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The role of ethylene in shoot gravitropism in sunflower (*Helianthus annuus*)

by

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ABSTRACT

The role of ethylene in sunflower shoot gravitropism was investigated in epicotyls of light-grown *Helianthus annuus* seedlings. Ethylene evolution was greatest in the lower half of the bending region of gravistimulated shoots. An interaction of ethylene and auxin was required for upward curvature of stems; ethylene may increase tissue sensitivity to auxin.

Gravistimulated epicotyls were wider at their base than upright plants. Ethylene was likely responsible for this increase in width which supported the upward bending stem. Cells along the lower side of the bending region of the plant elongated at a faster rate than cells along the upper side, resulting in the upward bending of the shoot. Auxins were likely responsible for this difference in elongation rate.

ACC synthase and ACC oxidase mRNA expression was not detected during gravistimulation until after ethylene evolution peaked, indicating that ethylene biosynthesis during gravistimulation was from the enzymes already present in the tissue.

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LIST OF ABBREVIATIONS

ACC	1-aminocyclopropane-1-carboxylic acid
AIBA	α-aminoisobutyric acid
AOA	α-(aminooxy)acetic acid
AVG	aminoethoxyvinylglycine
BTP	bis-tris propane
DEPC	diethylpyrocarbonate
DTT	dithiothreitol
G/S	gravistimulated
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
NPA	N-(1-naphthyl)phthalamic acid
PVPP	polyvinylpolypyrrolidone
SAM	s-adenosylmethionine
SDS	sodiumdodecylsulphate
SE	standard error
SSC	sodium citrate, sodium chloride
SSPE	sodium chloride, sodium phosphate, EDTA
STS	silver thiosulphate
TIBA	2,3,5-tri-iodobenzoic acid

We should not pretend to understand the world only by the intellect; we apprehend it just as much by feeling.

Carl G. Jung, Psychological Types

CHAPTER 1 - INTRODUCTION

Gravity plays an important role in the development and growth of plants. Two main responses of gravity on plants are gravitropism and gravimorphism. Gravitropism is the overall orientation of plant organs relative to the force of gravity, such as stems growing upward and roots downward. Gravimorphism involves more subtle alterations in plant form due to gravity, for example, reaction wood helping to support a lateral branch of a pine tree at a particular angle away from the main trunk. Although gravimorphism is an important aspect of the effects of gravity on plants, it is not discussed here. My main interests lie in understanding how plants, specifically stems, respond to changes in the direction of the force of gravity, otherwise known as shoot gravitropism. Stems are usually negatively orthogravitropic (oriented and growing 180° away from the force of gravity), while petioles and lateral branches are often diagravitropic (oriented and growing 90° from the direction of gravity) or plagiogravitropic (between ortho- and diagravitropic). Primary roots are usually positively orthogravitropic but lateral roots may be diagravitropic or plagiogravitropic.

Gravity may not directly influence plant yield but it directs shoot growth upward toward sunlight to increase light absorption, photosynthesis and thus yield and reproductive success, and allows roots to grow downward away from sunlight to increase nutrient uptake and prevent dehydration. The direction of root growth is also affected to some degree by the location of water (hydrotropism) (Loomis and Ewan, 1936) and nutrients (chemotropism) (see Hart, 1990; Ziegler, 1962). Roots must also be able to change their direction of growth to avoid compact soil or dense underground objects and must subsequently reorient themselves downward. For more background information on gravity and plants see Evans et al. (1986), Gordon and Cohen (1971), Hart (1990), and Wilkins (1979).

Three stages are involved in plant gravitropism: gravity perception, signal transduction and asymmetric growth response (Fukaki et al., 1996). In shoots, it is believed that gravity is perceived by statoliths, gravity-sensitive starch grains, located in parenchyma cells of the cortex. The signal transduction pathway following gravity perception has not been well documented. Signal transduction begins as a chain of events following the statolith sedimentation and ends with an asymmetric auxin distribution in the shoot (Barlow, 1995; Fukaki et al., 1996). The result of the signal transduction pathway is an asymmetric growth response which is responsible for upward bending of the shoot.

Many researchers are now focusing on the events taking place during signal transduction. These areas of study encompass biochemistry, anatomy, molecular biology and physiology, the latter of which includes plant growth hormones and metabolism. My own research focuses mainly on the roles that plant growth hormones, specifically ethylene, play in shoot gravitropism.

Ethylene is a deceptively simply hydrocarbon molecule consisting of two carbon atoms which are joined by a double bond. It is unlike the other well known phytohormones (auxins, gibberellins, cytokinins and abscisic acid) in that it is gaseous.

Ethylene is often considered to be a "stress" phytohormone, active mainly when plants are under duress. It is most famous for the triple response discovered by Neljubov in 1911 on pea seedlings; in the presence of ethylene, these seedlings show inhibited stem elongation, increased stem thickening and horizontal growth. Ethylene is also associated with wilting, leaf epinasty, leaf abscission, leaf chlorosis (the latter two often occur during pathogenic attack), inhibition of flowering (with the exception of mangos and bromeliads), adventitious root formation and fruit ripening (Abeles et al., 1992).

The complete ethylene biosynthesis pathway has only recently been determined by Yang and Hoffman in 1984. L-methionine is converted to s-adenosylmethionine (SAM), a

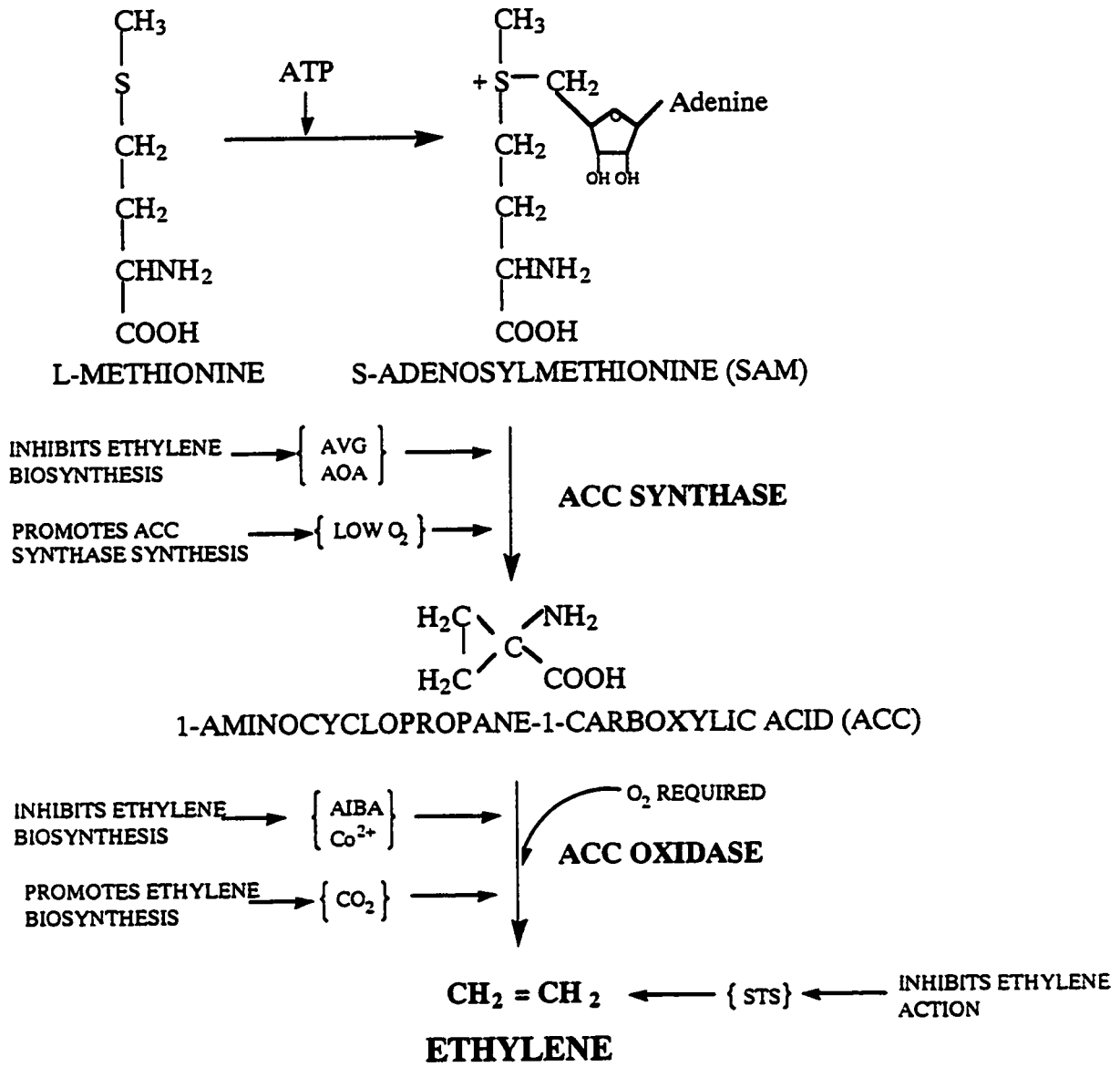

Figure 1.1 Ethylene biosynthesis pathway (modified from Yang, 1980).

process requiring ATP (figure 1.1). SAM is then transformed to 1-aminocyclopropane-1-carboxylic acid (ACC) with the aid of the enzyme ACC synthase. ACC is converted to ethylene with ACC oxidase, an oxygen-requiring reaction. The sulphur molecule from methionine which is incorporated into SAM is recycled to make more methionine, hence the demand of sulphur is not rate-limiting (Yang, 1980).

Ethylene is synthesized in all parts of the seed plant, although the exact location of synthesis is not fully understood. Much research has been done on determining the site of ACC oxidase activity. It has been postulated that ACC oxidase functions in cellular membranes; the plasmalemma and to a lesser extent, the tonoplast (Bouzayen et al., 1990).

Another group of phytohormones of great importance are the auxins. There are four naturally occurring auxins in plants; indole-3-acetic acid (IAA), the most famous of the four, indole-3-butyric acid (IBA), 4-chloroindoleacetic acid, and phenylacetic acid. The biosynthesis of auxins is not as straightforward as that of ethylene. There can be *de novo* synthesis from tryptophan via a number of pathways or hydrolysis of auxin conjugates (Bandurski et al., 1995). The main sites of auxin synthesis are the shoot apex, including young leaves, and in developing fruits, although there is some auxin production in root tips. Auxins are distributed via basipetal polar transport in shoots and acropetal polar transport in roots.

Auxins have long been associated with many aspects of plant development, including the promotion of adventitious rooting, stem and coleoptile elongation and, in low concentrations, root elongation. Auxins are also responsible for the inhibition of lateral bud development via apical dominance and in large concentrations, root elongation. This phytohormone is of great importance in gravitropism and phototropism. Auxins are also able to induce ethylene biosynthesis (Morgan and Hall, 1962), often making it difficult to isolate the independent actions of auxins and ethylene in various responses. Although my primary objective is to study the role ethylene plays in shoot gravitropism, the close

relationship between auxin and ethylene make it necessary to also examine auxin's role, with respect to ethylene, in gravistimulated stems.

I decided to use the light grown sunflower *Helianthus annuus* as my experimental model. Sunflowers are readily available, easy to plant and maintain, and develop relatively quickly such that they are ready for experimentation within approximately three weeks from planting. In addition, a great deal of the previous research in Dr. Reid's laboratory has involved sunflowers, thus much is already known about their development and response to various treatments, especially those pertaining to ethylene and auxins.

In past gravitropism experiments, researchers have often used etiolated sunflowers of approximately 6-8 days old. At this stage the plants have shoots consisting only of a hypocotyl and cotyledons, and no true leaves. As well, this stage only represents plants during a short period of their entire life cycle. I am more interested in how plants respond to gravity (with respect to ethylene) throughout a majority of their life. I have chosen to use older, light grown plants because of their increased similarity to plants grown in nature. Etiolated plants are physiologically and biochemically different than wild-grown plants; they contain etioplasts in place of chloroplasts, have different phytohormone levels, differ in gravitropic sensitivities and as recently discovered, may have different responses to auxins (Bown et al., 1975; Hart and MacDonald, 1981; Jensen et al., 1997). Plants grown in nature have shoots consisting of a hypocotyl, an epicotyl and true leaves (remembering that the latter of which is known to be a major auxin source) for the majority of their life cycle.

When searching the literature I found that although there is much to suggest that ethylene is involved in gravitropism, it is still virtually ignored in this field of study. It is known that ethylene levels do increase in many gravistimulated shoots (Clifford et al., 1983; De Wit et al., 1990; Kaufman et al., 1985; Philosoph-Hadas et al., 1996), but many researchers downplay a role for ethylene in gravitropism or dismiss it altogether. Much of

the growth regulator research in gravistimulated plants until now focuses instead on the role of auxins. In a recent supplement of the journal Planta (Sievers et al., 1997) dedicated entirely to the study of plants in space with a majority of the articles pertaining to plants and gravity, there is absolutely no mention of ethylene's role in gravitropism. I was curious to determine why gravistimulated plants expend energy producing the potent plant hormone ethylene - there must be a payoff; it seems logical to presume that ethylene either has a direct effect on gravitropism or is the result of some other action, but is important to gravitropism nonetheless. From the literature and from some preliminary experiments which are expanded on in chapter two, I hypothesized that ethylene does play a role in dicotyledonous shoot gravitropism. I discuss my investigation of this role at the physiological level in chapter two, the histological level in chapter three and at the molecular and biochemical levels in chapter four.

CHAPTER 2 - IS THERE A ROLE FOR ETHYLENE IN SUNFLOWER SHOOT GRAVITROPISM?

In the past century there have been extensive studies on the internal hormonal changes that control gravitropic growth in plants, yet no conclusive theory prevails. A commonly cited theory explaining the upward bending of shoots following gravistimulation is the Cholodny-Went hypothesis. This hypothesis states that auxins, which are synthesized in young stem tissues, shoot meristems and growing leaves are normally symmetrically distributed throughout the plant. When gravistimulated, the auxin is distributed asymmetrically, with the lower side of the stem receiving greater concentrations of the phytohormone than the upper side. This results in asymmetric growth: the lower side of the shoot increases its rate of growth while the upper side shows a decrease in normal shoot growth. (Hart, 1990).

Although the Cholodny-Went hypothesis has remained the most popular theory on shoot curvature, three main doubts challenge its validity: (1) despite the shoot apex being a major site of auxin synthesis, there is evidence indicating it may not be required for gravibending (Firn et al., 1981), (2) if the shoot is split in half lengthwise, theoretically preventing asymmetric distribution of auxin from the upper side to the lower side of the stem, upward curvature still occurs in the lowe half, (3) careful studies of auxin distribution have lead to suspicions regarding the quantity and timing of auxin gradients. It is still unknown whether a sufficient auxin gradient exists and whether such an asymmetry develops quickly enough to induce gravicurvature (Hart, 1990), however, it must be kept in mind that each of these challenges to the Cholodny-Went hypothesis can be rebutted to some extent.

Despite these uncertainties regarding the specifics of the Cholodny-Went hypothesis, it is still believed by many that auxins do play an important role in gravibending but that their role must be further examined (Harrison and Pickard, 1989;

Hart, 1990; Philosoph-Hadas et al., 1996). One theory becoming popular with researchers is that of changes in auxin sensitivity. With gravistimulation, it is likely that there are changes in lateral auxin distribution (Harrison and Pickard, 1989; Migliaccio and Rayle, 1989), but it is now thought that the upper and lower shoot tissues change their sensitivity to these auxins (Rorabaugh and Salisbury, 1989; Salisbury et al., 1988). Recently the importance of the shoot epidermis has been re-examined almost a century after it was discovered that its removal resulted in a decrease in gravibending (Hart, 1990). Further research has indicated that there are differences in auxin sensitivity between the epidermal and sub-epidermal tissues in both the upper and lower sides of shoots; epidermal tissues respond to increased auxin concentrations with increases in the rate of growth while sub-epidermal tissues decrease the rate of stem growth in the presence of increased auxin concentrations (Masuda and Yamamoto, 1972; Thimann and Schneider, 1938). Thus in a gravistimulated shoot, auxins in the upper side of the stem flow from the epidermal tissues to the sub-epidermal tissues and auxins in the lower stem flow from the sub-epidermal tissues to the epidermal tissues. This would result in a decrease in the growth rate in the upper side and an increase in the rate of growth in the lower side of the shoot (MacDonald and Hart, 1987).

Another phytohormone has also been implicated in gravibending. Some authors have shown that ethylene concentrations increase following gravistimulation of shoots (Clifford et al., 1983; De Wit et al., 1990; Harrison and Pickard, 1984; Kaufman et al., 1985; Philosoph-Hadas et al., 1996). With the exception of one group, these researchers proposed that in lieu of a primary response, ethylene is responsible for controlling the later stages of gravibending. Harrison and Pickard (1989) dismiss any role for ethylene in shoot gravitropism based on their research within the first three hours of gravistimulation of tomato hypocotyls. This supports the likelihood that changes in ethylene concentration are not involved in the early stages of gravibending, but it does not rule out any possibility

of a secondary role in shoot gravitropism as three hours is not a very long investigation time compared to twelve (Clifford et al., 1983), twenty-five (Philosoph-Hadas et al., 1996) and forty-eight hours (Kaufman et al., 1985) of gravistimulation.

Some researchers have found that the addition of ethylene or the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) increases shoot gravibending and that ethylene inhibitors delay shoot gravicurvature. Blake et al. (1980) induced branch hyponasty in conifer seedlings with exogenous ethylene. Philosoph-Hadas et al. (1996) were able to inhibit gravicurvature of snapdragon spikes by treatment with the ethylene inhibitors silver thiosulphate, cobalt chloride and 2,5-norbornadiene. Upward bending was again inhibited when cockleburs were treated with cobalt chloride, silver nitrate, carbon dioxide or aminoethoxyvinylglycine (AVG) (Salisbury and Wheeler 1981; Wheeler and Salisbury 1981). Unfortunately none of the above researchers reapplied ethylene or any of its precursors to the ethylene-inhibited plants to ensure that the inhibitors did not simply prevent gravicurvature due to toxicity of these chemicals to the plant. Kaufman et al. (1985) did not observe any changes in gravibending of oat seedling stems when ethylene was either added or inhibited. When Clifford et al. (1983) treated dandelion peduncles with ethylene synthesis inhibitors, the early stages of gravibending was unaffected. Thus all the above experiments indicate that ethylene may have a role in gravitropism, but that this role may be confined to certain groups or species of plants or during the later stages of gravistimulation.

It has been known for some time that auxins promote ethylene synthesis (Morgan and Hall, 1962). Wright et al. (1978) observed an auxin-mediated increase in ethylene production in grass nodes following gravistimulation. Philosoph-Hadas et al. (1996) suggested, based on their research of snapdragon spikes, that with gravistimulation comes either asymmetric auxin distribution or asymmetric auxin sensitivity. This then produces auxin-induced ethylene, precipitating another chain of events resulting in gravicurvature.

There has also been evidence to suggest that ethylene may influence auxin activity. Liu and Reid (1992) proposed that the effect of ethylene on adventitious rooting in derooted sunflower hypocotyls was mediated by auxin but that the presence of ethylene enhanced the response of exogenous auxin. From this they proposed that ethylene increased tissue sensitivity to auxin. Burg and Burg (1966) and Lyon (1970) have shown that ethylene can inhibit both polar and lateral transport of IAA in pea stems and tomato leaves. However Liu and Reid (1992) were not able to show any ethylene-mediated inhibition in radiolabeled IAA in sunflower hypocotyls, indicating that this function may be species- or tissue-specific.

I wished to determine why ethylene levels increase in gravistimulated shoots. Why do plants expend energy producing this growth regulator when removed from the upright position? With my research I attempted to show that ethylene is important for gravicurvature to take place in dicotyledon shoots. In this chapter I investigated ethylene's role in gravitropism in light-grown sunflower epicotyls at the physiological level. Based on the early research described above I assumed that auxins also play an important and possibly a primary role in gravitropism, but that they are not the only phytohormones responsible. While it seems plausible that ethylene and auxins somehow interact in order for upward bending of the stem to occur, I did not dismiss the possibility that ethylene production is a result of shoot gravicurvature.

METHODS AND MATERIALS

Growth Conditions

Sunflower (*Helianthus annuus* L. var. Dahlgren 131) seeds were germinated in 50% peat moss, 5% Terragreen, 22.5% vermiculite and 22.5% perlite, watered daily and given 0.3g of 20:20:20 (N-P-K) fertilizer plus micronutrients (0.005% Mg, 0.05% Fe, 0.003% Mn, 0.0068% B, 0.0025% Zn, 0.0013% Co, 0.0036% Cu and 0.0009% Mo) twice weekly. All plants were grown with GE Lighting wide spectrum plant and aquarian fluorescent lamps (40 Watts) with a photon flux density of 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$, set for a 16h photoperiod. Some plants were grown in a growth chamber in the greenhouse at a temperature of 24°C light:18°C dark, while other plants were grown in the laboratory at a temperature of 23.5°C light:21°C dark.

Plants

Experiments were conducted when plants reached 17-23 days in age (because not all batches of sunflowers grow identically every time, despite similar growth conditions, I relied on visual observation of the plants' features rather than age to determine at what time I used them for experiments) and had stems consisting of a hypocotyl, epicotyl, first leaves outstretched and other sets of leaves at a young stage of growth but still located close to the apex (plate 2.1).

Treatments

Intact plants were treated with application of the appropriate chemical to cotton wool which was wrapped around the epicotyl just below the first petioles one day prior to the

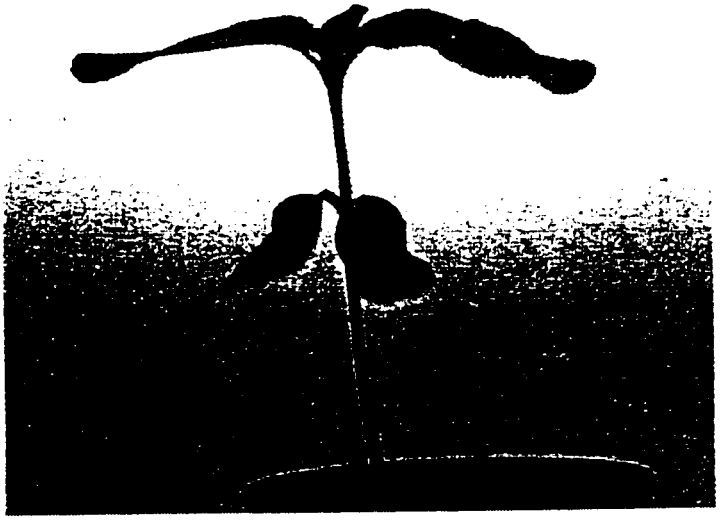

Plate 2.1 Intact sunflower stem representative of the age of the plants used in these experiments.

experiment. In experiments with decapitated plants, the plants were always decapitated the day prior to treatment. In completely decapitated plants, the epicotyl was cut just below the first petioles (plate 2.2). Any other methods of excision are explained in the figures. Decapitated plants received their treatments via cotton wool wrapped around the epicotyl immediately below the excision site (plate 2.2). Multiple chemicals were applied to plants one immediately after the other in the fashion described above.

All plants were treated the same day as they were gravistimulated. Plants were treated in the light prior to gravistimulation with 1mL of various concentrations of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (ICN), (α-aminooxy)acetic acid (AOA) (Sigma), an inhibitor of ACC synthase (Yang, 1980), N-(1-naphthyl)phthalamic acid (NPA) (Pfaltz and Bauer Inc.), an auxin transport inhibitor (Morgan and Söding, 1958), and ethanolic indole-3-butyric acid (IBA) (Sigma), an auxin, at pH 5.9. All of these treatments contained minute amounts of the surfactant polyoxyethylene sorbitan monolaurate (Tween 20) (ICN) to allow for easier penetration of the treatments into the plants. "Untreated" control plants were wrapped with cotton wool and treated with double distilled water and Tween to ensure neither the Tween nor the method of application had any significant effect on the results. For gravistimulation plants were placed in a dark room and kept on their sides at 90° to the force or gravity. Upright control plants were also placed in the dark room. Plants were kept out of the light to prevent any phototropic effects.

Gravicurvature Measurements

Gravicurvature was measured with a protractor from negatives (Kodak Tri-X pan black and white ISO 400 negative film, Kodak D-76 developer and Kodak Rapid Fixer)

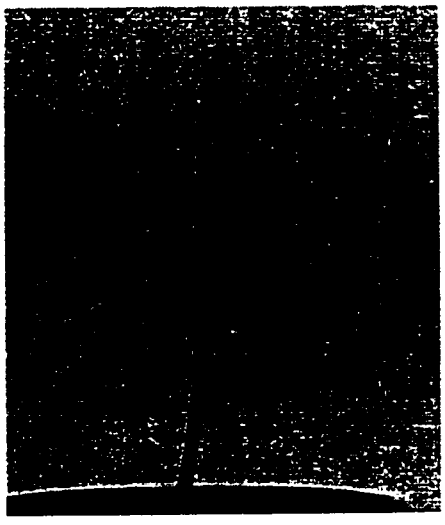

Plate 2.2 Sunflower epicotyl with complete decapitation.

taken in the dark with a green safe light (two layers of plastic green correction filter emitting not more than 5$\mu mol \cdot m^{-2} \cdot s^{-1}$ with a 40W GE incandescent light bulb). The degree of curvature was measured with respect to the horizon, with 0° representing no curvature and 90° being directly opposite the force of gravity.

Ethylene Evolution Measurements

To determine ethylene evolution, epicotyl samples were collected by cutting the tissues with a sharp razor blade and immediately incubating in 5mL syringes for exactly 20 minutes in the dark. Following incubation 1mL air samples were drawn with a 1mL syringe attached to the 5mL syringe via a three-way valve. After weighing the tissue, the air samples were injected for ethylene analysis into a Photovac 10S Plus gas chromatograph with the oven set at 45°, a 3.2mm × 2.45 m 60/80 Carbopack B column (1.5% XE-60 - 1% H_3PO_4; Supelco Canada, Oakville ON) and a photoionization detector. In all figures the concentrations of ethylene are in picomols of ethylene per gram of fresh weight of tissue per hour (pmol/fw/h).

Statistics

All experiments were repeated at least once, often twice. Experiments normally consisted of 10 replicates per treatments Most data are shown in means ± standard error (SE). When the SE bar is not shown, the error is smaller than the symbol.

RESULTS AND DISCUSSION

The role of ethylene in gravitropism has been elusive; while some researchers believe that ethylene is somehow associated in shoot gravitropism, others dismiss ethylene's involvement or deem it unimportant as it does not appear to play a primary role in upward bending of the shoot. The main goal of my research was to see if ethylene plays a role in gravitropism.

Ethylene in shoot gravitropism

Gravistimulated sunflower epicotyls show an increase in ethylene evolution which is not observed in upright control plants (figures 2.1 A and B). Initially there is a small decrease in ethylene evolution following gravistimulation but thereafter ethylene production increases for several hours following gravistimulation, corresponding with an increase in upward curvature of the epicotyl. There is a direct, positive relationship between ethylene production and upward bending of the sunflower epicotyl within the first four hours of gravistimulation (figure 2.1A). Figure 2.1B shows the relationship between ethylene evolution and stem curvature over the first 36 hours of gravistimulation. Ethylene production peaks at 22.46 pmol/fw/h after 4 hours of gravistimulation, rapidly declining to 8.70 pmol/fw/h by 12 hours, then decreasing to near zero levels by 24 hours. Gravicurvature does not peak until between 8 to 12 hours after gravistimulation. There is a slight drop in upward curvature between 12 and 24 hours after which time the degree of curvature plateaus. Thus the shoot remains curved even after ethylene levels drop. The results in these figures display the same trends as shown by others; Clifford et al. (1983), Harrison and Pickard (1984) and Philosoph-Hadas et al. (1996) all observed increases in

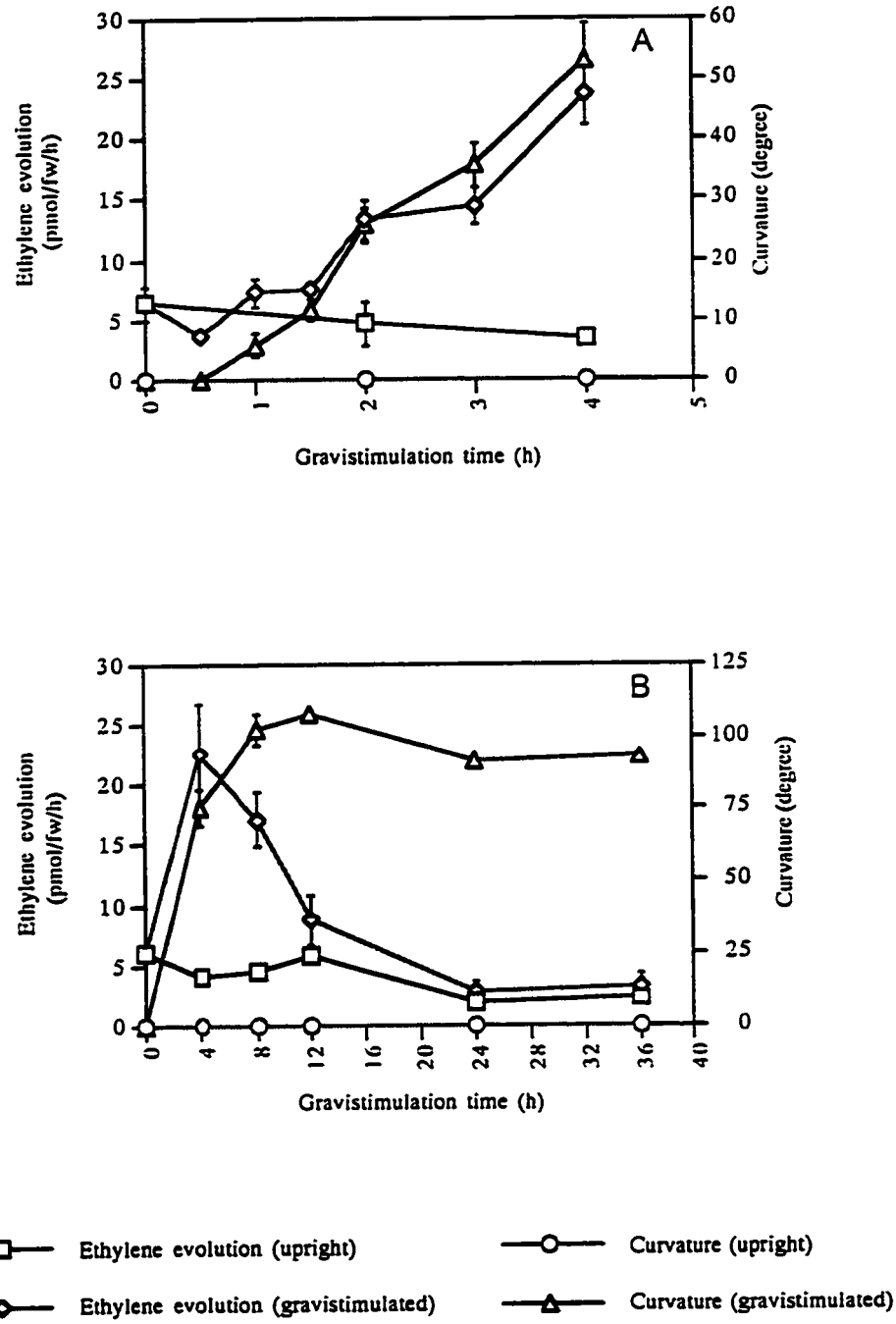

Figure 2.1 Ethylene evolution and curvature of 4 hour (A) and 36 hour (B) gravistimulated sunflower epicotyls (means ± SE).

ethylene production in gravistimulated dandelion peduncles, tomato seedlings and snapdragon spikes respectively.

To rule out the possibility that ethylene evolution during gravistimulation is not incidental, ethylene production must be inhibited and then restored. A variety of chemicals are capable of inhibiting ethylene biosynthesis and action. I have tried AOA and AVG, both inhibitors of ACC synthase (Boller et al., 1979; Yang, 1980), silver thiosulphate (STS), an inhibitor of ethylene action (Veen, 1983), and α-aminoisobutyric acid (AIBA) which blocks ACC oxidase activity (Edwards et al., 1983). All of these inhibitors did prevent epinasty thus they do inhibit ethylene biosynthesis or activity. Of the many that I have tried, I have found AOA to work the most effectively. I have decided not to use the other inhibitors; STS is known for its toxicity in plants, AVG for its high cost and I did not find AIBA to be as effective as AOA at inhibiting ethylene biosynthesis.

10mM AOA proves effective at inhibiting both ethylene evolution and upward curvature of gravistimulated shoots (figures 2.2 and 2.3). While 10mM ACC dramatically increases ethylene production in plants (figure 2.2), it has little effect on gravicurvature (figure 2.3). These results also imply that *de novo* synthesis of ethylene is involved in gravitropism. To rule out the possibility that AOA inhibits curvature by toxicity to the plant, ACC is added back to the plant, with the expectation that some curvature will be restored. When stems are treated with both AOA and ACC, ethylene production is greater than in untreated gravistimulated plants but almost half that of ACC-treated stems (244 pmol ethylene/fw/h versus 455 pmol ethylene/fw/h). Stems in these plants ultimately curve upward almost as much as untreated stems, however at a much slower rate, indicating that the concentration of AOA used is not toxic to the plant. These experiments indicate that ethylene is not only produced during gravistimulation, but that it appears to be correlated with the upward bending of the epicotyl. The simple fact that inhibition of ethylene

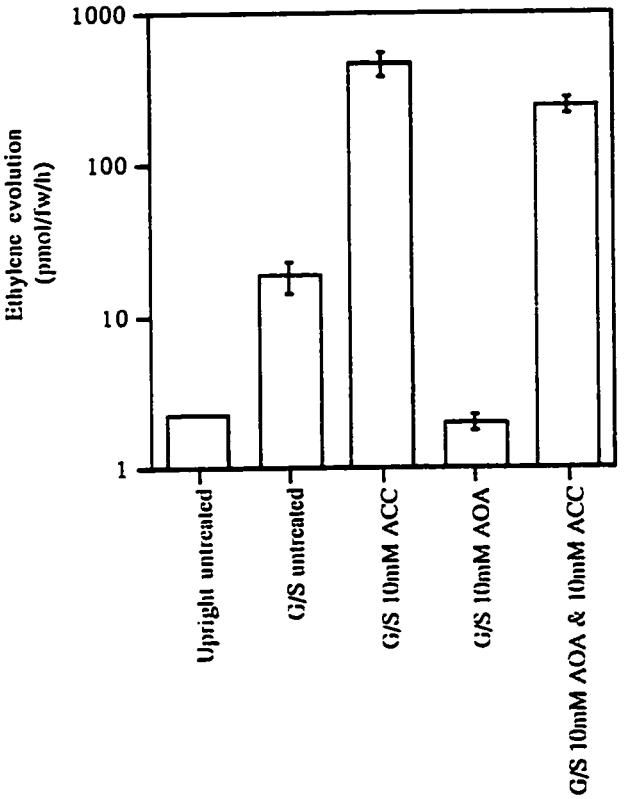

Figure 2.2 Effectiveness of ACC and AOA on ethylene evolution of sunflower epicotyls gravistimulated (G/S) six hours (means ± SE).

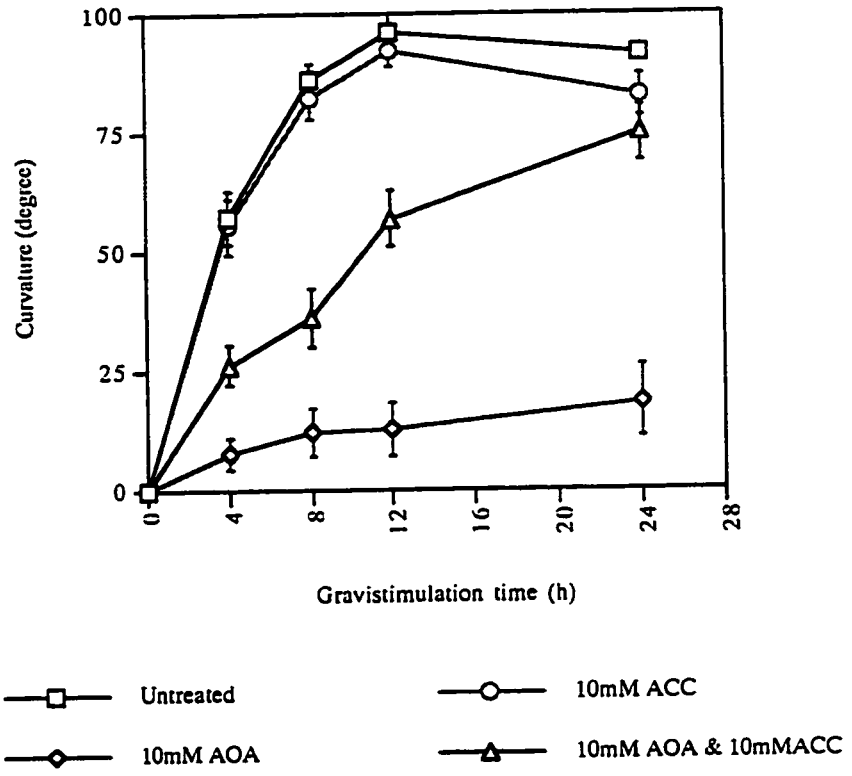

Figure 2.3 Curvature of AOA- and ACC-treated gravistimulated sunflower epicotyls (means ± SE).

synthesis results in the prevention of gravicurvature is good evidence that ethylene is required for curvature of the stem during gravistimulation.

One of the first researchers with data suggesting that ethylene causes gravitropic bending in shoots was Neljubov in 1911 (Abeles et al., 1992) and his observations of negatively gravitropic stems becoming diagravitropic with exogenous ethylene. In 1973 Zobel studied the normalization of diagravitropic shoot and root development in the tomato mutant *diageotropica* in the presence of ethylene. In 1980, based on their research of ethylene and gravitropism and cocklebur stems, Wheeler and Salisbury also proposed that ethylene might be responsible for gravicurvature. Clifford et al. (1983) did not believe that ethylene was involved in gravibending in dandelion peduncles but that perhaps it played a role in the later stages of bending, that is, autotropism (bending arrest). Philosoph-Hadas et al. (1996) further investigated the correlation between ethylene and bending in gravistimulated snapdragon spikes. They found that ethylene production rates decreased with increasing distance from the shoot apex; the greatest amounts of ethylene evolved in the shoot bending zone. From this they concluded that the presence of ethylene in the bending region of the stem was essential for upward curvature to take place.

To further investigate this correlation between ethylene and gravicurvature, I measured ethylene production in gravistimulated plants at three different locations along the epicotyl and in one location in the hypocotyl (figure 2.4). Region 1 is the portion of the epicotyl between the apex and the first petioles, and region 2 comprises the epicotyl just below the first petioles. Region 3 includes the portion of the epicotyl above the cotyledons and region 4 consists of the portion of the hypocotyl located directly below the cotyledons. The greatest amount of ethylene evolution is found in the epicotyl between the cotyledons and the first petioles (regions 2 and 3), which is also the site of the bending region of the stem. The hypocotyl portion (region 4) produces significantly less ethylene than the

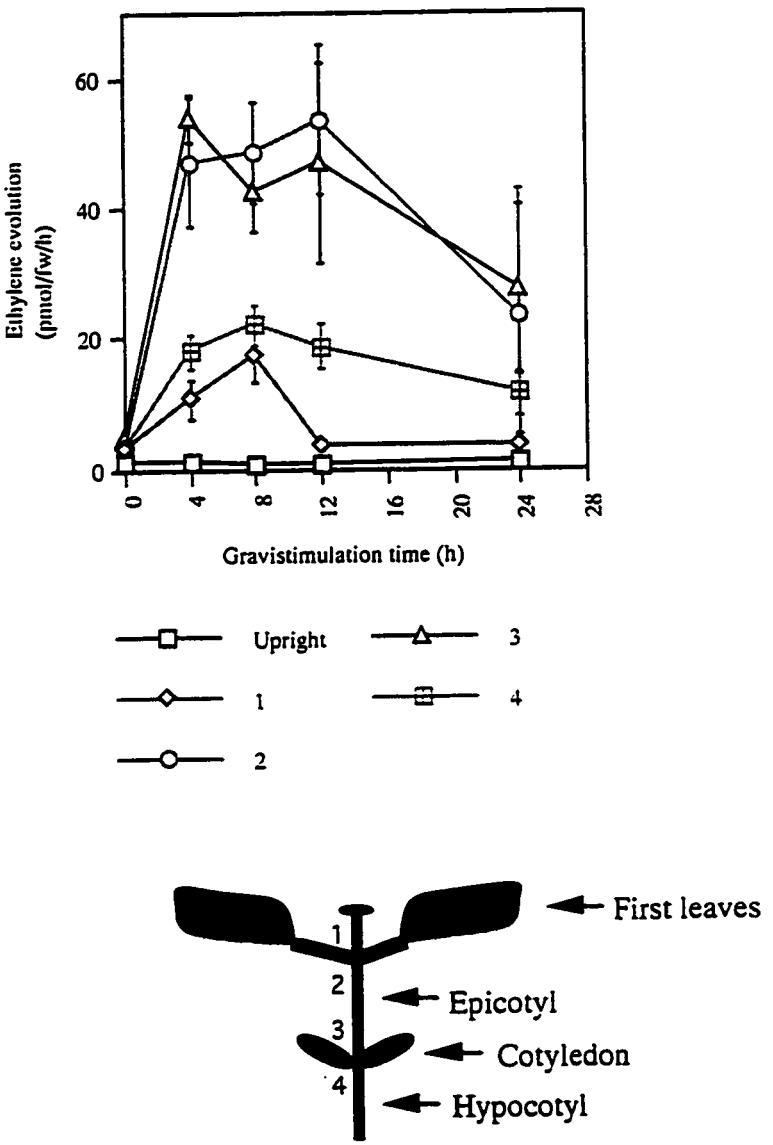

Figure 2.4 Ethylene evolution of the entire upright and various sections of gravistimulated sunflower epicotyls (1-3) and hypocotyls (4) (means ± SE).

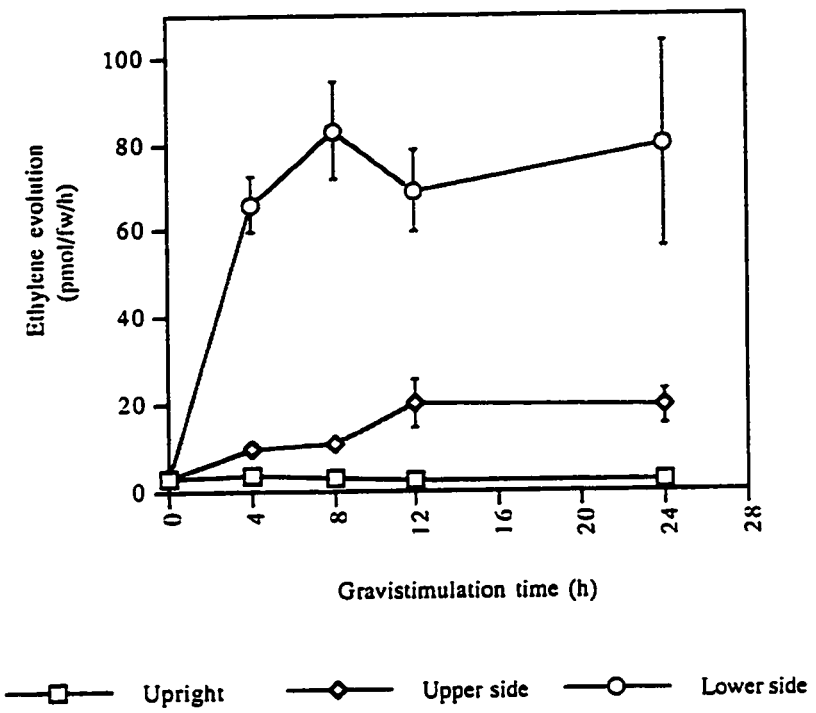

Figure 2.5 Ethylene evolution of upper and lower sides of gravistimulated intact sunflower epicotyls (means ± SE). All samples (including upright control) are longitudinal sections.

bending region, but slightly more than the apical portion of the epicotyl (region 1). Like Philosoph-Hadas et al. (1996), I found ethylene production to be greatest in the bending zone of the gravistimulated stem. This is further evidence that ethylene is required for gravicurvature in shoots

Having determined that ethylene evolution is greatest in the bending region of the gravistimulated epicotyl, I sought to examine this region in greater detail. I measured the differences in ethylene production in the upper and lower sides of the bending region in gravistimulated epicotyls and the corresponding region in upright stems (figure 2.5). There is negligible ethylene evolution in upright plants. The upper side of the gravistimulated stems have greater ethylene production than the upright plants but the greatest amount of ethylene evolution is in the lower side of the gravistimulated epicotyls. This is in agreement with results by Clifford et al. (1983), Philosoph-Hadas et al. (1996) and Woltering (1991).

If ethylene production is greatest in the lower side of a gravistimulated stem, and the ethylene inhibitor AOA is effective at inhibiting gravicurvature, it stands to reason that AOA would be more effective at inhibiting upward bending of gravistimulated shoots when applied to the lower side of the stem than the upper side. Indeed this is the case; when plants are treated unilaterally with 10mM AOA there is a dramatic difference in gravicurvature (figure 2.6). All plants treated with AOA, regardless of the area of application, have less gravicurvature than the untreated plants. However, application of AOA to the lower side of the stem results in a greater inhibition of gravicurvature than AOA treatment of the upper side of the gravistimulated shoot. In untreated plants there is little difference in gravicurvature whether double distilled water was applied to either the upper or lower sides of the stem.

Just as I expected different degrees of gravibending in unilaterally-applied AOA, I also hypothesized that there would be no differences in the upward curvature in

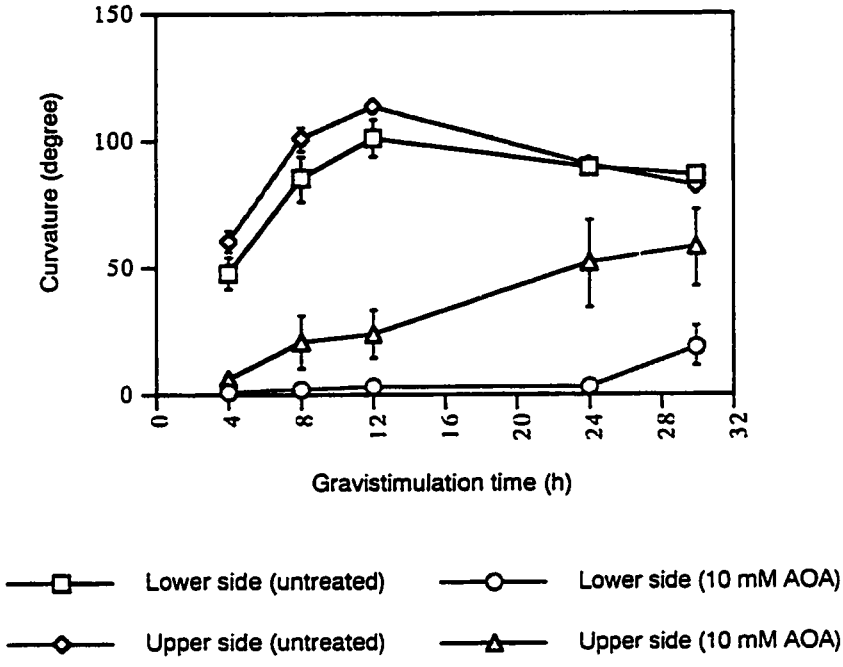

Figure 2.6 Curvature of intact gravistimulated sunflower epicotyls with unilateral AOA application (means ±SE).

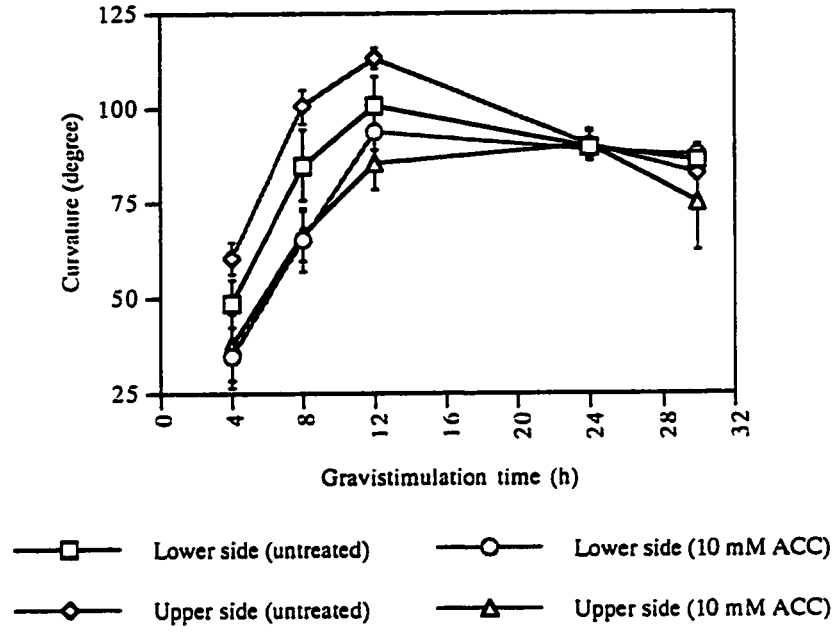

Figure 2.7 Curvature of intact gravistimulated sunflower epicotyls with unilateral ACC application (means ± SE).

gravistimulated stems with unilateral application of ACC to either the upper and lower sides as ACC did not appear to have any effect on bending in figure 2.3. Again, this is the case (figure 2.7). In this experiment it was observed that, like in untreated plants, there is no difference in the upward curvature of either upper and lower ACC-treated gravistimulated stems.

All of the above experiments fulfill the criteria to implicate a role for ethylene in gravitropism: (1) plants produce ethylene when gravistimulated, (2) gravitropism is inhibited when ethylene biosynthesis is inhibited, and (3) upward curvature of the stem is restored in ethylene-inhibited plants with the application of ethylene precursors.

It is difficult to determine ethylene's exact role in gravitropism. Because ethylene production is greatest in the lower half of the bending region of the gravistimulated stem (also the site where curvature suppression due to inhibition of ethylene biosynthesis is greatest), and the timing of increases in ethylene evolution and gravicurvature is almost identical, it is not unreasonable to conclude that ethylene production during gravistimulation is responsible for the upward bending of the shoot, however I do not know exactly how an increase in ethylene production results in gravicurvature. Does ethylene simply initiate gravibending or is it responsible for controlling and/or maintaining the degree of curvature? Could ethylene also be responsible for signaling to the plant to desist with bending (autotropism), thus preventing the plant from bending over onto itself?

It is well documented that ethylene inhibits cell division, and hence growth, resulting in the inhibition of such processes as shoot and root elongation and leaf expansion. Recently there is evidence indicating that low concentrations of ethylene may actually stimulate growth while higher doses of ethylene inhibit growth (Lee and Reid, 1997; Liu et al., 1990; Zobel and Roberts, 1977). Ethylene is also known to promote elongation in many aquatic plant stems and in various seedlings still at the hook stage (Abeles, 1992; Goto and Esashi, 1974; Suge, 1971). I too, have observed that low

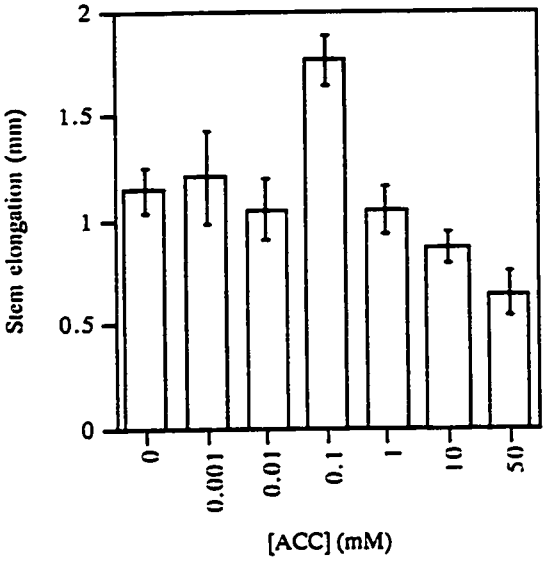

Figure 2.8 Change in elongation of ACC-treated upright intact sunflower epicotyls. Plants were placed in a darkroom for 24 hours after ACC treatment (means ± SE).

concentrations of ethylene promote elongation of stems which are inhibited by higher concentrations of ethylene. Figure 2.8 shows that when intact plants are treated with different concentrations of ACC and left upright in the dark for 24 hours there are noticeable changes in stem elongation; plants treated with the lower concentrations of ACC do not have any difference in growth compared to control plants, however plants treated with 0.1mM ACC have greater stem elongation than all other plants. Plants treated with the greatest concentration of ACC, 50mM, show the smallest increase in epicotyl elongation. Obviously there are concentrations of ethylene that are so low that they have no effect at all on upright epicotyl growth (keeping in mind that not all of the applied chemical penetrates the plants' cuticle). This experiment raises another possibility of how ethylene may play a role in gravibending: changes in either ethylene levels or ethylene sensitivities may control both the bending process and bending arrest. Perhaps the low concentration of ethylene evolving in the lower side of the epicotyl shortly after gravistimulation takes place stimulates elongation, resulting in upward bending of the shoot. As the ethylene concentration continues to increase over time then possibly cell elongation is arrested and curvature stops, preventing overbending of the stem.

Auxins in shoot gravitropism

The bulk of studies on the role of phytohormones in gravitropism have been on auxins. As outlined in the introduction to this chapter, it is generally accepted that auxins do play a major and possibly a primary role in the curvature of gravistimulated plants (Harrison and Pickard, 1989; Hart, 1990; Migliaccio and Rayle, 1989; Philosoph-Hadas et al., 1996; Rorabaugh and Salisbury, 1989; Salisbury et al., 1988). My own results also show that auxins appear to be involved in gravitropism. If basipetal auxin transport is inhibited with N-(1-naphthyl)phthalamic acid (NPA), gravicurvature is inhibited (figure

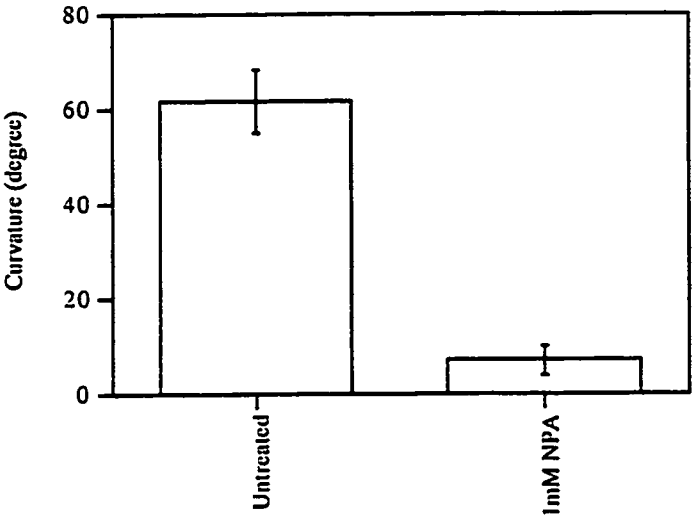

Figure 2.9 Curvature of NPA-treated gravistimulated sunflower epicotyls (means ± SE).

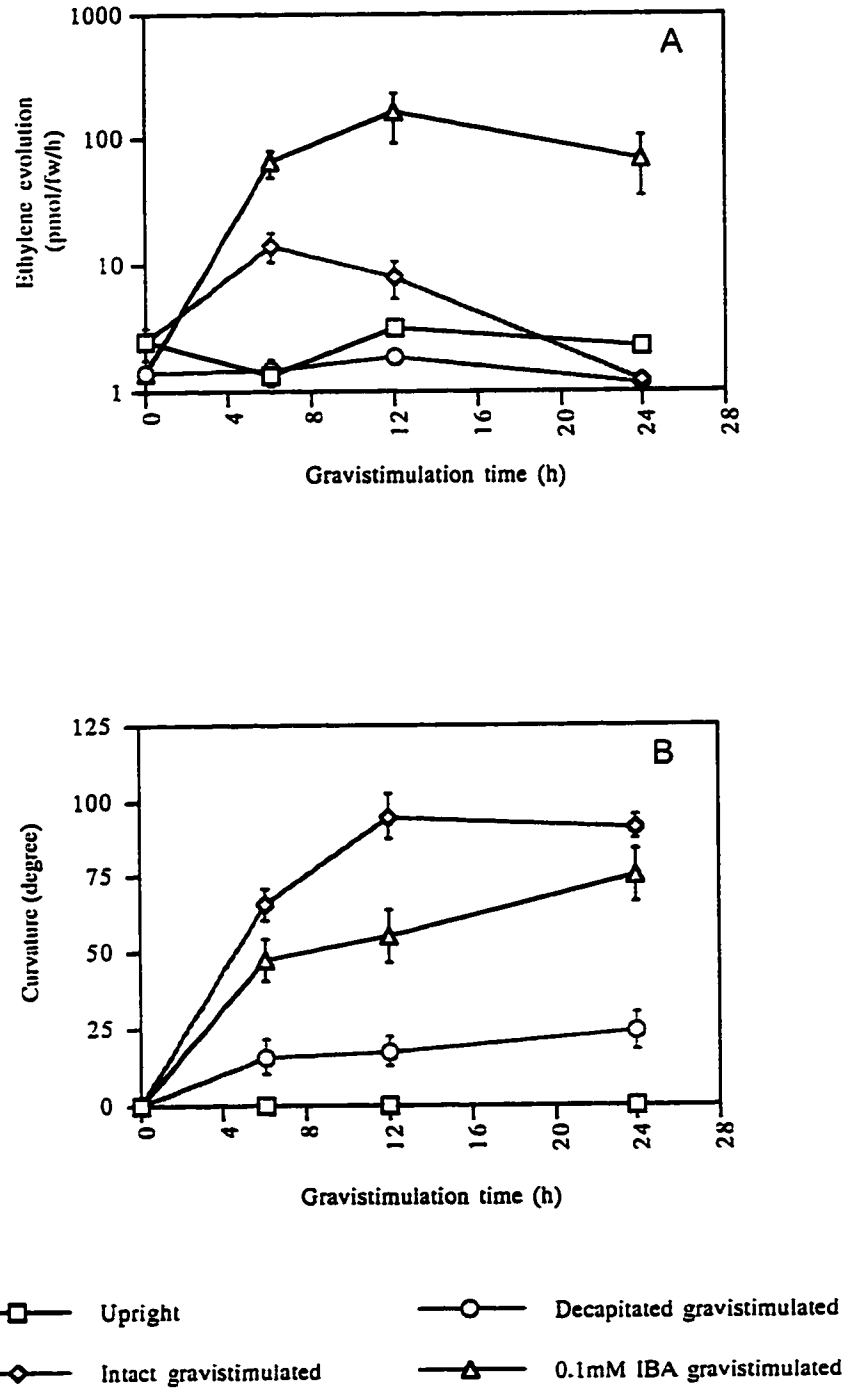

Figure 2.10 Ethylene evolution (A) and curvature (B) of decapitated IBA-treated gravistimulated sunflower epicotyls (means ± SE).

2.9). While untreated stems curve upward 61.4°, NPA-treated plants only curve 6.8°. If the majority of the auxin source of the epicotyl is removed via excision of the shoot apex and of young petioles/leaves, there is very little curvature during gravistimulation as compared to intact gravistimulated epicotyls (figure 2.10B). The addition of 0.1mM IBA to these excised epicotyls eventually restores their degree of curvature to levels comparable to those in intact plants. Whether auxins work by polar or lateral transport, or instead by changes in cells' sensitivity to auxins as outlined in the introduction is not conclusive with these experiments.

As with ethylene, steps have been taken in my work to show that auxins are required for upward bending of gravistimulated shoots; if most of the auxin sources are removed from the stems or if basipetal auxin transport is inhibited, then gravicurvature is inhibited. If auxin is reapplied to decapitated stems, then gravibending is restored. I decided not to measure auxin levels in the stems, both because I am focusing on the importance of ethylene in gravitropism, and because such an undertaking would be a whole project unto itself.

To further implicate a role for auxins in gravitropism, I removed various auxin sources (shoot apex, young petioles/leaves) and examined the resulting changes in gravicurvature of the epicotyls. Figure 2.11B depicts the curvature in gravistimulated epicotyls that have had their apex, petioles, leaves, and apex/petioles excised. Intact stems and epicotyls with excised apices show the greatest amount of gravicurvature (62.12° and 55.88° respectively). While there is no significant difference in gravicurvature between plants with detached petioles (40.4°) and those with detached leaves (52.4°), plants with excised petioles curve upward slightly less than both those with intact stems and epicotyls with detached apices. Plants removed of both their apex and petioles show hardly any upward curvature (1.5°). This experiment shows that not only do auxins play a role in

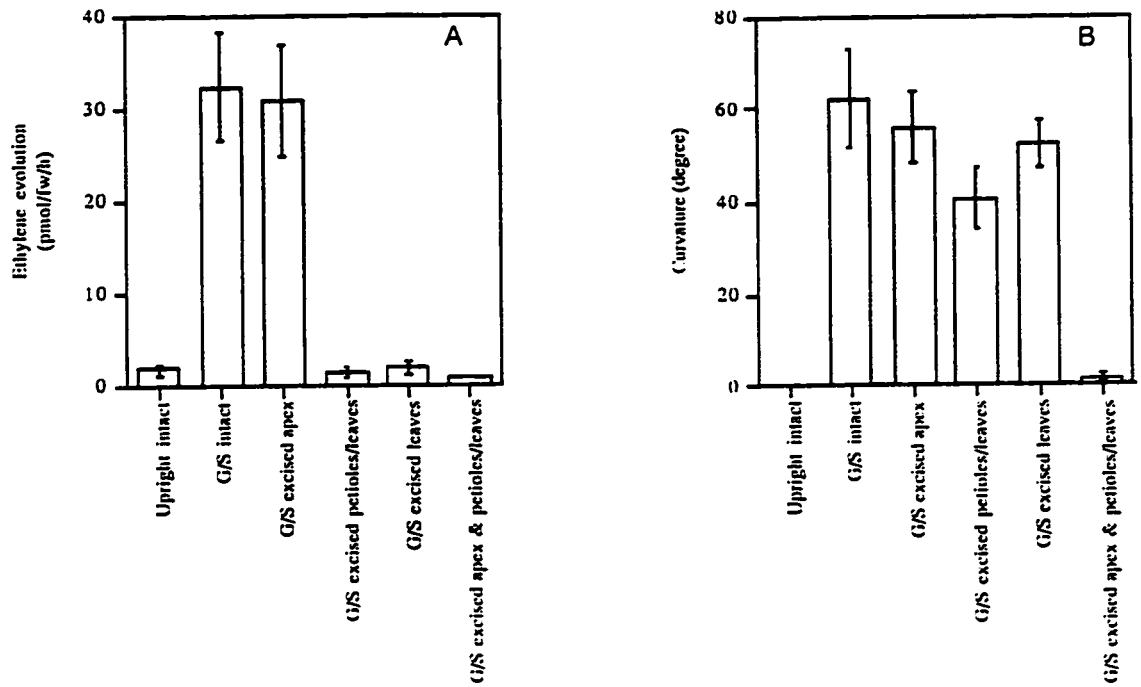

Figure 2.11 Ethylene evolution (A) and curvature (B) of gravistimulated (G/S) sunflower epicotyls with excised apex, petioles/leaves, leaves and apex with petioles/leaves (means ± SE).

gravitropism, which is indicated by the lack of curvature in gravistimulated epicotyls removed of the majority of their auxin source, but that the removal of only one of the major auxin sources does not have a significant inhibitory effect on gravibending, providing other auxin sources remain intact. Perhaps only trace amounts of auxins are required for gravicurvature. This also suggests that plants have a "backup" system to respond to gravity should there be a problem with one auxin source.

Ethylene/auxin interaction in shoot gravitropism

After establishing that both ethylene and auxins are required for gravitropism, the next step was to investigate whether a relationship exists between these two phytohormones. Because auxins are capable of inducing ethylene biosynthesis by increasing the production of ACC synthase (Sato and Theologis, 1989), it was logical to examine ethylene production in gravistimulated auxin-manipulated plants. In figure 2.10A ethylene evolution is measured both in plants removed of their auxin source via decapitation, and in IBA-treated decapitated plants. Decapitated plants, which have very little gravicurvature (figure 2.10B) produce only small amounts of ethylene. Decapitated plants treated with 0.1mM IBA evolve large amounts of ethylene, greater even than intact gravistimulated plants. IBA treatment also restores gravibending of the shoot (figure 2.10B). The results in this figure indicate that there is a relationship between ethylene and auxin in gravitropism.

This association between auxin and ethylene can be examined further by looking at ethylene production in epicotyls that have had their various auxin sources removed (figure 2.11A). As expected, upright plants produce very little ethylene, especially compared with the intact gravistimulated control plants. Plants with excised shoot apices produce as much ethylene as the intact plants, and show the same degree of curvature (figure 2.11B). While plants excised of their first leaves or petioles/leaves curve upward approximately as much

as the intact gravistimulated plants, they produce significantly less ethylene, in fact almost as much as the intact upright plants. Gravistimulated stems with excised apex and petioles/leaves show neither much ethylene evolution nor gravibending. At first glance it would appear that ethylene is not necessary for gravibending in epicotyls. However, experiments with the ethylene inhibitor AOA have shown this to be otherwise. Perhaps only trace amounts of ethylene are required for gravitropism. There is always the likelihood with phytohormones that only minute changes in concentration, changes too small for us to measure with current technology, are sufficient for carrying out a specific action.

If the actions of auxins and ethylene are somehow interrelated in gravitropism, then it stands to reason that auxins would be more effective at promoting gravicurvature if applied only to the lower side of a gravistimulated decapitated stem, where ethylene production is greatest, then if applied to the upper side of the epicotyl. In fact this is the case (figure 2.12). Curvature is greater when 0.1mM IBA is applied to the lower side of the decapitated shoot than when applied only to the upper side.

Based on the following, it appears that ethylene and auxin not only share a role in gravicurvature of epicotyls, but that their roles are somehow interdependent: (1) auxins increase ethylene production, (2) a removal of the majority of auxin sources in stems results in a severe reduction in ethylene production, (3) auxins work most effectively at restoring upward curvature in decapitated gravistimulated stems when applied to the site where ethylene production is greatest.

We do not know how ethylene and auxins interact. It seems likely, since auxins are capable of enhancing conversion of SAM to ACC by increasing ACC synthase synthesis (Sato and Theologis, 1989), that auxins stimulate ethylene production when stems are gravistimulated, although auxin may instead act by altering the stem cells' sensitivity to ethylene. In studying abscission of pepper (*Capsicum annuum*) reproductive organs,

Huberman et al. (1997) proposed that a decrease in endogenous auxins in the abscission zone results in an increase in sensitivity of the zone to ethylene-induced abscission. Another possibility is that ethylene stimulates auxin activity, perhaps by changing the sensitivity of tissues to auxins, as suggested by recent work by Visser et al. (1996) on *Rumex palustris* shoots and by Liu and Reid (1992) on sunflower hypocotyls. In *R. palustris*, endogenous levels of ethylene increased prior to adventitious rooting while the concentration of endogenous auxin did not change, although its presence was required. In sunflower hypocotyls auxin-induced adventitious rooting was more effective in the presence of exogenous ACC than without this precursor to ethylene. These results suggest that ethylene increases the tissue's sensitivity to auxin. The results in figure 2.13 suggest that a similar interaction exists between auxin and ethylene in gravitropism. Decapitated plants treated with 0.05 mM ACC and a range of IBA concentrations show an increase in curvature compared to epicotyls treated only with IBA. Stems treated with both IBA and 5mM AOA show hardly any gravibending. These results indicate that the presence of ethylene may render the tissue more sensitive to the auxins present. However one must view this conclusion with some reservations for the following reasons: (1) auxins promote ethylene biosynthesis, so the exogenous ethylene may not actually be entirely responsible for the change in curvature, (2) figures 2.3 and 2.7 show that exogenous ACC has no effect on gravicurvature in stems, (3) repeated trials of this experiment showed similar trends, although there was some variability in the gravistimulation time required to observe maximal response.

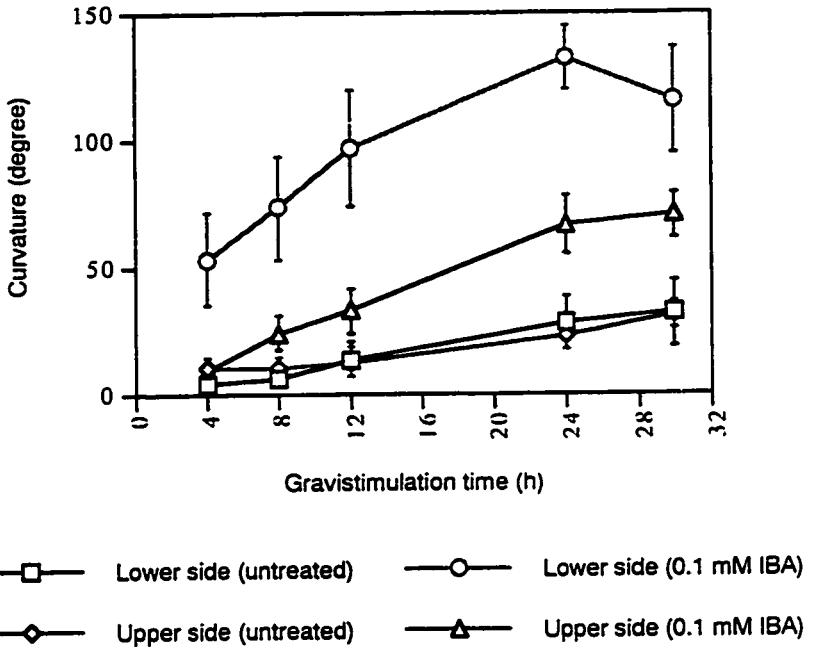

Figure 2.12 Curvature of gravistimulated decapitated epicotyls with unilateral IBA application (means ± SE).

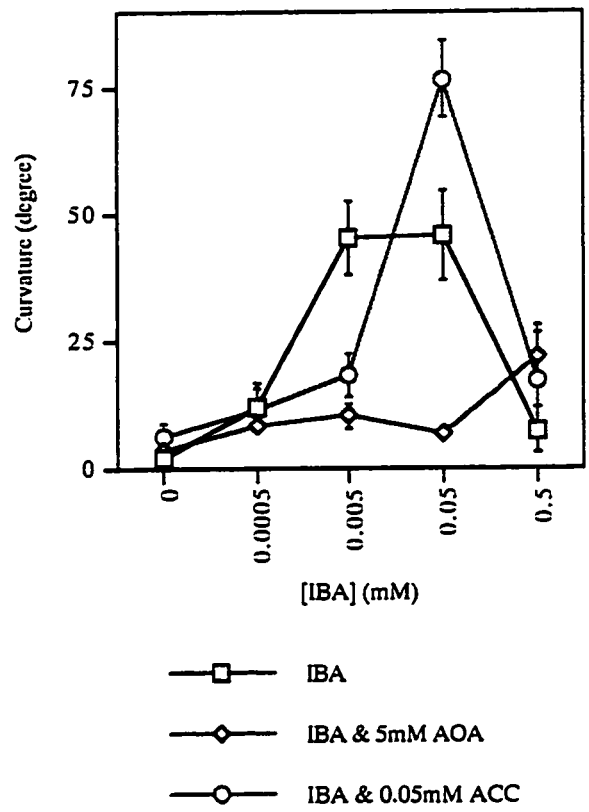

Figure 2.13 Curvature of decapitated epicotyls treated with various concentrations of IBA, IBA with AOA, and IBA with ACC, and all gravistimulated 12 hours prior to curvature measurements (means ± SE).

SUMMARY

The results from the experiments described in this chapter provide convincing evidence of ethylene's importance in gravitropism. Until now ethylene's role in gravitropism has been largely downplayed or dismissed. My results show that ethylene is definitely required for gravitropism, as are auxins, and that these two phytohormones interact with one another, most likely in the lower half of the bending region of the gravistimulated epicotyl. At this point in time, however, I do not know the exact role of ethylene in gravicurvature, although I proposed several ideas.

In the future, plants with mutations such as auxin resistance (aux, axr), tryptophan (precursor in auxin biosynthesis) requiring (trp), ethylene insensitivity (ein, and etr), ethylene overproducers (eto), constitutive triple response mutants (ctr), *diageotropica* mutants (dgt), root hypocotyl gravitropic mutants (rhg), and shoot gravitropic mutants (sgr) may be able to provide greater insight into how phytohormones bring about changes in stem curvature during gravistimulation. Many of these mutants exist today, the majority of them being *Arabidopsis* and tomato plants, however most of the gravitropic research with these mutants focuses on signal transduction with less examination of the physiology of the plant growth substances. Future advances in gas chromatography and mass spectrophotometry will hopefully one day allow for more sensitive measurements of phytohormones. A great deal could be learned about the roles ethylene and auxins play in gravicurvature if these phytohormones could be measured within individual cells or small groups of cells as opposed to large amounts of tissue. Recent research indicates calcium also has an important role in gravitropism (Philosoph-Hadas et al., 1996). There are still many avenues remaining to be explored in the physiology of dicotyledon shoot gravitropism.

CHAPTER 3 - ETHYLENE AND ANATOMY IN SUNFLOWER SHOOT GRAVITROPISM

The ability to modify cell shape and size is imperative for plants to interact efficiently with their environment. Examples of this form of adaptation are evident throughout our environment: trees shaped by persistent wind forces, stem height differences between alpine and prairie ecotypes of the same species and aerenchyma formation in roots of nonwetland flooded grasses. Gravity is no exception; when shoots bend in response to gravity (or light), cellular changes take place. In the case of gravistimulated dicotyledonous stems it was initially believed that curvature began at the shoot apex and traveled basipetally over time (Dolk, 1936). However Digby and Firn (1979) and Meicenheimer and Nackid (1994) showed in both etiolated and light-grown dicotyledonous plants that curvature begins simultaneously along the entire stem. It is believed that stems curve upward due to different rates of cellular elongation (Hart, 1990). Careful measurements by Digby and Firn (1979) lead them to conclude that gravicurvature results from a cessation of growth along the upper side of a gravistimulated stem, while the growth rate of the lower side is equal to or greater than that of upright controls. Firn and Digby (1977) proposed that the differential rates of elongation resulting in gravicurvature occur within the upper and lower peripheral cell layers, noting that epidermally peeled sunflower hypocotyls are capable of growth but not gravibending. These results are supported by the work of Migliaccio and Rayle (1984) who also observed a lack of gravibending in epidermally peeled sunflower hypocotyls. However, one must keep in mind that removing the epidermis is a drastic procedure resulting in the initiation of wounding responses which may also have an effect on stem curvature. Although there has been much recent information obtained regarding the theories of differential growth rates in gravibending stems, very few experiments have examined the histological changes that take

place within the cells of gravistimulated dicotyledon shoots. There is a dearth of information regarding specific changes in cell shape and cell numbers within these stems.

It is generally accepted that ethylene inhibits cell and stem elongation. This is actually one of the tenets of the triple response observations of behaviour in plants treated with exogenous ethylene (Burg, 1973; Osborne, 1977; Sanchez-Bravo et al., 1992). There are, however, many instances where exogenous ethylene does not inhibit elongation. In submerged aquatic plants ethylene actually stimulates stem elongation (Osborne, 1977) and evidence has suggested that ethylene enhances shoot growth in the early stages of seedling development in such plants as bean and cocklebur (Goto and Esashi, 1974), oat and rice (Suge, 1971) and wheat (Suge et al., 1997). It is likely that ethylene at very low concentrations can promote elongation while at higher concentrations inhibit elongation.

Auxins have long been associated with stimulating cell and stem elongation, a belief that is the basis of the Cholodny-Went hypothesis (Hart, 1990). There is still no general consensus on how auxins stimulate cell elongation. One popular theory is the acid-growth hypothesis (Rayle and Cleland, 1970) which states that auxins cause the cells in question to secrete protons into their cell walls, thus lowering the cell pH which in turn activates certain cell wall-degrading enzymes. The result is cell wall loosening and cell elongation.

Ethylene is believed to stimulate isodiametric cell expansion (Eisinger, 1983; Osborne, 1977; Sanchez-Bravo et al., 1992), a process involved in actively growing seedling tissue, epinastic movements in petioles (Abeles et al., 1992), leaf expansion (Lee and Reid, 1997) and cells in leaf abscission zones (Osborne, 1977; Sexton, 1982). Burg (1973) proposed that ethylene does not directly stimulate cell expansion, but rather delays cell differentiation, allowing radial growth to continue unabated.

Lateral cell expansion is thought to be due to changes in microtubule and cellulose microfibril orientation. In upright stems both microfibrils and microtubules are normally arranged in a transverse direction within the cell. When these stems are treated with

ethylene the microfibrils and microtubules run in both longitudinal and oblique arrays (Burg, 1973; Lang et al., 1982; Roberts et al., 1985). Work by Nick et al. (1990) showed that in sunflower hypocotyls only the microtubules located subjacent to the outer epidermal wall changed direction when gravistimulated. These authors state that longitudinal orientation of microtubules is associated with a low rate of cell elongation while transverse microtubule arrays are associated with a high rate of cell elongation. In their experiments, Nick et al. (1990) found that the microtubules in the lower side of gravistimulated sunflower hypocotyls were oriented in a transverse fashion, while those on the upper side were positioned in a longitudinal direction. These results were duplicated with exogenous IAA, leading the authors to propose that auxins might mediate the microtubule orientation in gravistimulated stems.

In order for increases in cell elongation and radial cell expansion to take place, there must also be increases in cell wall formation to support this new growth. Such cell wall formation is thought to be affected by ethylene. Research suggests that ethylene stimulates the synthesis and incorporation of cell wall materials. (Ingemarsson, 1995; Ingemarsson et al., 1991; Sanchez-Bravo et al., 1992).

In the last chapter I showed that both ethylene and auxins are required for gravibending to occur in sunflower epicotyls. Exactly how ethylene and auxins interact and their exact roles in gravitropism are not known at this time. Perhaps a closer examination at the histological level can provide more answers. The purpose of the research in this chapter was to study changes in cell size and number in gravistimulated sunflower epicotyls with the hopes that I could better elucidate a role for ethylene, and maybe also auxins, in shoot gravitropism. I hypothesized that in the bending region of the epicotyl, the cells in the lower side of gravistimulated stems elongate at a greater rate than the cells in the upper half, resulting in the upward bending of the shoot and that this increase in the rate of elongation is due to an interaction of ethylene and auxin (figure 3.1). Furthermore, I

believed it likely that there could be minimal elongation in the cells of the basal region of the epicotyl as energy is likely diverted to the more actively growing cells in the lower side of the bending region.

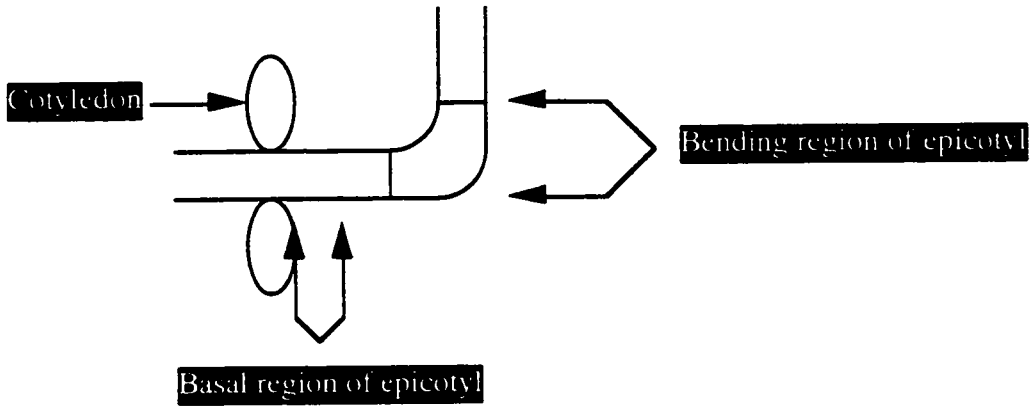

Figure 3.1 Gravistimulated sunflower epicotyl illustrating the basal and bending regions.

METHODS AND MATERIALS

Growth Conditions / Treatments

All sunflower plants were grown and treated under the same conditions as specified in the second chapter of this thesis.

Stem Diameter and Stem Elongation Measurements

One day prior to treating and/or gravistimulating the plants, the stem diameter was measured with calipers at the base of the epicotyl or approximately midway up the epicotyl. The latter was measured at a location marked with india ink, an inert substance which has no effect on plant stem growth. Initial stem elongation measurements were taken with calipers between two vertically positioned marks of india ink along the epicotyl. Following treatment and 24 hours of gravistimulation, these same areas were remeasured and the change in stem elongation and radial growth calculated.

Histology

Histological examination of epidermal and pith cells was conducted in 19 day old gravistimulated sunflower epicotyls following 48 hours of gravistimulation in the dark. For epidermal examination, watchman's forceps were used to take epidermal peels along the upper and lower sides at both the basal and bending region of the epicotyl. In upright control plants, epidermal peels were taken from both the base and a site midway along the epicotyls (the approximate region where bending occurs in gravistimulated plants) but obviously no distinction was made about the side the peels were collected. The peels were then fixed for a minimum of 24 hours in 85% lactic acid (BDH) to remove pigmentation. Following fixation the peels were placed on microscope slides and covered with coverslips

which were then sealed with nail polish to prevent evaporation of the lactic acid and desiccation of the tissue.

In order to examine the pith cells, a sagittal hand section was taken at both the base and the bending region of the gravistimulated sunflower epicotyls and at the base and midway along the epicotyl in upright plants. The tissues were placed under a partial vacuum and fixed for 24 hours in a solution of 1.6% paraformaldehyde (Sigma) and 2.5% glutaraldehyde (Sigma) in a 0.05M K_2Na phosphate buffer (pH 6.8) (Sigma). The tissue was then dehydrated with methyl cellosolve (BDH) for another 24 hours followed with a change of 100% ethanol for 24 hours. The tissues were placed under a partial vacuum a second time and left for another 24 hours in new 100% ethanol. Over the next three days the tissue was gradually infiltrated with historesin (Leica Canada) until the tissues were immersed only in the infiltration solution. The tissue was then embedded according to Yeung and Law (1987) in a 15:1:0.6 ratio of infiltration solution, hardener and PEG 400 solution (Sigma) for 24 hours, sectioned 5 μ using a Reichert-Jung 2040 autocut retracting microtome with Ralph glass knives and stained with toluidine blue O (TBO) (Sigma).

The length and width of both the epidermal cells and the pith cells were measured under 10X and 40X magnification on a microscope.

Statistics

All experiments were repeated at least once. In cell measurement experiments n=180 and in stem measurements n=10-14. Data are shown in means ± standard error (SE) in the form of error bars, and in 95% confidence t-tests in the form of lower case letters and symbols placed above the columns. When the SE bar is not shown, the error is smaller than the symbol.

RESULTS AND DISCUSSION

In order for organs to change their orientation, they must undergo a change in either turgor or growth. Changes in turgor are responsible for reversible movement such as nyctinasty in leaves (changes in leaf positioning due to circadian rhythms), heliotropism (solar tracking of leaves and flowers) and leaf thigmonasty (for example: closing of *Mimosa* leaves in response to touch). In many cases plant movement is due to differential growth patterns between or within organs. This is the case in phototropism, gravitropism and thigmotropism (Hart, 1990). Although it is already known that gravistimulated shoots bend upward due to differences in growth rates between the upper and lower sides of the organ (Digby and Firn, 1979), this knowledge is based on observing changes in distance between marks placed equidistantly along gravistimulated sunflower hypocotyls and not from histological examination of the tissue involved in the growth. It is unknown whether these changes in growth rates are due to alterations in cell shape or adjustments in the rates of cell division. With my experiments I hope not only to clarify which anatomical changes take place in shoot gravitropism, but to relate these changes to ethylene and possibly auxin.

Before discussing the effects of gravitropism on epicotyls at the histological level, it is useful to examine the changes in radial expansion and elongation of the entire epicotyl. Although not statistically significant, the data in figure 3.2A suggest an increase in the width of the base of gravistimulated epicotyls compared with upright plants within a 24 hour period. During this same time period, there is elongation of the epicotyls, but there is no difference in the rate of elongation between upright and gravistimulated plants (figure 3.2B).

It is useful to have a general understanding of the changes in stem width and length, but it does not reveal much about how these changes come about. For this reason I investigated the growth patterns of the epicotyl in greater detail by examining the alterations

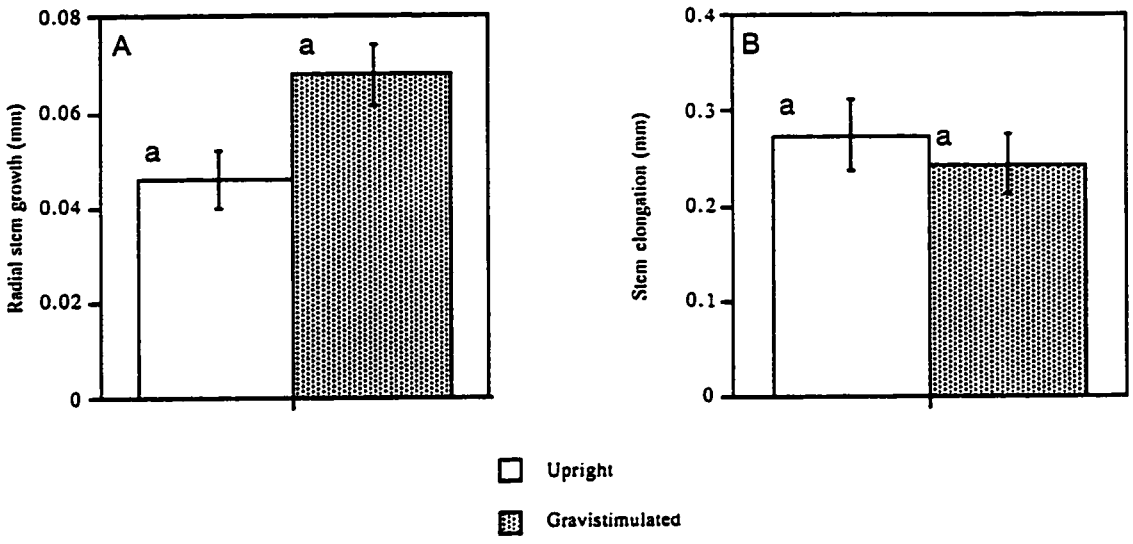

Figure 3.2 Radial stem expansion (A) and stem elongation (B) in upright and gravistimulated sunflower epicotyls. Radial stem growth measured at the base of the epicotyls (means ± SE). Columns with different lower case letters are significantly different (p = 0.05).

in the size of the cells involved in stem width and elongation. Despite the many different cell types in shoots, I decided to concentrate on the epidermal cells as they are believed to be of great importance in gravibending (as mentioned in the introduction to this chapter), and pith cells because they make up a large portion of the stem, and because their large size makes it easy to detect any changes in growth. In addition, the location of the pith cells and the epidermal cells make for a good comparison between the central and peripheral areas of the shoot. I examined these two cell types in two different areas of the upright and gravistimulated epicotyl: the region where the majority of the bending takes place (bending region) and for comparison the lower portion of the epicotyl beneath the bending region where no visible curvature takes place but where much of the epicotyl is supported (basal region) (figure 3.1).

Changes in epidermal cell width

In upright sunflowers, the epidermal cells are wider at the base than midway along the epicotyl (in upright plants measurements made midway along the epicotyl are for comparison with the bending region of gravistimulated epicotyls) (figure 3.3A). In gravistimulated sunflowers the location of the epidermal cells must be taken into consideration; there may be differences in shape and growth of the epidermal cells depending whether they are found along the upper, lateral or lower sides of the stem. For simplification, I only examined the epidermal cells along the upper and lower sides of the epicotyl. In fact, in both the bending and basal regions of the gravistimulated epicotyl, the epidermal cells along the lower side are significantly wider than the epidermal cells along the upper side, especially in the basal region. The epidermal cells along the lower side in both regions of the gravistimulated stem are also significantly wider than the epidermal cells in upright plants. In the basal region of the gravistimulated stems, the epidermal cells along the upper side are of the same width as those in upright plants. However in the bending

region, the epidermal cells along the upper side actually have less radial growth than in upright stems. In short, figure 3.3A shows that gravistimulated epicotyls have wider epidermal cells along the lower side of the stem compared to the cell along the upper side and in upright plants.

Changes in pith cell width

Pith cells in the base of upright epicotyls are significantly wider than those in the middle area of the stem (figure 3.4A). In gravistimulated epicotyls, the pith cells in the basal region are not significantly wider than those in the bending region. There is also no significant difference in width between the pith cells in the basal region of gravistimulated epicotyls and those in the basal region of upright epicotyls. The pith cells in the bending region of gravistimulated epicotyls are significantly wider than those in the corresponding area of upright epicotyls. Thus in gravistimulated epicotyls there appears to be an increase in girth of the pith cells in the bending region while the pith cells of the basal region grow radially at the same rate as in upright plants.

Summary of changes in cell width

I have not found any mention of studies of radial cell expansion in gravistimulated shoots in the literature, yet it seems reasonable that the cells of the basal and bending regions of the epicotyl are wider in gravistimulated stems than in upright epicotyls. As a shoot curves upward, the plant's centre of gravity shifts basally along the stem. In the basal region of a gravistimulated epicotyl, an increase in cell width of the epidermal cells along the lower side of the shoot likely provide greater anchorage to the upward-bending stem. The increased width of the pith and epidermal cells in the bending region probably also provides greater anchoring support to the gravibending stem. Similar support

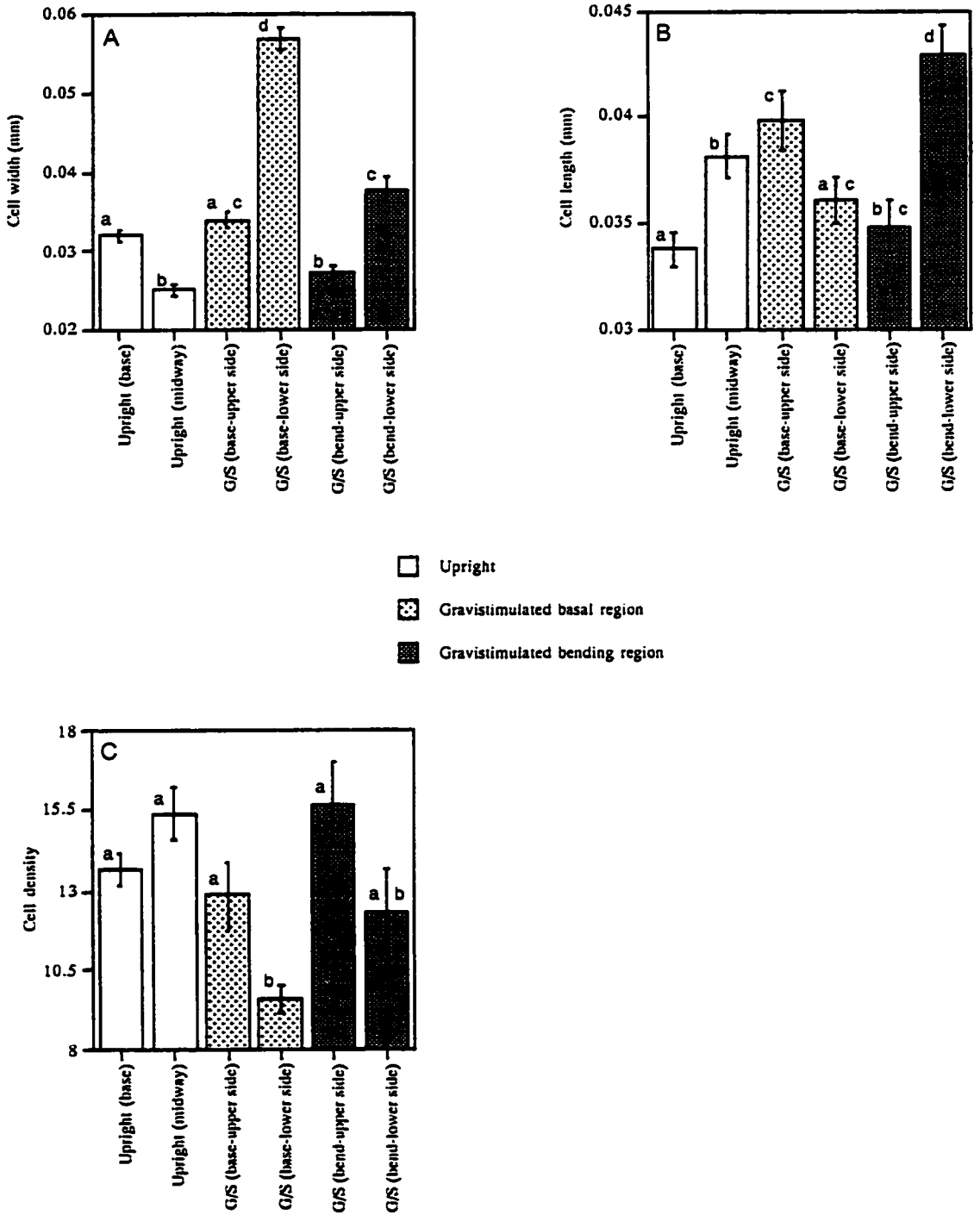

Figure 3.3 Width (A), length (B) and cell density (C) of sunflower epidermal cells in the basal and middle regions of upright epicotyls and both the upper and lower sides of the basal and bending regions of gravistimulated (G/S) epicotyls (means ± SE). Columns with different lower case letters are significantly different ($p = 0.05$).

mechanisms are seen elsewhere in plants: cells along the lower side of the lamina of bean leaves increase in area causing leaves to grow upward (hyponasty) (Hayes and Lippincott, 1981), and in gymnosperms, compression wood resulting from increased activity of the vascular cambium along the lower side of radial branches causes the vascular cells to increase in size thus pushing branches upward (Westing, 1965).

Changes in epidermal cell length

The variations in epidermal cell length within a 24 hour period are more subtle than those in cell width (figure 3.3B). In upright plants, the epidermal cells in the middle region of the epicotyl are significantly longer than those at the base of the epicotyl. In gravistimulated stems, the difference in length between the cells in the basal and bending regions is not as straightforward as in upright plants. There is no significant difference in length of the epidermal cells along the upper side of the stem between the basal or bending regions. However the epidermal cells along the lower side of the gravistimulated stem are significantly longer in the bending region than in the basal region.

There is no significant difference in epidermal cell length between the upper and lower sides of the basal region of gravistimulated epicotyls (figure 3.3B). In the bending region of gravistimulated plants, epidermal cells along the lower side are significantly longer than those along the upper side.

In the basal region, only the epidermal cells along the upper side of the gravistimulated stem are significantly longer than the corresponding epidermal cells in the upright plants (figure 3.3B). In the bending region, only the epidermal cells along the lower side are significantly longer than the corresponding cells in upright plants.

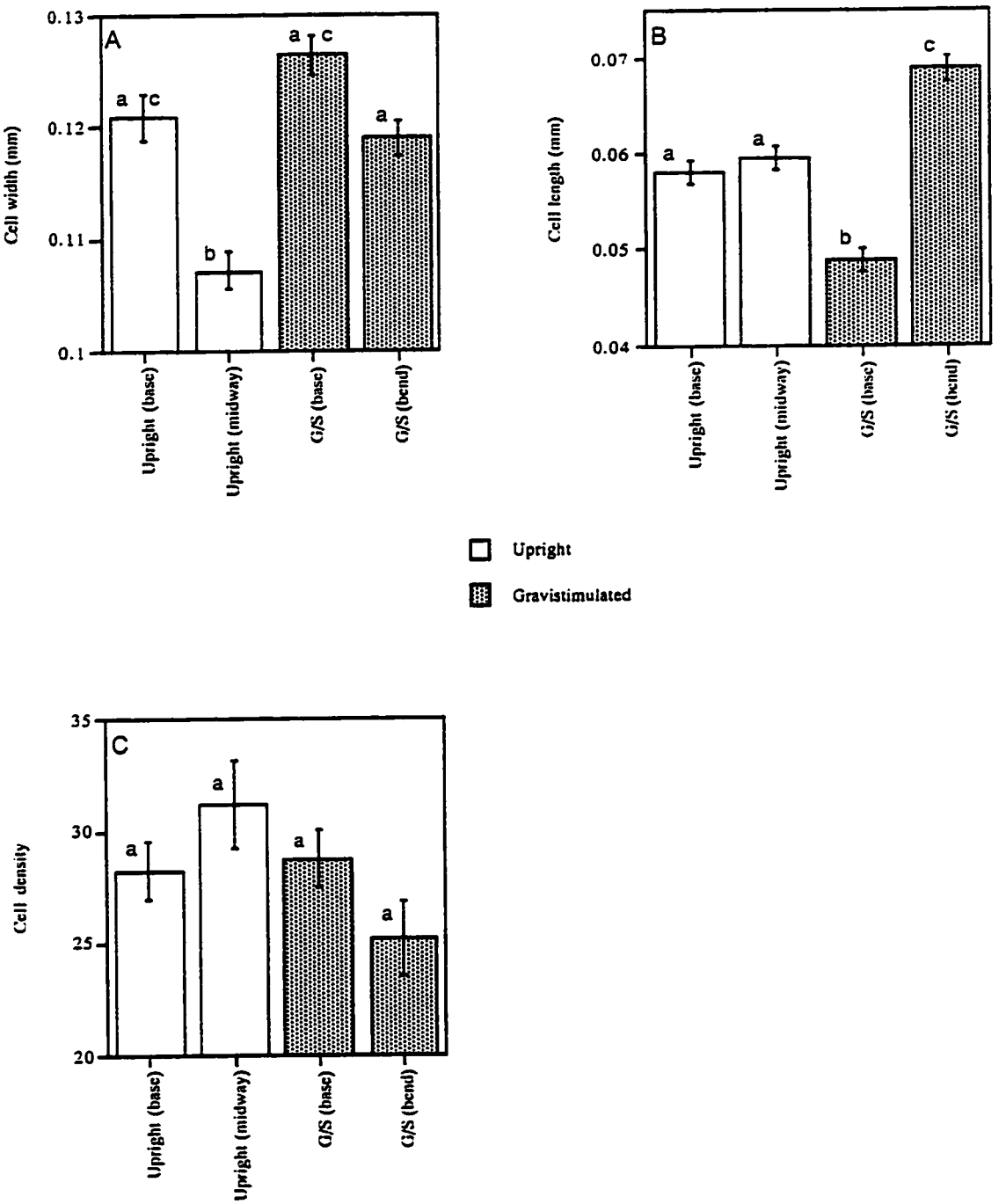

Figure 3.4 Width (A), length (B) and cell density (C) of sunflower pith cells in the basal and middle regions of upright epicotyls and basal and bending regions of gravistimulated (G/S) epicotyls (means ± SE). Columns with different lower case letters are significantly different (p = 0.05).

Changes in pith cell length

There is no significant difference in pith cell length between the basal and middle regions of upright epicotyls (figure 3.4B). In gravistimulated plants, the pith cells in the bending region of the epicotyls are significantly longer than those in the basal region. The pith cells at the base of the upright epicotyls are longer than those at the base of gravistimulated epicotyls. The pith cells in the bending region of gravistimulated stems are significantly longer than those in the corresponding region of upright plants.

Summary of changes in cell length

In the bending region of gravistimulated stems, it is likely that the greater rate of elongation of the epidermal cells along the lower side than the upper side is responsible, at least in part, for the upward curvature of the stem. This is in agreement with the observations of Firn and Digby (1977) and Digby and Firn (1979). The increase in the rate of elongation of the pith cells in the bending region may also contribute to gravibending. Perhaps, keeping in mind that Firn and Digby (1977) and Migliaccio and Rayle (1984) found that the removal of the peripheral layers of the shoot prevented gravicurvature, the increased elongation of the pith cells may simply be a response to gravicurvature.

In the basal region of the gravistimulated stem, the pith cells elongate at a lesser rate than the corresponding cells in upright plants. Only the epidermal cells along the upper side of gravistimulated epicotyls elongate at a greater rate than the corresponding epidermal cells in upright stems. The base of the epicotyl does not perceptibly curve upward when gravistimulated so there is little need for differential elongation between the upper and lower sides in this region. The decrease in the rate of elongation of the pith cells of the base of the gravistimulated epicotyl may reflect a shift in energy allocation; because these cells appear to serve as an anchoring/support system for the entire gravitropic shoot, perhaps they divert energy from their "elongation potential" to allow for an increase in

radial growth, thus increasing the load which they can support. It seems odd that the epidermal cells along the upper side at the base of the gravistimulated shoot elongate at a greater rate than the cells along the lower side and the corresponding epidermal cells in upright plants. Perhaps this slight, yet significant increase in length along the upper side of the stem also contributes to the support of the upward bending shoot or it may be involved in autotropism, signaling the arrest of gravicurvature and preventing overbending of the stem.

Cell density

Along with investigating the changes in cell width and elongation, it is useful to examine the rate of division of the pith and epidermal cells. Rather than determine the change in cell division over time, I compared the density (the number of cells within a fixed area) of the pith and epidermal cells (figures 3.3C and 3.4C). There is no significant difference in cell density of the epidermal cells along the upper side of the basal region of the gravistimulated epicotyl and the basal region of the upright epicotyl (figure 3.3C). The epidermal cells along the lower side of the basal region of the gravistimulated epicotyl are significantly fewer in number than the same region in upright plants. There is no significant difference in cell number between the epidermal cells of the bending region and the corresponding epidermal cells in the upright plants. There is no significant difference in pith cell number between upright and gravistimulated plants (figure 3.4C).

Essentially the cell measurements (width and length) between gravistimulated and upright stems of figures 3.3C and 3.4C indicate that there is little difference in the cell division rate within pith and epidermal cells. The epidermal cells which are of lower number per area (the cells along the lower side of the basal region of the stem) than the other epidermal cells are the same cells which are significantly wider than the other epidermal cells.

Ethylene and auxins in stem width

Treatment of gravistimulated shoots with phytohormone regulators can provide a clearer understanding of the role of phytohormones involved in gravicurvature. Neither ethylene nor auxins appear to have much of an effect on stem diameter (figure 3.5A). There is no significant difference in stem width between the gravistimulated ACC-treated and gravistimulated untreated plants as well as between the upright ACC-treated and upright untreated plants. Gravistimulated ACC-treated plants have significantly more radial stem growth than ACC-treated upright plants, much greater than the insignificant difference in stem width between the upright and gravistimulated untreated plants. In AOA-treated plants there is no difference in stem width between the upright and gravistimulated plants. However, the gravistimulated AOA-treated plants have less radial expansion than untreated gravistimulated stems while the upright AOA plants have the same rate of radial growth as the upright controls. Both the upright and gravistimulated IBA-treated plants have similar rates of radial growth and these rates are also similar with those of the untreated upright and gravistimulated shoots.

I was surprised to observe that ACC has no effect on cell width, however the fact that exogenous ethylene does not appear to have a role in radial cell expansion in gravistimulated plants does not mean that endogenous ethylene is also without a role. When ethylene biosynthesis is inhibited with AOA, radial stem expansion (figure 3.5A) and shoot curvature (figure 3.5C) in gravistimulated plants are also inhibited [exogenous ACC does not affect gravibending in sunflower epicotyls (figure 3.5C); these results are consistent with data in chapter two (figures 2.3 and 2.7)]. Just as only one particular concentration of ACC stimulated cell elongation in figure 2.8, perhaps only a narrow range of concentrations of ACC will enhance radial cell expansion. A more likely scenario is that ethylene action involves a stimulus threshold and any additional ethylene has no additive effect. In this case, the endogenous levels of ethylene produced by gravistimulation alone

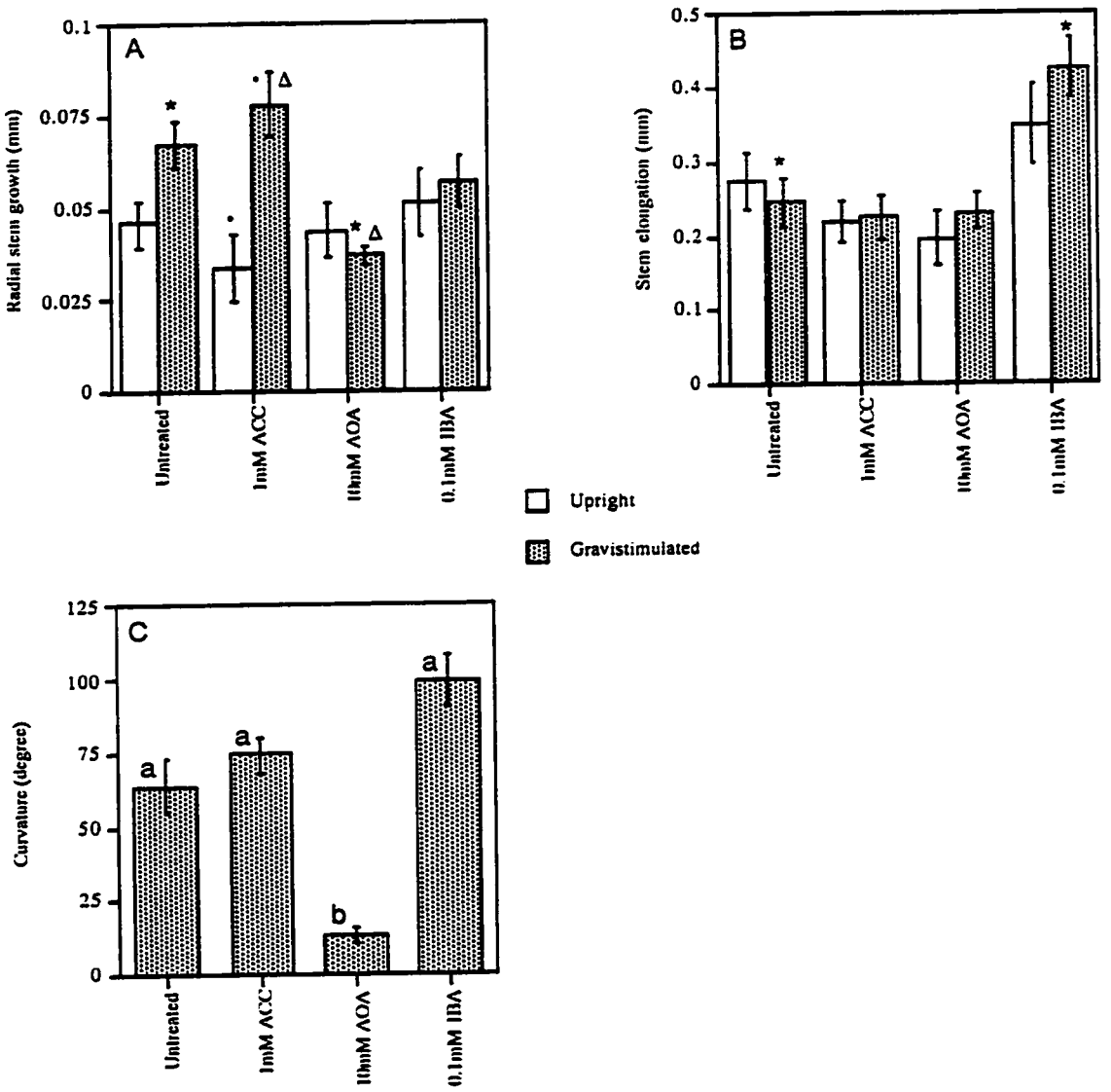

Figure 3.5 Radial stem expansion (A), stem elongation (B) and curvature (C) in untreated, ACC-, AOA- and IBA-treated upright and gravistimulated sunflower epicotyls. Radial stem expansion measured at the base of the epicotyls (means ± SE). Columns with same symbols (figures A and B) and columns with different lower case letters (figure C) are significantly different (p = 0.05).

result in increases in cell width and additional ethylene has no further effect on these cells. This theory is supported by the fact that the ethylene inhibitor AOA reduces radial cell expansion in gravistimulated shoots.

As auxins are associated with stem elongation, it is not unexpected that exogenous auxins have little effect in manipulating cell width. Perhaps, like ethylene, endogenous auxin levels are sufficient for regulating cell width and exogenous applications are simply not required. Maybe experiments with auxin transport inhibitors such as NPA or 2,3,5-tri-iodobenzoic acid (TIBA) (Niedergang-Kamien and Skoog, 1956) would confirm whether or not endogenous auxins have a role in radial cell expansion.

Ethylene and auxins in stem elongation

As already mentioned in the introduction to this chapter, auxins are normally associated with promotion of cell elongation and ethylene with isodiametric cell expansion and in some cases, with cell elongation. The results in figures 3.5A, 3.5B and 2.8 support these beliefs. There is no significant difference in the rate of stem elongation between upright and gravistimulated plants within treatments (figure 3.5B). There is also no significant difference in the rate of stem elongation in all upright plants. In the gravistimulated plants, only the IBA-treated stems are significantly longer than the untreated gravistimulated plants. There is no significant difference in the degree of curvature of these auxin-treated stems compared to the other plants (figure 3.5C).

The 1mM ACC-treated plants in this experiment do not have significantly greater stem elongation than untreated plants or the AOA-treated plants. However the results in figure 2.8 show that upright epicotyls treated with 0.1mM ACC have a significantly greater amount of stem elongation compared to untreated shoots and epicotyls treated with other concentrations of ACC. This suggests that low (but detectable) levels of ACC can stimulate stem elongation. It must also be kept in mind that auxins, which are capable of

stimulating ethylene biosynthesis (Morgan and Hall, 1962), may initiate cell and stem elongation via increased ethylene production.

AOA-treated plants do not show any significant difference in stem elongation compared to untreated plants in either upright or gravistimulated shoots (figure 3.5B). However these plants have significantly less gravicurvature than the untreated and the other treated stems (figure 3.5C).

Summary of the role of ethylene and auxin in controlling cell shape

Understanding how ethylene and auxins affect cell shape begs the question of how they relate to gravitropism. As observed in chapter two, both ethylene and auxins are required for upward bending of the shoot to take place. Ethylene may increase radial cell expansion, indicating that increases in width of pith cells and epidermal cells along the lower side in both the basal and bending regions of gravistimulated epicotyls are due to increases in ethylene levels.

In the basal region of gravistimulated epicotyls, the epidermal cells along the lower side have greater cell width than in upright plants. These epidermal cells have an average length similar to those in upright plants. This emphasis on increased cell width as opposed to cell length indicates that, unlike the bending region of the gravistimulated epicotyl, the basal region is mainly acted upon by ethylene, and not auxins. It would be interesting to follow up on the action or inaction of auxins in the basal region of the epicotyl, perhaps by inhibiting auxin transport with NPA or TIBA, or by using auxin-deficient mutants.

The association of auxins with cell elongation suggests that this phytohormone is responsible for the increase in epidermal cell length along the lower side of the bending region, and pith cell length, also in the bending region, of gravistimulated epicotyls. As already mentioned, certain concentrations of ethylene are also believed to promote cell elongation, indicating that perhaps it is an interaction of both auxins and ethylene that

enhance cell length in the bending region of horizontal shoots. In support of this theory is the observation that ethylene and auxin activity appear to be most effective in the bending region (see chapter two).

Both auxins and ethylene are believed to modify cell shape via changes in cellular microfibril and microtubule orientation (Burg, 1973; Nick et al., 1990). In addition auxins are believed to soften cell walls allowing for cell elongation (Rayle and Cleland, 1970). It is possible that ethylene, which has been implicated in the building of cell wall materials (Ingemarsson, 1995), re-establishes cell wall growth in these auxin-induced elongated cells once elongation is complete and perhaps prevents these cells from elongating too much.

SUMMARY

The research in this chapter implicates both ethylene and auxins in the changes in cell shape and size in gravistimulated epicotyls. Auxins are more commonly associated with cell elongation and are likely responsible for the increase in cell elongation along the lower side of the bending region of the epicotyl, resulting in upward bending. Low concentrations of ethylene have also been implicated with stimulating cell elongation. It is possible then that cell and stem elongation in the bending region are due to both auxins and ethylene, and maybe even an interaction of the two phytohormones. This would agree with the findings in the second chapter of this thesis.

Ethylene is also associated with radial cell expansion. An increase in girth both in the bending region of the stem, and especially at the base of the epicotyl is most likely responsible for providing anchorage or increased support for the gravistimulated shoot.

There are many possibilities for future work in this field of research. My work only begins to discriminate between cell sizes and possibly roles in divers cell types and regions of gravistimulated shoots. More in-depth examinations of the effect of auxins and ethylene on individual cell shape and growth, as well as studies of turgor pressure changes, cell wall reconstruction and changes in the pattern of microfibril and microtubule orientation in stems would contribute significantly to the understanding of the anatomical changes taking place during shoot gravistimulation.

CHAPTER 4 - ACC OXIDASE AND ACC SYNTHASE ACTIVITY AND mRNA EXPRESSION IN SUNFLOWER SHOOT GRAVITROPISM

It has been known for little under a century that ethylene has a biological role in plants (Abeles et al., 1992). Initially ethylene production was quantified by measuring the amount of biological changes in response to ethylene exposure, for example the degree of petiole bending with epinasty. These relatively crude measuring techniques continued until the late 1950's when gas chromatographs were found to detect levels as low as 10 to 100μL/liter of ethylene with a thermal conductivity detector and 5 to 10μL/liter of ethylene with a flame ionization detector (Abeles et al., 1992). To this day, gas chromatographs are still used to quantify ethylene levels, although detectors have become more sensitive. Technological advances in biochemistry and molecular biology have allowed for a greater understanding of ethylene biosynthesis. This in turn is resulting in increases in the study of ethylene precursors as well as activity and expression of enzymes involved in ethylene biosynthesis, specifically ACC synthase and ACC oxidase.

Information from the analysis of activity and expression patterns of ACC synthase and ACC oxidase, in conjunction with ethylene production, may further interpret how ethylene is synthesized and functions in plants. For example, Emery et al. (1994) and Kathiresan et al. (1996) observed directly correlating daily fluctuations in ethylene production and activity and mRNA abundance of ACC oxidase in the ramets of *Stellaria longipes*. Finlayson et al. (1996) found a discrepancy between the amount of ethylene evolution, ACC oxidase mRNA's and ACC oxidase activity in sunflower roots, indicating that ethylene biosynthesis is perhaps more complex than earlier imagined.

ACC synthase and ACC oxidase are two enzymes important in the biosynthesis of ethylene. ACC synthase converts SAM into ACC while ACC oxidase synthesizes ethylene from ACC (Yang and Hoffman, 1984). The amount of ACC synthase in plants often corresponds with the levels of ACC and ethylene evolution, suggesting that it is the rate-

limiting enzyme in the biosynthesis of ethylene (Abeles et al., 1992). Although not the rate-limiting enzyme, ACC oxidase is also important in regulating ethylene production (Blume and Grierson, 1997; Emery et al., 1994; Kathiresan et al., 1996).

The data in chapter two show a peak in ethylene production in sunflower epicotyls after four hours of gravistimulation, after which time the ethylene evolution slowly drops, reaching it's lowest level by 24 hours of gravistimulation. Only trace amounts of ethylene production were detected in upright plants. Measuring ethylene production during gravistimulation is very useful for examining how plants respond to this form of stress. In addition analysis of ethylene biosynthesis at the biochemical and molecular levels might be able to provide even more specific information regarding the timing of ethylene production.

In this chapter I examined the patterns of activity and mRNA abundance of ACC synthase and ACC oxidase in 24 hour gravistimulated sunflower epicotyls. I hypothesized that the timing of expression and activity of these two enzymes should correlate positively with the rate of ethylene production observed in chapter two.

METHODS AND MATERIALS

Growth Conditions

All sunflower plants were grown under the same conditions as specified in the second chapter of this thesis.

RNA extractions

Total RNA was extracted from sunflower epicotyls according to Lee Downing et al. (1992) with some modifications. Approximately 0.5g of fresh weight tissue from the epicotyl between the cotyledons and the first leaves (this includes the bending region) was ground in liquid N_2 in a 50mL mortar. The tissue was further ground in 1.4mL of RNA extraction buffer [25mM Tris (ICN) (pH 8.0), 25mM EDTA (Sigma), 75mM NaCl (BDH), 1% sodiumdodecylsulphate (SDS) (BDH)], 950µL phenol (BDH) and 500µL chloroform (Caledon Laboratories LTD):iso-amyl alcohol (BDH). After centrifugation at 4°C the supernatant was further extracted with 25:24:1 phenol:chloroform:iso-amyl alcohol then 24:1 chloroform:iso-amyl alcohol. The RNA in the aqueous layer was precipitated at -20°C overnight in 4mM LiCl (BDH). The pellet was washed with cold 2mM LiCl and resuspended in 150µL diethylpyrocarbonate (DEPC) (Calbiochem-Novachem Corporation) water, 1/10 volume 3M sodium acetate (BDH) and 2 volumes 95% EtOH at -80°C for 2 hours. The pellet was washed with 250µL 70% EtOH, dried and resuspended in 35µL DEPC water. The RNA was then quantitated by optical density (Hitachi U-2000 spectrophotometer) and a small sample electrophoresed for observation.

RNA gel blotting/hybridization

The RNA samples were electrophoresed on a 0.8% agarose-formaldehyde gel and blotted onto nylon Hybond N+ membranes (Amersham). The membranes were probed and washed twice with 50mL 2X sodium citrate and sodium chloride (SSC) with 0.1% SDS and once with 50mL 0.2X SSC and 0.1% SDS, each time for 15 minutes at 65°C. The membranes were exposed to X-ray film (Kodak XAR) for one week in -80°C. The membranes were stripped with 50mL 50% formamide and 2X SSPE (300mM sodium chloride, 23mM sodium phosphate and 2mM EDTA, pH 7.4 with NaOH) for 1 hour at 65°C and rinsed with 0.1X SSPE and subsequently rehybridized. The DNA probes were ^{32}P-labeled (Amersham) using the random primer method according to Sambrook et al. (1989). The following probes were used: ACC oxidase probe (ACCO1) from the pGEXACO plasmid from *Helianthus annuus* cDNA clone (Liu and Reid, 1994) and a partial genomic fragment ACC synthase probe (HACS.1) from *Helianthus annuus* (Kathiresan et al. 1996b).

ACC oxidase assay

ACC oxidase was extracted and assayed according to the protocol by He et al. (1996) with minor modifications. Approximately 0.5g (fresh weight) of frozen sunflower epicotyl tissue was ground in 50mL mortars. The tissue was further ground in 5% (final volume) polyvinylpolypyrrolidone (PVPP) (Sigma) to absorb phenolics, then again in 7 times w/v cold extraction buffer [100mM buffer base [bis-tris propane (BTP) (Sigma) and 10% glycerol (Sigma), pH 6.6) and 2mM dithiothreitol (DTT) (Sigma)]. The brie was microfuged at 4°C for 10 minutes and supernatant collected, microfuged again for 3 minutes and frozen in liquid N_2.

To assay the enzyme, 0.5mL fresh assay buffer (buffer base, 10mM sodium ascorbate (Sigma), 5mM ACC (ICN) and 10μM F_2SO_4 (Sigma)) was added to 6.2mL test tubes. After capping with rubber septa, 1.4mL air was removed from the tubes and replaced with 1.15mL 100% CO_2 (Linde Union Carbide) and 0.25mL 100% O_2 (Linde Union Carbide). After incubating the assay buffer for 15 minutes in 35°C shaking water, 100μL of the enzyme prep was injected into each tube and incubated in 35°C shaking water for 30 minutes. A 1mL headspace was collected from each tube and the ethylene levels analyzed with the 10S Plus Photovac described in chapter 2 of this thesis.

ACC synthase assay

ACC synthase was extracted as described by Chi et al. (1991) and assayed according to the methods by Lizada and Yang (1979) with some modifications. Sunflower epicotyl tissue (approximately 1.5g) was ground in a 50mL mortar with liquid N_2 and 2mL extraction buffer (200mM sodium phosphate, pH 8.0, 5mM DTT and 10μM pyridoxal-5-phosphate). The brie was microfuged for 20 minutes at 4°C and the supernatant collected. Prior to elution, PD-10 Sephadex G-25M (Pharmacia) columns were equilibrated with 3 column volumes of extraction buffer. 2mL of enzyme extract was added to the column and eluted with 2.5mL extraction buffer. To prepare the enzyme for assay, 1mL of the eluate, 200μL 1M sodium phosphate, pH 8.0 and 50μL 0.2M SAM were added to a test tube, vortexed and incubated for 1.5 hours in a 30°C waterbath. To assay the enzyme, 400μL of the enzyme prep was added to a 6.2mL test tube along with 5μL $HgCl_2$ and 450μL water. The tubes were sealed with rubber septa through with 100μL of 2:1 cold bleach:saturated NaOH was injected. The tubes were vortexed for 5 seconds, kept on ice for 2.5 minutes, vortexed again and a 1mL headspace was collected and the ethylene levels analyzed with the 10S Plus Photovac (see chapter two for details).

RESULTS AND DISCUSSION

The ability to study and manipulate plants at the molecular level offers an understanding of how plants develop, function and flourish within their environments. Gene therapy, mutations, and RNA and DNA expression are just some of the many tools of plant research being used today. For my own research purposes molecular techniques may provide more answers regarding the role of ethylene in shoot gravitropism. As already mentioned, two enzymes of importance in ethylene biosynthesis are ACC synthase, the rate-limiting enzyme which converts SAM to ACC, and ACC oxidase which produces ethylene from ACC (Abeles et al., 1992; Yang and Hoffman, 1984). By observing the timing of expression of these enzymes' mRNA more may be understood about ethylene evolution during gravistimulation.

Figure 4.1 shows the timing of ACC synthase mRNA expression during gravistimulation. No expression was observed until the sixth hour of gravistimulation (note the blot is very faint, yet detectable at this time period). By 8 hours mRNA expression was at its strongest. Expression of mRNA by 12 hours of gravistimulation was stronger than at 6 hours, but not as much as at 8 hours. No ACC synthase mRNA was detected after 12 hours of gravistimulation. No ACC synthase mRNA expression was detected in upright plants during this time period.

Expression of ACC oxidase mRNA occurred slightly earlier than ACC synthase mRNA (figure 4.2). ACC oxidase mRNA was first detected after 4 hours of gravistimulation. The strongest expression of this mRNA was recorded after 6 hours of gravistimulation. By 8 hours, mRNA expression was stronger than at 4 hours but less than that at 6 hours. No ACC oxidase mRNA was detected after 8 hours of gravistimulation. No ACC oxidase mRNA expression was detected in upright plants during this time period.

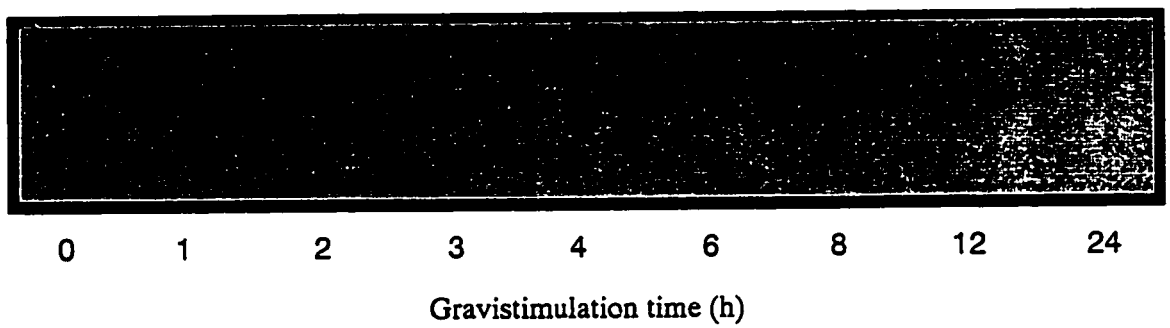

Figure 4.1 ACC synthase mRNA expression in sunflower epicotyls at various gravistimulation times. No ACC synthase mRNA expression was detected in upright plants during this time period.

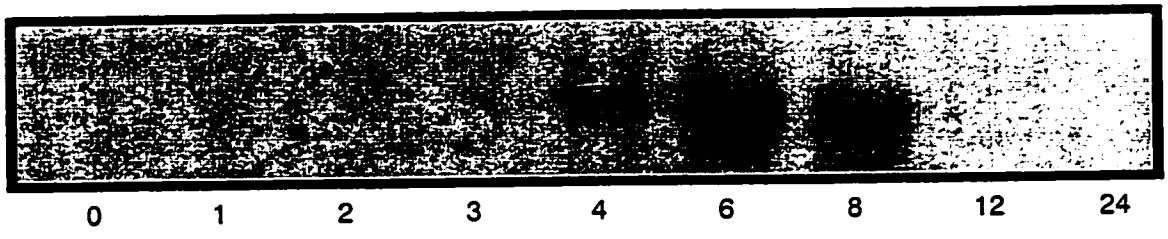

Figure 4.2 ACC oxidase mRNA expression in sunflower epicotyl at various gravistimulation times. No ACC oxidase mRNA expression was detected in upright plants during this time period.

The expression of ACC synthase and ACC oxidase mRNA during gravistimulation supports my findings from chapters two and three that ethylene is indeed involved in gravitropism. ACC synthase mRNA expression was first detected after 6 hours of gravistimulation, indicating the approximate time of synthesis of this enzyme in the shoot. Figure 2.1 shows that ethylene evolution begins shortly after gravistimulation, well before the 6 hour mark. In fact ethylene production peaks after 4 hours of gravistimulation. A similar trend is seen in ACC oxidase mRNA expression. This enzyme's mRNA is expressed after approximately 4 hours of gravistimulation, also long after ethylene production has begun. The synthesis of ACC synthase and ACC oxidase following the peak of ethylene evolution and the relatively small (yet statistically significant) increase in ethylene production [compared to the immensely greater increase in ethylene evolution in fruit ripening (Rothan, 1989)] suggest that ethylene is synthesized from the ACC synthase and ACC oxidase enzymes already present in the tissue prior to gravistimulation.

Synthesis of these two enzymes may be triggered when their endogenous levels begin to deplete. Another possibility is that the production of these two enzymes and ethylene evolution are involved in a positive feedback cycle. Perhaps once ethylene production has reached a certain threshold, ACC synthase and ACC oxidase expression are triggered in the event that the plant may require even larger amounts of ethylene to handle whatever stress it is under. In the case of 90° gravistimulation of young sunflower epicotyls, it appears that additional ethylene production from the newly transcribed ACC synthase and ACC oxidase is not required for gravitropism. At any rate, such a positive feedback cycle may control ethylene production, preventing overbending of the shoot. Other similar examples of positive feedback of ethylene on its own synthesis exist in plants. Positive feedback regulation of ACC synthase and ACC oxidase transcripts with exogenous ethylene was observed in ripening tomato fruit (Nakatsuka et al. 1997),

ripening cantaloupe fruit (Liu et al., 1985) and senescing carnation (Woodson et al., 1992) and morning glory flowers (Suttle and Kende, 1980).

An examination of the levels of ACC synthase and ACC oxidase activity might offer greater insight into the timing of ethylene production. Unfortunately after repeated attempts the enzyme activity levels remained undetectable. Finlayson et al. (1996) did find differences between ethylene production, ACC oxidase mRNA expression and ACC oxidase activity; the regions of the sunflower seedlings with the greatest ethylene evolution and ACC oxidase mRNA abundance had the lowest levels of ACC oxidase activity. For my research however, it is not a case of low levels of ACC oxidase or ACC synthase activity, but no detectable activity at all. It is unlikely that these enzymes are simply not active in the epicotyl, as ethylene is produced in this region of the plant. Rather it is conceivable that these methods are simply not sensitive enough to quantify the enzyme activity levels in this tissue. The protocols for measuring ACC synthase and ACC oxidase activity are fairly new and were developed for tissues containing high amounts of ACC oxidase (leaves, roots) (Finlayson et al., 1996; He et al., 1996)and ACC synthase (fruits, cotyledons) (Chi et al., 1991; Lizada and Yang, 1979). Stems are not associated with high levels of either of these two enzymes. As informative as it would be to obtain data on ACC synthase and ACC oxidase activity, it would probably take a considerable amount of time to modify the protocols for sunflower epicotyl tissue.

SUMMARY

The changes in expression of ACC synthase and ACC oxidase mRNA during gravistimulation supports my hypothesis that ethylene has a role in gravitropism. Detectable expression of ACC synthase mRNA begins following 6 hours of gravistimulation (there is probably some minimal expression prior to this time which is simply too low to measure) and ends after 12 hours of gravistimulation. ACC oxidase mRNA expression begins after 4 hours of gravistimulation and ends following 8 hours of gravistimulation. Ethylene production peaks after 4 hours of gravistimulation.

There was no detectable activity of ACC oxidase and ACC synthase in the epicotyls. The absence of enzyme activity is probably due to a lack of protocols specific for young sunflower epicotyls.

It is likely that ethylene production, which begins within the first hour following gravistimulation, is from the low levels of ACC synthase and ACC oxidase enzymes already present in the tissue at the time of gravistimulation. Expression of these enzymes' mRNA following a peak in ethylene production during gravistimulation is probably in case additional ethylene is required for the plant to deal with this form of stress.

With the recent advances in molecular biology and biochemistry, there are many prospects for further research in this field. The role of ethylene in gravitropism may be elucidated even further with the advent of more sensitive assays of ACC synthase and ACC oxidase activity, with ethylene, auxin and gravitropic mutants, with more precise location and timing of ACC synthase and ACC oxidase mRNA expression in shoots as well as with a greater understanding of the genes involved in gravitropism.

CHAPTER 5 - FINAL SUMMARY AND CONCLUSIONS

The main objective of my research was to see if ethylene has a role in gravitropism. I hypothesized that ethylene is required for the upward bending of sunflower shoots, although based on the overwhelming evidence that auxins are essential for gravicurvature, ethylene is clearly not the only phytohormone involved.

Chapter two focused on the physiology of the possible interaction between gravicurvature and ethylene. From these experiments I found that ethylene is required for gravitropism because: (1) the sunflower shoots produce ethylene when gravistimulated, with the greatest amount of evolution in the lower half of the bending region of the epicotyl, (2) when ethylene biosynthesis is inhibited with AOA, shoot gravibending is also inhibited, with AOA-inhibition being most effective when applied to the lower half of the bending region of the epicotyl, (3) gravicurvature is restored in plants with reduced ethylene levels when an ethylene precursor is applied. Auxins are also required for gravitropism; removal or inhibition of auxin prevented bending of the stem which, consequently, was restored when auxin was added back to the plant. As auxins are known to induce ethylene evolution, it prompted me to question whether these two phytohormones interact in gravitropism. I have found this to be the case as auxin removal or inhibition not only prevents gravicurvature, but also reduces ethylene production. As well, auxin is most effective at restoring curvature in decapitated plants (plants removed of their auxin source) when applied to the lower half of the bending region of the epicotyl, which, as mentioned above, is the site where ethylene production is greatest. My data thus indicates that an interaction of ethylene and auxins is required for shoot gravitropism. Decapitated gravistimulated shoots treated with a variety of auxin concentrations had a greater rate of upward curvature when also treated with a narrow range of concentrations of ethylene, suggesting that ethylene may be involved in increasing tissue sensitivity to auxin.

The experiments in chapter three examined the role of ethylene and auxin on cell shape resulting in shoot gravitropism. Gravistimulated epicotyls are wider than upright stems. Specifically it is the epidermal cells along the lower side of the basal and bending region, and pith cells in the bending region of the stem and which are wider in gravistimulated than upright plants (note that only the pith cells and the epidermal cells were observed). Although exogenous ACC did not affect the radial growth of the shoot, I found evidence suggesting that ethylene is responsible for controlling stem width: gravistimulated stems treated with an inhibitor of ethylene biosynthesis are narrower than untreated gravistimulated shoots.

I also observed changes in the rate of elongation between gravistimulated and upright plants. In the basal region the epidermal cells along the upper side of the gravistimulated stem elongate at a greater rate than in upright stems, while pith cells elongate at a slower rate in gravistimulated than upright plants. In the bending region, epidermal cells along the lower side and pith cells elongate at a greater rate than in upright plants. Auxins increase the rate of elongation of the gravistimulated epicotyls, although I found that low concentrations of ethylene also have some effect in promoting elongation in epicotyls.

The role of ethylene on shoot gravitropism was examined from a molecular and biochemical perspective in chapter four. I examined the expression of mRNA and the enzyme activity of ACC synthase, the primary rate-limiting enzyme that converts SAM into ACC and ACC oxidase which converts ACC into ethylene. Although the protocols were not sensitive enough to allow me to obtain accurate measurements of enzyme activity, I was able to determine the timing of expression of the mRNA for these enzymes during gravistimulation. While ethylene production begins within the first hour of gravistimulation, peaking after 4 hours, ACC synthase and ACC oxidase mRNA do not begin to show expression until after 6 and 4 hours of gravistimulation respectively. ACC

synthase mRNA was expressed from 6 to 12 hours following gravistimulation, while ACC oxidase mRNA was expressed from 4 to 8 hours after gravistimulation. The fact that both ACC synthase and ACC oxidase mRNA's were expressed during gravistimulation supports my conclusions from chapters two and three that ethylene is indeed involved in gravitropism.

While the previous paragraphs support my hypothesis that ethylene is somehow required for shoot gravitropism, they do not explain precisely how ethylene is involved. Although my research does not offer a obvious role for ethylene, I propose the following tentative scheme of how this phytohormone, as well as auxin, might be involved in gravicurvature (figure 5.1).

When plants are gravistimulated, auxins are asymmetrically distributed, resulting in an increase in auxin concentration in the epidermal tissues along the lower side of the gravistimulated stem and the subepidermal tissues along the upper side of the stem (MacDonald and Hart, 1987). Consequently there is a decrease in auxin concentration in the epidermal cells along the upper side of the stem and the subepidermal tissues along the lower side of the stem. The increase in auxin concentration in the epidermal tissue along the lower side of the shoot initiates ethylene production [elevated auxin is known to promote ethylene synthesis (Morgan and Hall, 1962)], perhaps initially by somehow increasing ACC synthase activity (with the ACC synthase already present in the tissue) and later by increasing ACC synthase transcription and translation (Abeles et al., 1992). The increased activity of ACC synthase, along with the ACC oxidase also already present in the tissue, results in the increased biosynthesis of ethylene, especially along the lower side of the gravistimulated stem. The rapid production of ethylene (detectable within the first hour following gravistimulation) along the lower side of the stem increases the sensitivity of that tissue to the additional auxin present due to gravistimulation, keeping in mind MacDonald and Hart's (1987) suggestion that epidermal and sub-epidermal tissues are differentially

sensitive to auxin, with epidermal tissues increasing their rate of elongation and sub-epidermal tissues decreasing their rate of elongation. Others have also shown that auxin can increase the sensitivity of tissues to auxin (Liu and Reid, 1992; Visser et al., 1996).

Although ethylene production begins to decrease after peaking at approximately four hours after gravistimulation, the elevated levels of ethylene concentration exist for a sufficiently long period, along with other factors, to initiate the signal transduction pathway which will result in upward bending of the stem. The presence of auxin and ethylene in the shoot signals the epicotyl cells to increase their rate of elongation, mostly along the lower side of the epicotyl. The increase in the rate of cell elongation likely occurs according to the acid-growth hypothesis proposed by Rayle and Cleland (1970). All the while, the rate of cell elongation in the upper side of the shoot is unchanged or reduced. The difference in the rate of elongation between the upper and lower sides of the epicotyl result in upward bending of the shoot. The ethylene present in the tissue, again especially along the lower side of the stem, also induces cell radial growth by inducing changes in the direction of microtubule and microfibril orientation from a transverse pattern to longitudinal and oblique directions. The widening of the stem increases anchorage and support of the upward bending shoot. Once the stem curves upwards, the rate of cell elongation along the "lower" side of the shoot slows down, becoming similar to the rate of the rest of the stem and bending stops. Any fine adjustments to be made in stem curvature are made via differential cell elongation of the stem via auxins or low concentrations of ethylene. Ethylene may also be responsible for initiating cell wall formation following cell elongation and radial expansion (Ingemarsson, 1995). The role of ethylene and auxin in diagravitropic and plagiogravitropic organs would be the same as with negatively orthogravitropic organs. The only difference would be the preprogrammed final degree of curvature, which is probably controlled at the genetic level.

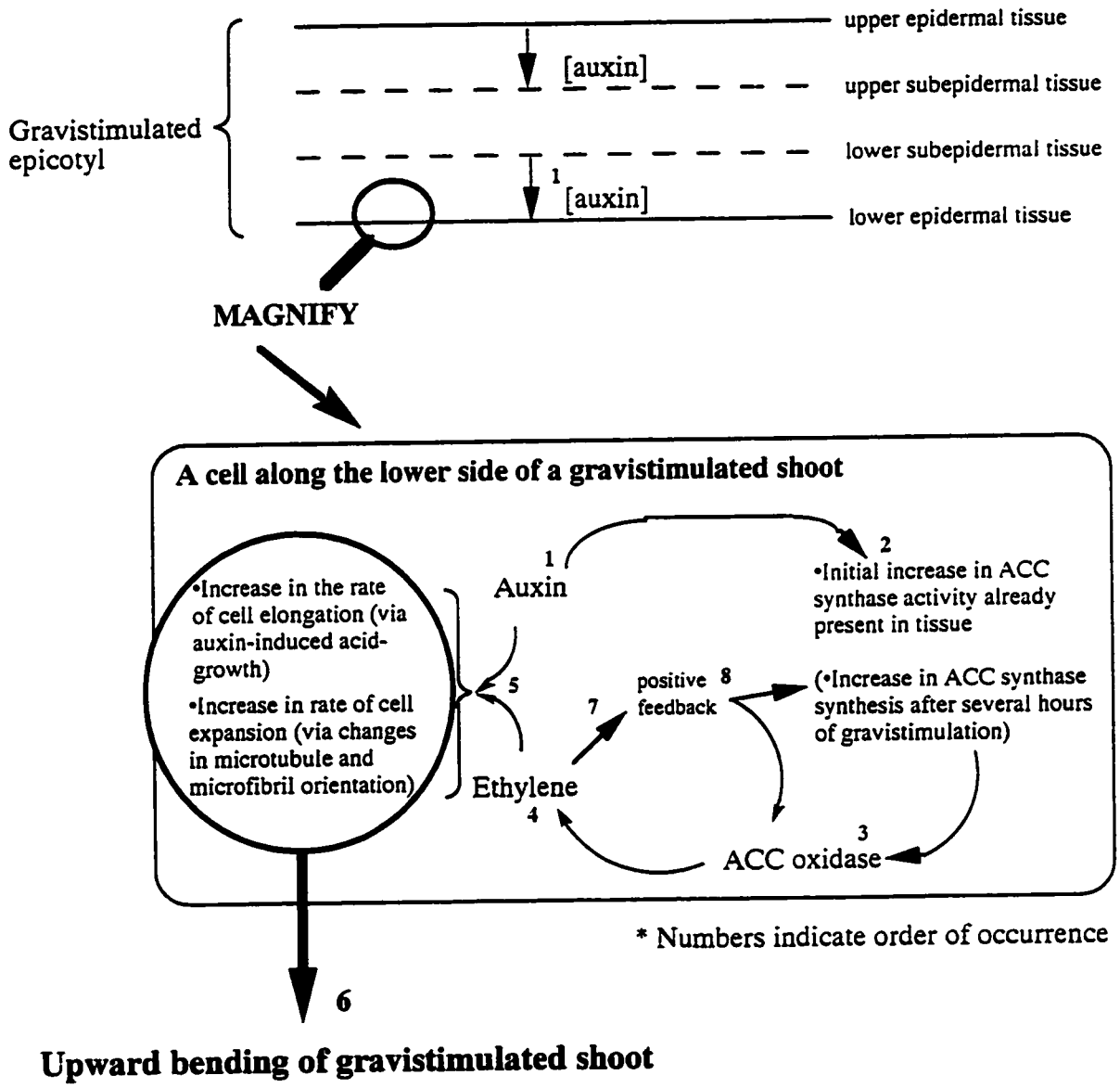

Figure 5.1 Model of proposed theory of the roles of ethylene and auxin in shoot gravitropism.

The increase in endogenous levels of ethylene during gravistimulation stimulate additional ACC synthase and ACC oxidase synthesis 4 to 6 hours, respectively, following gravistimulation in a positive feedback cycle in the event that large amounts of ethylene are required to reorient shoot growth in the negatively orthogravitropic direction, including autotropism, as indicated by Clifford et al. (1983). If these large concentrations of ethylene are not required, ethylene production and ACC synthase and ACC oxidase mRNA transcription decrease. Obviously there is much more involved in shoot gravitropism but the purpose of this model is to focus on the overall roles played by ethylene and auxins.

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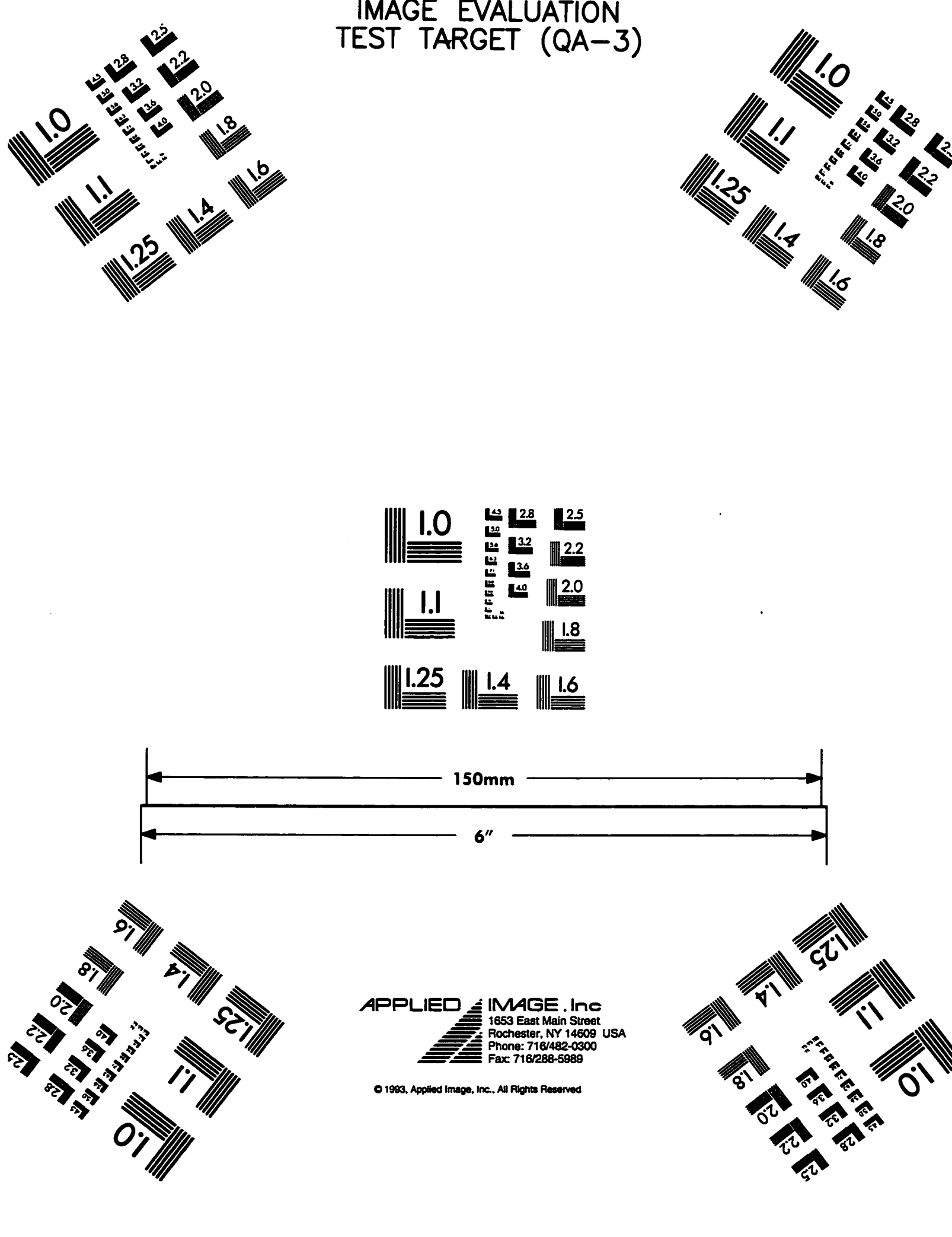
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