. (1983) showed by immunofluorescence techniques that specific parenchyma cells associated with vascular bundles contained proteins having affinity for NPA. These sites were predominantly located at the basal end of these cells. It was assumed that this protein represents the auxin anion transporter which is responsible for auxin efflux at the basal end of the cell and results in polar auxin transport. This site is inhibited by 2,3,5-TIBA and NPA (Thomson et al., 1973).

Although the evidence supporting the chemiosmotic polar diffusion model is mostly indirect in nature, the evidence is very convincing. As stated by Cleland (1973), the acid growth theory implies that the action of IAA is to cause proton secretion which results in enhanced cell elongation. This action is probably accompanied by changes in extracellular as well as intracellular pH. Hodges (1973) has presented evidence that a plasmalemma ATPase utilizes ATP as the energy source for proton pumping. The ATPase inhibitor DCCD was found to inhibit auxin-induced proton efflux of Avena coleoptiles (Cleland, 1982). These findings lend strong support to the view that ATPase causes proton efflux. Furthermore, DCCD has been found to inhibit the translocation of 2,4,5-T injected into bean cotyledonary nodes (Thesis, C.W. Corbett 1977). The loading of sucrose

into the phloem has been shown to be regulated by pH gradients between the phloem (about pH 8-8.5) and the surrounding apoplast (about pH 5.0) (Giaquinta, 1977). Recently, pH gradients have been shown to be important in the uptake of 2,4-D into suspension-cultured Acer pseudoplatanus L. cells (Leguay and Guern, 1977) and the uptake of IAA into Avena coleoptile segments (Hasenstein and Rayle, 1984). Furthermore, pH gradients are believed to control the uptake of IAA into Cucurbita pepo L. membrane vesicles (Hertel et al., 1983).

This thesis will discuss some further work on auxin translocation. Various types of evidence have been found that imply that compounds can affect the loading and unloading of auxins into the veins of bean plants. This evidence suggests the hypothesis that one factor regulating auxin vein loading is the pH gradient between the apoplast and symplast. Many plants are known to have a pH stat mechanism which regulates intra and extra cellular pH. It seems possible that the differences in the pathways of auxin transport in our various experiments may be due to effects on the pH stat mechanism. It has been found that the protein synthesis inhibitor cycloheximide has a profound effect on auxin translocation, which seems to indicate a strong relationship between protein synthesis and long distance auxin translocation. Perhaps cycloheximide has some effect on the maintenance of the pH gradient between the symplast and the apoplast. Robert Cleland (1982) also

noted that protein synthesis is a requirement for auxin-induced proton excretion. He proposed two possible physiological reasons for this phenomena. First, that the auxin sensitive ATPases on the plasma membrane are very labile. Thus protein synthesis is needed to make the specific enzyme ATPase. Second, perhaps a specific protein is not what is required, but rather protein synthesis in general may use up some compound which acts as an inhibitor to the ATPase.

In this thesis evidence will also be presented that an increase in apoplastic translocation of auxins is obtained by the treatment of plants via the nutrient solution with NH_4HCO_3. The NH_4HCO_3 probably has some effect on the pH stat mechanism. Through the use of the PEP carboxylase enzyme system, the pH stat mechanism controls cellular pH by the synthesis and degradation of malate in the cytoplasm. Recently, Schweizer and Erismann (1985) have shown that the cell sap from primary leaves of Phaseolus vulgaris L. has a lower pH, when plants are given nutrient solution containing ammonium as the nitrogen source, than when nitrate is the nitrogen source. They were also able to show that the extractable activity of PEP carboxylase in the primary leaves was higher under nitrate nutrition than under ammonium nutrition.

Previous work showed that the stem injection technique used to apply auxins to plants resulted in both acropetal and basipetal movement (Basler et al., 1970). However many

factors such as environmental and chemical inhibitors have been found to have an influence on the direction of the translocation of the injected auxins (Basler et al., 1961; Basler et al., 1970; Long and Basler, 1973; and Basler, 1977). It is important to study auxin transport not only for a better understanding of growth and development but also to learn about factors which determine how growth regulators and herbicides are translocated and to develop new ideas for the improvement of crop production.

This study was conducted to examine the ways the form of nitrogen given to bean plants affects the translocation of auxins and amino acids. The effects of protein synthesis inhibitors and amino acid analogs were also studied to clarify the relationship between protein synthesis and auxin translocation. Whether protein synthesis is needed to maintain pH gradients used in auxin uptake, or as an auxin carrier will be discussed.

CHAPTER II

MATERIALS AND METHODS

Bush bean seeds (Phaseolus vulgaris L. CV Stringless Green-Pod) were imbibed by soaking in distilled water with aeration for two to three hours. The seeds were then placed in vermiculite moistened with one-half strength Hoagland's nutrient solution and germinated in a growth chamber with continuous light of 222 $\mu E \cdot m^{-1} \cdot s^{-2}$ and a constant temperature of 33° C. After five days the seedlings were transplanted to amber jars containing 400 ml of aerated one-half stength Hoagland's nutrient solution and placed in the growth chamber in a completely randomized manner. Plants were grown to the age of nine days with 14 hour, 29° C days and 10 hour, 27° C nights.

Plants grown with nitrogen deficient nutrient solution were treated as above except that prior to the last two days before treatment they were transferred to a nutrient solution in which the nitrate ion was replaced with chlorine. Treatments to study the effect of certain salts in the nutrient solution on the translocation of auxins were carried out by transferring plants to one-fourth strength Hoagland's nutrient solution containing the indicated concentration of salts. The pH of these solutions was

adjusted to pH 7 with the appropriate acid or base (HCl, NaOH, or NH_4OH).

Plants were treated by injecting 1-µl of [^{14}C] auxins or amino acids mixed in 95 % ethanol. The chemicals were injected into plants and deposited below the cotyledonary node in the following manner. The needle of a 1-µl pipet was inserted at the cotyledonary node and forced down the center of the stem in the pith tissue to about 1 cm below the cotyledonary node. The treated plants were then returned to the growth chamber for four or twenty-four hours before being harvested.

At harvest the plants were separated into young shoots including all tissue above the primary leaves, primary leaves including the petioles, epicotyl including all tissue above the cotyledonary node to the primary leaves, treated area including all tissue 0.5 cm above the cotyledonary node and tissue 1.5 cm below the cotyledonary node, hypocotyl including the remainder of the stem down to the roots, and roots. A five ml sample was taken from each nutrient solution. All the plant parts were freeze-dried and homogenized in 95 % ethanol with a high speed homogenizer. Samples of 0.5 ml or less were taken from the homogenate and were assayed for radioactivity by liquid scintillation counting.

The data were analyzed using either the DPM or the percent of the recovered ^{14}C label to calculate the vein loading and apoplastic/symplastic transport ratio of the

injected radioactive material. The percent vein loading was determined using the following equation:

$$\% \text{ vein loading} = \frac{\text{Total } ^{14}C \text{ recovered} - \text{Treated area } ^{14}C}{\text{Total } ^{14}C \text{ recovered}} \times 100$$

This is actually just a measurement of the percent of the label that was translocated out of the treated area. This is thought to be a measure of vein loading since previous work showed that metabolic inhibitors such as DCCD inhibited the movement of stem injected [1-^{14}C]2,4,5-T from the treated area (Basler, 1977). Metabolic inhibitors should not have any affect on simple diffusion. Thus, translocation of the label out of the treated area must involve uptake into parenchyma or cells of the vascular bundles. Therefore movement out of the treated area may be used as a measure of vein loading.

The apoplastic/symplastic transport ratio was determined using the following equation:

$$\text{apoplastic/symplast transport ratio} = \frac{\text{primary leaves } ^{14}C}{\text{hypocotyl, roots and nutrient } ^{14}C}$$

It is believed that this is a measure of vein unloading since previous work has shown that the metabolic inhibitor DCCD enhanced the apoplastic transport of [^{14}C]IAA to the primary leaves (Basler 1977). Since a metabolic inhibitor should neither inhibit nor enhance simple diffusion in the apoplast, this enhanced apoplastic translocation must result from efflux from parenchyma or phloem cells adjacent to the water transpiration stream in the xylem. Since the enhanced apoplastic translocation was accompanied by a simultaneous

decrease in basipetal translocation, the ratio between these two types of translocation (the apoplastic/symplastic transport ratio) is a convenient expression of vein unloading.

Statistical analyses included standard F tests and LSD at the 5 % level.

The radioactive chemicals used include: 2,4,5-T[1-^{14}C] (54 ci/mole), IAA[1-^{14}C] (48 ci/mole), 2,4-D[1-^{14}C] all of which were obtained from Amersham, glycine[1-^{14}C] (52.4 ci/mole), L-glutamic acid[U-^{14}C] (200 ci/mole), and L-arginine[U-^{14}C] (300 ci/mole) obtained from Research Products International and L-leucine[U-^{14}C] (270 ci/mole) obtained from ICN Pharmaceuticals, INC. Other chemicals used include cordycepin, puromycin, chloramphenicol, DL-ethionine, beta-2-thienyl alanine, cycloheximide, iminodiacetic acid, and bis tris propane all obtained from Sigma.

CHAPTER III

RESULTS

The Effect of Protein Synthesis Inhibitors on the Translocation of Auxins

Various protein synthesis inhibitors were used to determine the requirement of protein synthesis for auxin translocation. The protein synthesis inhibitors puromycin and chloramphenicol as well as the RNA synthesis inhibitor cordycepin all significantly increased vein loading of the auxin 2,4,5-T, while vein unloading was significantly increased by chloramphenicol, cordycepin and cycloheximide (Table I). Injections with a solution containing 5 µg of cordycepin and 2,4,5-T [1-^{14}C] increased vein loading to 118 % of the control and increased vein unloading to 230 % of the control. Injection of 15 µg chloramphenicol also increased both loading and unloading, while injection of 10 µg puromycin increased loading but not unloading. The protein synthesis inhibitor cycloheximide also had an effect on auxin translocation. Injection of 10 µg of cycloheximide along with 2,4,5-T[1-^{14}C] resulted in a significant increase in vein unloading. These data indicate an important role for protein synthesis in auxin vein loading and unloading.

The Effect of Nitrogen Deficiency on the Translocation of Auxins and Amino Acids

All proteins contain nitrogen, and a lack of nitrogen as a nutrient should inhibit the synthesis of new proteins. The results (Table II) show that a two day period of nitrogen starvation caused a significant increase in the vein unloading of 2,4,5-T[1-^{14}C]. Two days of nitrogen starvation did not cause a significant increase in vein unloading of IAA[1-^{14}C]. Nitrogen deficiency in the nutrient solution seems to cause a larger proportion of the 2,4,5-T[1-^{14}C] to accumulate in the primary leaves as well as to cause a decrease in the downward translocation of 2,4,5-T[1-^{14}C] (Table III). IAA did not act in this manner.

Amino acids can be in either the ionized or unionized forms depending on the pH of the surroundings. Thus, transport of amino acids possibly can be influenced by a pH gradient in a manner similar to auxins. When bean seedlings were injected with ^{14}C-labeled amino acids, the amino acid arginine showed a significant decrease in vein unloading when exposed to two days of nitrogen deficiency (Table II). However, observation of the radioactivity in each plant part (Table III) shows that only leucine exhibits an increase accumulation of the label in the nutrient solution in the nitrogen deficient plants as compared to control plants that recieved complete nutrient solutions. When the data is expressed as a percent of the control and all treatments are compared, leucine and arginine accumulate to a larger extent

in the roots and nutrient solution than 2,4,5-T. Plants grown under nitrogen deficient conditions typically have stunted tops and well developed roots (Miller, 1938).

The Effect of Amino Acid Analogs on the Translocation of Auxins

To further investigate Cleland's two hypotheses on the possible relationship between protein synthesis and auxin-induced proton excretion, we injected beans with various amino acid analogs and traced the effect this treatment had on auxin translocation. If protein synthesis is needed to make a specific protein such as ATPase, then the presence of an amino acid analog should have an effect on loading and unloading since the proteins made would contain the amino acid analog and would thus be inactive. On the other hand, if the requirement is just protein synthesis in general then the presence of the analogs should not affect loading or unloading. The plants injected with the amino acid analog ethionine exhibited a decrease in the loading of 2,4,5-T [1-^{14}C] while the treatments with the amino acid analog beta thienyl alanine exhibited a significant increase in unloading of 2,4,5-T[1-^{14}C] (Table IV). There were no significant differences in loading or unloading in the bean plants given amino acid analogs and injected with IAA [1-^{14}C].

The Effects of Various Combinations of High and Low Levels of Nutrient Solution Nitrate, Phosphate,and Sulfate on the Translocation of IAA

The translocation of auxins were affected by the levels of nitrate, phosphate and sulfate in the nutrient solution. Bean seedlings were given a pretreatment of either high or low levels of nitrate, phosphate and sulfate four hours before injection with 0.5 µg of IAA[1-^{14}C]. The results showed that when nitrate was deficient and phosphate and sulfate were low the value for vein unloading was high (1.08) which was a significant increase in vein unloading of IAA as compared to unloading (0.46) when all three of the anions were kept at a high value (Table V). When one of the nutrients was kept at a high level in relation to the other two there was a tendency for a decrease in vein unloading. For example, when sulfate was high in relation to the others, the value for vein unloading was 0.56, and when nitrate was high, the value for vein unloading was 0.63. Both of these values are significantly lower than 1.08; the value for unloading when all anions were kept low. When phosphate was high the value for vein unloading was 0.71 which, while not significant, showed a tendency towards a decrease in vein unloading. The effects of the three anions appear to be somewhat additive.

The Effects of Ammonium Bicarbonate on the Translocation of Auxins and Amino Acids

Some of the most striking effects on loading and unloading of auxins were obtained by treatment of bean seedlings with ammonium bicarbonate via the nutrient solution. The 15 mM ammonium bicarbonate treatment caused a significant increase in the vein unloading of [1-^{14}C]2,4,5-T (Table VI). The effects of 15 mM sodium bicarbonate and 15 mM ammonium chloride were also examined to determine if bicarbonate or ammonium was the active ion. The increase in vein unloading seemed to occur specifically in response to ammonium bicarbonate since neither ammonium chloride nor sodium bicarbonate significantly affected 2,4,5-T[1-^{14}C] translocation. Ammonium bicarbonate significantly increased the vein unloading of both the auxins IAA and 2,4,5-T while there was a slight decrease in vein unloading of leucine. However, the effect on leucine was not significantly different from the control (Table VII).

Vein unloading of auxins was also significantly increased when the 15 mM ammonium bicarbonate was placed in the nutrient solution four hours before treatment and samples harvested four hours after treatment (Table VIII) as compared to the previous experiments in which the 15 mM ammonium bicarbonate was placed in the nutrient solution at the time of injection and harvested twenty-four hours later (Table VII). Vein loading of 2,4,5-T[1-^{14}C] was significantly increased only in this experiment (Table III).

The translocation of the amino acids glycine, arginine, and glutamic acid were not significantly changed by the 15 mM ammonium bicarbonate treatments. The plants treated with ammonium bicarbonate demonstrated a tendency for a decrease in vein unloading of amino acids. This is in contrast to the increased vein unloading of auxin in similarly treated plants. It was also noted that there was a significant increase in the ^{14}C-labeled amino acids recovered in these treatments as compared to the control Table VIII).

The Effects of Various Ammonium Compounds on the Translocation of IAA

The effect of 15 mM concentrations of various ammonium compounds on the translocation of IAA[1-^{14}C] was examined (Table IX). Bean seedlings were transferred to one-fourth strength Hoagland's nutrient solution containing 15 mM concentrations of either ammonium chloride, ammonium acetate, ammonium bicarbonate, ammonium sulfate, ammonium phosphate, ammonium nitrate or ammonium vanadate. When vein unloading was calculated in the usual manner only the ammonium chloride treatment caused a significant increase in vein unloading. Ammonium bicarbonate in this experiment caused a decrease in vein loading and no significant change in vein unloading of IAA. This was not noted in any of the previous experiments (Table IX). The young shoots were well developed on these plants so it is possible that this tissue acted as a sink for apoplastic translocation. The results

were recalculated to determine the shoot/root ratio. This value was determined by dividing the total DPM recovered in the young shoot by the sum of the DPM recovered in the hypocotyl, roots and nutrient solution. When the data are calculated in this manner there was a significant increase in the translocation of IAA upwards to the shoot for the ammonium bicarbonate treatments as well as for the ammonium acetate and ammonium phosphate treatments. Also noted was an increase in apoplastic transport with ammonium chloride. The ammonium nitrate treatment showed a tendency towards a decrease in vein unloading however this value is not significantly different from the control.

The Effects of Ammonium Bicarbonate, Potassium Nitrate and Interaction with Cycloheximide on Auxin Translocation When pH is Controlled by CO_2/Air Aeration.

After several of the described experiments had been completed, it was discovered that when the nutrient solutions containing ammonium bicarbonate were aerated the pH rose to 8.3. The pH of the nutrient solutions containing potassium nitrate remained at pH 6.7. To control this variable in this experiment nutrient solutions containing ammonium bicarbonate were aerated with a carbon dioxide-air mixture. This proceedure caused the pH to stay at a fairly constant pH 6.2.

There were no significant differences in vein loading or vein unloading of IAA caused by the ammonium bicarbonate treatments as compared to the potassium nitrate treatments. This was in contrast to the results of previous experiments in which ammonium bicarbonate significantly increased vein unloading of IAA. Cycloheximide increased loading only in the presence of potassium nitrate. Cycloheximide enhanced unloading of IAA in both ammonium and nitrate nutrient solutions but to the greatest extent in the presence of ammonium as the nitrogen source.

Ammonium bicarbonate, when compared to potassium nitrate treatments, caused a significant increase in the vein unloading of 2,4-D (Table X) an effect which was not noticed when IAA was used as the auxin. There were no significant differences in vein loading of 2,4-D between the ammonium and nitrate treatments. Cycloheximide caused a significant decrease in the vein loading in the presence of potassium nitrate. On the other hand cycloheximide enhanced unloading most in the presence of ammonium bicarbonate.

The Effects of Injection of Ammonium and Nitrate on the Translocation of Auxins

The plants were injected in the stem with auxin and with or without either ammonium, nitrate or chloride as the salts of large impermeable cations or anions. These treatments allowed examination of the effects of the ammonium, nitrate or chlorine independently since the

associated large cation or anion is impermeable. The injection of three micro-equivalents of ammonium caused a significant increase in the vein unloading of IAA, but injection with three micro-equivalents of nitrate did not cause a significant change in vein unloading as compared to the control (Table XI). Injection with three micro-equivalents of chlorine caused a significant increase in vein unloading of IAA. Vein loading of IAA was not affected by injection with nitrate, ammonium, or chlorine into the stem with the auxin. The vein unloading of 2,4,5-T was not significantly affected by the injection with nitrate, ammonium, or chloride. Vein loading of 2,4,5-T was decreased by ammonium injection.

TABLE I

EFFECTS OF PUROMYCIN, CHLORAMPHENICOL AND CORDYCEPIN ON THE TRANSLOCATION OF 2,4,5-T[1-^{14}C]

Plants were grown in one-half strengh Hoagland's nutrient solution. Inhibitors were injected with a 1-µl syringe along with the 0.5 µg 2,4,5-T[1-^{14}C]. Plants were harvested four hours after injection.

Treatment	Percent of the control: Vein loading	Vein unloading
EXPERIMENT 1		
10 µg/µl Puromycin	107*	143
15 µg/µl Chloramphenicol	114*	173*
5 µg/µl Cordycepin	118*	230*
EXPERIMENT 2		
10 µg/µl Cycloheximide	86	243*

* Indicates significant differences from the control at the 0.05 level.

TABLE II

EFFECTS OF NITROGEN DEFICIENCY IN THE NUTRIENT SOLUTION ON THE TRANSLOCATION OF ^{14}C AUXINS AND AMINO ACIDS

Plants were grown in one-half strength Hoagland's nutrient solution and at the time of treatment were transferred to nutrient solution in which the nitrate was replaced with chloride. Control plants were placed in one-half strength Hoagland's solution. Plants were injected with either 0.5 µg 2,4,5-T[1-^{14}C], 0.5 ug IAA[1-^{14}C], 0.022 µg Leucine[U-^{14}C], 0.092 µg Glutamic acid[U-^{14}C], or 0.073 µg Arginine[U-^{14}C]. Harvest was four hours after injection.

Treatment	Total ^{14}C Recovered (%)	Vein loading (%)	Vein unloading
2,4,5-T[1-^{14}C]			
Control	70	68	0.14
2 Day N Starvation	63	60	0.27*
IAA[1-^{14}C]			
Control	74	47	0.40
2 Day N Starvation	65	49	0.30
Leucine[U-^{14}C]			
Control	54	77	2.35
2 Day N Starvation	51	63	2.10
Glutamic Acid[U-^{14}C]			
Control	58	75	7.15
2 Day N Starvation	70	80	8.88
Arginine[U-^{14}C]			
Control	71	75	4.13
2 Day N Starvation	63	78	2.80*

* Indicates significant differences from the control at the 0.005 level. Vein unloading is expressed as the apoplastic/symplastic transport ratio.

TABLE III

THE EFFECTS OF TWO DAYS OF NITROGEN STARVATION ON THE TRANSLOCATION OF ^{14}C AUXINS AND AMINO ACIDS TO VARIOUS PLANT PARTS

Treatment	% RECOVERY						
	Young shoot	Pri. leaf	Epi-cotyl	Treat. area	Hypo-cotyl	Roots	Nutr. sol.
2,4,5-T[1-^{14}C]							
control	0.11	4.84	8.51	21.66	20.83	3.85	10.27
2 Day	0.09	6.51*	5.94	25.35	17.47	2.58	5.43*
% Control	84a	135c	70ab	117ab	84b	66a	53a
IAA[1-^{14}C]							
control	2.40	10.14	6.50	53.32	17.92	9.53	0.20
2 Day	1.53	9.19	6.93	51.16	21.90	9.07	0.22
% Control	64a	91ab	107bc	96a	122b	95a	112ab
Leucine[U-^{14}C]							
control	5.31	21.30	4.19	12.55	5.90	4.57	0.17
2 Day	3.09*	14.51*	5.15	20.30	1.48*	2.57*	3.55*
% Control	58a	68a	123c	162b	25a	56a	2044c
Glutamic acid[U-^{14}C]							
control	1.64	60.39	3.51	25.30	6.89	2.14	0.13
2 Day	0.74*	67.69	2.31*	20.05	7.19	1.93	0.10
% Control	45a	112bc	66a	79a	104b	90a	77a
Arginine[U-^{14}C]							
control	5.04	50.12	6.21	24.83	5.62	8.12	0.07
2 Day	5.87	46.00	6.23	22.24	5.89	13.31	0.46
% Control	116b	92ab	100abc	90a	105b	164b	633b

* Indicates significant differences from the control at the 0.05 level. Values followed by the same letter are not significantly different at the 5 % level.

TABLE IV

EFFECTS OF TREATMENTS WITH THE AMINO ACID ANALOGS ON THE TRANSLOCATION OF ^{14}C AUXINS.

Plants were grown in one-half strength Hoagland's nutrient solution. Nine day old plants were injected with 1-µl of 80 µg/µl solution of either DL-Ethionine or Beta-2-Thienyl-DL-Alanine. The injection site was marked and the plants were injected at the same site four hour later with 0.5 µg/µl of either IAA[1-^{14}C] or 2,4,5-T[1-^{14}C]. Plants were harvested four hours after injection.

	Percent of the control		
Treatment	Total ^{14}C Recovered	vein loading	vein unloading
IAA[1-^{14}C]			
DL Ethionine	96	83	58
Beta-2-Thienyl Alanine	98	96	127
2,4,5-T[1-^{14}C]			
DL Ethionine	90	72*	82
Beta-2-Thienyl Alanine	99	92	228*

* Indicates significant differences from the control at the 0.05 level.

TABLE V

EFFECTS WITH HIGH AND LOW LEVELS OF NITRATE, PHOSPHATE, AND SULFATE ON THE TRANSLOCATION IAA[1-^{14}C]

Twenty-four hours before treatment plants were transferred from one-half strength Hoagland's nutrient solution to nutrient solution in which the phosphate, sulfate, nitrate and sodium were varied by adding sodium salts to obtain either 1 or 5 mM phosphate, sulfate or nitrate anions. Other components of the nutrient solution were not varied from the one-half strength Hoagland's concentration. NO_3 was supplied as either KNO_3 or $NaNO_3$, PO_4 was supplied as either NaH_2PO_4, K_2HPO_3 , or H_3PO_4 and SO_4 was supplied as Na_2SO_4, K_2SO_4 or $MgSO_4$. The pH of these solutions was adjusted to 5.5 with 1 N NaOH and 1 M H_2SO_4. Four hours before harvest the plants were injected with 0.5 µg of IAA[1-^{14}C].

Treatment						
mM NO	mM PO	mM SO	mM Na	Total ^{14}C recovered (%)	Vein loading (%)	Vein unloading
5	5	5	12.0	85 ab*	51 a	0.46 a
5	5	1	8.0	77 a	58 a	0.61 a
5	1	5	8.0	93 ab	57 a	0.73 ab
5	1	1	4.0	81 ab	52 a	0.63 a
0	5	5	7.5	66 a	52 a	0.49 a
0	5	1	3.5	88 ab	53 a	0.71 ab
0	1	5	3.5	81 ab	55 a	0.56 a
0	1	1	0.0	72 a	61 a	1.08 b

* Values followed by the same letter are not significantly different at the 5% level. Vein unloading is expressed as the apoplastic/symplastic transport ratio.

TABLE VI

EFFECTS OF AMMONIUM BICARBONATE, SODIUM BICARBONATE AND AMMONIUM CHLORIDE IN THE NUTRIENT SOLUTION ON THE TRANSLOCATION OF 2,4,5-T [1-^{14}C]

Plants were transferred at the time of injection, from one-half strength Hoagland's nutrient solution to one fourth-strength Hoagland's nutrient solution containing the concentration of the ammonia salt indicated below. Control plants recieved one-fourth strength Hoagland's nutrient solution. Plants were injected with 0.5 µg of 2,4,5-T [1-^{14}C]. Plants were harvested twenty-four hours after injection.

	Percent of the control		
Treatment	Total ^{14}C Recovered	Vein loading	Vein unloading
15 mM AMMONIUM BICARBONATE	83*	100	268*
15 mM SODIUM BICARBONATE	93	107	122
15 mM AMMONIUM CHLORIDE	85*	95	198
15 mM SODIUM CHLORIDE	95	105	76

* Indicates significant differences from the control at the 0.05 level.

TABLE VII

EFFECTS OF 15 mM AMMONIUM BICARBONATE IN THE NUTRIENT SOLUTION ON THE TRANSLOCATION OF ^{14}C AUXINS AND LEUCINE

Plants were transferred from one-half strength Hoagland's nutrient solution to one-fourth strength Hoagland's solution containing 15 mM ammonium bicarbonate at the time of injection. Control plants recieved one-fourth strength Hoagland's nutrient solution. Plants were injected with 0.5 µg/µl of IAA [1-^{14}C], 0.5 µg/µl 2,4,5-T [1-^{14}C] or 0.022 µg/µl L leucine [U-^{14}C]. Plants were harvested twenty-four hours after injection.

	Percent of the control		
Treatment	Total ^{14}C Recovered	Vein loading	Vein unloading
IAA [1-^{14}C]	108	100	276*
2,4,5-T [1-^{14}C]	83*	100	268*
L Leucine [U-^{14}C]	95	102	92

* Indicates significant differences from the control at the 0.05 level.

TABLE VIII

EFFECTS OF A FOUR HOUR TREATMENT OF 15 mM AMMONIUM BICARBONATE IN THE NUTRIENT SOLUTION ON THE TRANSLOCATION OF ^{14}C AUXINS AND AMINO ACIDS

Plants were transferred from one-half strength Hoagland's nutrient solution to one-fourth strength Hoagland's nutrient solution containing 15 mM ammonium bicarbonate four hours before injection. Control plants recieved one-fourth strength Hoagland's nutrient solution. Plants were injected with either 0.5 µg IAA[1-^{14}C], 0.5 µg 2,4,5-T[1-^{14}C], 0.092 µg glutamic acid [U-^{14}C], or 0.073 µg arginine[U-^{14}C], 0.179 µg glycine[1-^{14}C]. Plants were harvested four hours after injection.

	Percent of the control		
Treatment	Total ^{14}C Recovered	Vein loading	Vein unloading
IAA[1-^{14}C]	108	87	201*
2,4,5-T[1-^{14}C]	94	129*	444*
Glycine[1-^{14}C]	140*	92	59
Arginine[U-^{14}C]	127*	92	89
Glutamic acid[U-^{14}C]	159*	116	73

* Indicates significant differences from the control at the 0.05 level.

TABLE IX

EFFECTS OF AMMONIUM WITH VARIOUS ANIONS IN THE NUTRIENT SOLUTION ON THE TRANSLOCATION OF IAA[1-^{14}C]

Four hours before treatment plants were transferred from one-half strength Hoagland's nutrient solution to one-fourth strength Hoagland's nutrient solution also contained 15 mM solutions of the various ammonia salts. Control plants were transferred to one-fourth strength Hoagland's nutrient solution. Treatments consisted of injection with 0.5 µg IAA[1-^{14}C]. Plants were harvested four hours after injection.

Treatment	Percent of the control			
	Total ^{14}C Recovered	Vein loading	Vein unloading	Shoot/Root Ratio
Ammonium bicarbonate	113	58*	134	345*
Ammonium meta vanadate	96	102	132	122
Ammonium acetate	123*	98	196	365*
Ammonium chloride	91	99	227*	282*
Ammonium phosphate (dibasic)	111	92	160	314*
Ammonium sulfate	126*	86	53	100
Ammonium nitrate	110	105	51	82

*Indicates significant differences from the control at the 0.05 level.

TABLE X

THE EFFECTS OF 15 mM AMMONIUM BICARBONATE AND 15 mM POTASSIUM NITRATE IN THE NUTRIENT SOLUTION AND THE INTERACTION OF CYCLOHEXIMIDE ON THE TRANSLOCATION OF IAA[1-^{14}C] AND 2,4-D[1-^{14}C]

Plants treated with ammonium bicarbonate were transferred from one-half strength Hoagland's nutrient solution, four hours before injection, to nutrient solution in which nitrate was replaced with chlorine and 15 mM ammonium bicarbonate was added. The pH was maintained at 6.2 in these treatments by aeration with a carbon dioxide-air mixture. Plants treated with potassium nitrate were transferred from one-half-strength Hoagland's nutrient solution four hours before treatment, to one-half strength Hoagland's in which nitrate was increased to 15 mM by adding excess potassium nitrate. Plants were injected with 2.0 µg IAA[1-^{14}C] or 2.0 µg 2,4-D[1-^{14}C] and or 2.5 µg of cycloheximide.

Treatment	Vein loading (%)	Vein unloading
IAA[1-^{14}C]		
Ammonium Bicarbonate	43.38 a *	.148 a
Potassium Nitrate	43.64 a	.141 a
Ammonium Bicarbonate + Cycloheximide	47.41 ab	.566 c
Potassium Nitrate + Cycloheximide	54.17 b	.345 b
2,4-D[1-^{14}C]		
Ammonium Bicarbonate	48.82 ab	.286 b
Potassium Nitrate	56.35 b	.089 a
Ammonium Bicarbonate + Cycloheximide	42.57 a	.934 c
Potassium nitrate + Cycloheximide	44.88 a	.127 ab

* Values followed by the same letter are not significantly different at the 5% level. Vein unloading is expressed as the apoplastic symplastic transport ratio.

TABLE XI

THE EFFECTS OF INJECTION WITH BIS TRIS PROPANE NITRATE, AMMONIUMIMINODIACETATE OR BIS TRIS PROPANE CHLORIDE ON THE TRANSLOCATION OF ^{14}C AUXINS INJECTED IN TO BEANS

Plants were grown in one-half strength Hoagland's nutrient solution. Treatment was by injection with 0.5 µg of [1-^{14}C]IAA or 0.5 µg of [1-^{14}C] 2,4,5-T and 3 µeq. of either bis tris propane nitrate, ammoniumiminodiacetate, or bis tris propane chloride.

	Percent Of the control	
Treatment	Vein loading	Vein unloading
IAA[1-^{14}C]		
Bis tris propane nitrate	96	62
Ammoniumiminodiacetate	107	309*
Bis tris propane chloride	104	406*
2,4,5-T[1-^{14}C]		
Bis tris propane nitrate	96	83
Ammoniumiminodiacetate	78*	112
Bis tris propane chloride	112	127

* Indicates significant differences from the control at the 0.05 level.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Substantial evidence has been accumulated to support the theory that the intracellular pH of many plant cells is regulated by a pH stat mechanism (Davies, 1973; Raven and Smith, 1976). This pH stat mechanism would not only maintain cytoplasmic pH at the optimum for proper functioning of enzymes, but it would also preserve the pH gradient within the cell and external to it. The pH stat mechanism, as explained by Davies (1973), would regulate the intracellular pH via the synthesis and degradation of malate. When the intracellular pH was high the activity of the enzyme PEP carboxylase would be stimulated resulting in the fixation of carbon dioxide into malate which would act to acidify the cytoplasm. When the intracellular pH is low, malate would be decarboxylated by the malic enzyme to pyruvate releasing a hydroxyl ion in the process.

In support of the theory that pH gradients regulate auxin translocation, it has been demonstrated that there was a correlation between IAA-stimulated proton excretion by coleoptile segments and increased malate synthesis in cells (Haschke and Luttge, 1975; 1977). The electropotential of these cells was unchanged due to the potassium uptake that

accompanies proton excretion (Haschke and Luttge, 1977). This potassium uptake also seems to be related to the pH stat mechanism since the increased potassium uptake was accompanied by accumulation of malate (Haschke and Luttge, 1975).

Auxin translocation thus may involve not only ATPase, but also the action of the enzymes PEP carboxylase and malic enzyme for the maintenance of a pH gradient across the cell. To influence auxin translocation the compounds used in this study could affect any one of these steps.

The assimilation of ammonium produces an excess of proton excretion by cells, while the assimilation of nitrate produces an excess of hydroxyl ions by cells (Raven and Smith, 1976). Schweizer and Erismann (1985) reported that bean plants fed via the nutrient solution with either ammonium or no nitrogen had a low pH in the cell sap of the primary leaves and showed a decrease in the extractable activity of PEP carboxylase. Bean plants fed via the nutrient solution with nitate had a higher pH and the extractable activity of PEP carboxylase was increased. The concentration of malate in Phaseolus vulgaris L. was found to decrease under ammonium nutrition (Marques et al., 1983). It is possible that a cytoplasmic pH increase, caused by IAA induced proton excretion, would stimulate PEP carboxylase resulting in an increase in malate in the cytoplasm (Davies, 1979). It appears possible that IAA does directly stimulate an ATPase-promoted proton secretion since it has been shown

that the Km for the enzymatic proton secretion was decreased from 0.7 to 0.35 by auxin treatment of a plasma membrane-enriched vesicle fraction (Gabathuler and Cleland, 1985). However, fusicoccin promoted proton secretion was accompanied by acidification of the cytoplasm (Bertl and Felle, 1985). This suggests that fusicoccin stimulates the proton pump by cytoplasmic acidification. Others, (Romani et al., 1985) also concluded that cytoplasmic acidification enhanced the activity of the plasmalemma proton pump. Thus these data imply that rather than IAA directly stimulating ATPase and proton secretion (Gabathuler and Cleland, 1985), IAA may enhance malate synthesis, decrease cytoplasmic pH, and thus stimulate proton secretion at the plasmalemma. Under the influence of ammonium bicarbonate in the nutrient solution, there was an increase in the vein unloading of injected auxins (Table VI through X). Based on the available literature it seems likely that the ammonium treatments decreased the intra and extra cellular pH resulting in a shift to the unionized form of auxins which can easily pass thru the plasmalemma or unload. The results reported in the Schweizer and Erismann paper were obtained using a 5 mM solution of ammonium sulfate in the nutrient solution. However, in this study the largest increases in vein unloading were seen to result from ammonium bicarbonate, ammonium acetate, ammonium chloride and ammonium phosphate (Table IX). The reason that these effects were only observed with certain ammonium compounds

has not been determined at this time. Possibly the associated anion overcame the effect of the ammonium in some manner. Ammonium nitrate fed plants have been shown to respond more like nitrate fed plants than ammonium fed plants in terms of malate synthesis and extractable activity of PEP carboxylase (Marques et al., 1983).

Although it was later determined that the pH of the nutrient solutions containing ammonium bicarbonate rose upon aeration, it can be demonstrated that when pH is held constant, by CO_2/air aeration, the vein unloading of 2,4-D is significantly higher (Table X). Furthermore, when ammonium is injected directly into the plant as ammoniumiminodiacetate the vein unloading of IAA is increased (Table XI). These data seem to indicate that the observed results were not due entirely to an increase in nutrient solution pH. The addition of 15 mM potassium nitrate to the nutrient solution resulted in a significantly lower rate of vein unloading of 2,4-D (Table X). Plants injected with bis tris propane nitrate showed a low value for vein unloading of IAA which was not significantly different from the control. This is what would be expected to happen if nitrate nutrition results in a higher cell sap pH as demonstrated by Schweizer and Erismann (1985). Cycloheximide, when injected into beans, increased vein unloading (Table I), as will be discussed later. The interaction of cycloheximide injection with nutrient solution treatments increased vein unloading more than just

nutrient solution treatments alone. However, not all of these data were significantly different (Table X).

Nitrogen deficiency caused a significant increase in the vein unloading of 2,4,5-T[1-^{14}C], but not of IAA[1-^{14}C] (Table II). The acropetal transport of 2,4,5-T[1-^{14}C] was stimulated by nitrogen deficiency (Table III). The reason that IAA does not translocate in the same manner as 2,4,5-T is not known at this time. Nitrogen is an essential component of all proteins. Thus, a lack of nitrogen could interfer with the synthesis of new proteins. This conclusion is supported by the report by Schweizer and Erismann (1985) of the decreased PEP carboxylase activity in beans when nitrogen is lacking in the nutrient solution. Nitrogen deficiency has been demonstrated to effect the translocation of the foliarly applied herbicides [1-^{14}C]2,4-D and [methyl-^{14}C]glyphosate in Convolvulus arvensis L. (Shaw et al., 1985). In this study, nitrogen deficiency also caused an increase in acropetal translocation of 2,4-D and an increase in basipetal translocation of glyphosate.

The ammonium bicarbonate treatments used in this study caused no significant differences in the translocation of the amino acids injected into the plants (Table VIII, and VII). However, the amino acids did show a tendency for decreased vein unloading due to the ammonium bicarbonate treatment. On the other hand, nitrogen deficiency caused a decrease in vein unloading of arginine while leucine showed a significant increase in vein unloading (Table II).

However, the overall tendency was for the amino acids leucine and arginine to translocate downward to the roots and nutrient solution. Nitrogen-deficient plants have small tops and more extensive root growth and it might be expected that amino acids would translocate downward like glyphosate as shown in the previous study (Shaw et al., 1985). It has been demonstrated that applications of 10 µl of glyphosate to the leaf of purple nutsedge (Cyperus rotundus L.) preceeded by applications of 20 µl of 0.1 M ammonium chloride stimulated the translocation of glyphosate to the newly maturing leaves and roots (Wills and McWhorter, 1985). One of the effects of ammonium nutrition in isolated mesophyll cells was to reduce the fixation of carbon into sugars and direct it into the synthesis of amino acids (Paul et al., 1978). It has been generally agreed that due to the pH stress associated with nitrogen assimilation, most of the ammonium is assimilated into amino acids in the roots. These amino acids are then transported to the shoots (Smith and Raven, 1979). Therefore, ammonium nutrition should result in some differences in the translocation of amino acids.

The majority of the protein synthesis inhibitors tested in this study caused increased vein loading and increased vein unloading of [1-^{14}C]2,4,5-T. Thus, it seems that the protein synthesis inhibitors cause the auxin to load into the vein and then to promptly unload from the vein (Table I). It has been known for some time that protein synthesis is a requirement for auxin-induced cell enlargement (Nooden

and Thimann, 1963). Treatments with cycloheximide only show an increase in vein unloading (Table I). Some researchers have noted that the protein synthesis inhibitor cycloheximide inhibited IAA-induced proton excretion but not IAA induced growth (Pope, 1983). Long and Basler (1973) found that cycloheximide also inhibited basipetal translocation of stem-injected 2,4,5-T in beans. These data seem to indicate that the inhibition of protein synthesis may affect the maintenance of the pH gradients needed for auxin translocation. The interruption of protein synthesis or perhaps some other effect of the protein synthesis inhibitors may cause a change in the electropotential across the plasmalemma which could account for the observed effects on auxin translocation. Treatment of beetroot (Beta vulgaris L.) storage tissue with 1 µg/ml cycloheximide has been shown to inhibit the development of the net uptake of the ions potassium, sodium, and chlorine (Van Stevenick and Van Stevenick, 1972). Chloramphenicol is usually thought to be a specific inhibitor of protein synthesis in microorganisms however, Nooden and Thimann (1963) reported that chloramphenicol also inhibits protein synthesis in plants. Puromycin treatments increased vein loading only (Table I). Other experiments (Van Stevenick and Van Stevenick, 1972) with puromycin were unsatisfactory because puromycin caused increased permeabilty of the plasma membrane as evidenced by leakage of pigments from beetroot.

The experiments with the amino acid analogs were an attempt to clarify the relationship between protein synthesis and auxin translocation. It is possible that protein synthesis is needed either to replace enzymes or carriers which have rapid turnover rates, or to use up some compound that inhibits proton excretion by the ATPase (Cleland, 1982). The amino acid analogs affect protein synthesis by allowing protein synthesis to occur. However, the new proteins are likely to be inactive since some of their amino acids will be replaced by the analogs. If protein synthesis is needed to make a specific protein that is involved in the translocation of auxins the presence of the amino acid analog would have an effect on auxin vein loading and unloading. If protein synthesis in general is needed for auxin translocation then the presence of the amino acid analogs would have no effect on auxin vein loading and unloading. There were significant differences in the translocation of 2,4,5-T as affected by the two amino acid analogs and the trends in the translocation of IAA were similar although not statistically significant. However, the results were not conclusive in as much as the amino acid analogs had different effects on translocation. Ethionine inhibited loading as would be expected if the synthesis of a new functional protein were required for vein loading. However, thienyl alanine did not inhibit loading but enhanced unloading as did protein synthesis inhibitors such as cycloheximide. One interpretation of the data is that

the amino acid analogs affect pH perhaps by affecting proton pumps. This conclusion is supported by the data which showed different magnitudes of effects of the amino acid analogs for the translocation of the auxins IAA and 2,4,5-T. If non-functional proteins or carriers were being made, the percentage change on treatment with the amino acid analogs should have been the same for both auxins since both auxins would most likely use the same carrier. Since the two auxins have different pK values, a given change in pH could result in different carrier affinity for each auxin and result in different level of change in translocation of each auxin. Thus, the effects may be due to changes in pH rather than the formation of non-functional proteins.

Various combinations of nitrate, phosphate and sulfur had definite effects on the translocation of IAA (Table V). The uptake of cations and anions by plants has been shown to occur at different rates. Synthesis and degradation of malate was believed to compensate for the imbalance (Jacobson, 1955; Hiatt, 1967). Excess cation uptake resulted in proton excretion and accumulation of malate while excess anion uptake resulted in hydroxyl ion excretion and malate degradation. Kirkby and Armstrong (1980) demonstrated that feeding plants with nitrate nitrogen caused an excess of anion uptake which would cause a decrease in the pH of the tissue. Perhaps these combinations of nutrients affect either the pH gradients and or charge distribution across the plasmalemma, resulting in

the observed patterns of vein loading and vein unloading of IAA.

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VITA

JEAN ALICE PITTMAN WINTERS

Candidate for the Degree of

Master of Science

Thesis: NITROGEN NUTRITION AND METABOLISM AS IT AFFECTS TRANSLOCATION PATTERNS OF AUXINS

Major Field: Botany

Biographical:

Personal Data: Born in Marshall, Wisconsin, November 12, 1959, the daughter of James J. and Janet E. Pittman. Married to Bobby N. Winters on January 4, 1986.

Education: Graduated from Poteau High School, Poteau, Oklahoma, in May, 1978; recieved Bachelor of Science Degree in Botany from Oklahoma State University in December, December 1982; completed requirements for the Master of Science degree at Oklahoma State University in May, 1986.

Professional Experience: Graduate teaching assistant, Department of Botany, Oklahoma State University, January, 1983 to May, 1983; Graduate Research Assistant, Department of Agronomy, Oklahoma State University, May, 1983, to May, 1986.

Professional Organizations: Member American Society of Plant Physiologist; member of Oklahoma Academy of Science; Member of Phi Kappa Phi; Member of Gamma Sigma Delta.