THE UNIVERSITY OF CALGARY

Ethylene Biosynthesis in Roots

by

Scott Andrew Finlayson

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Ethylene Biosynthesis in Roots" submitted by Scott Andrew Finlayson in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Abstract

Ethylene is a plant growth regulator involved in many aspects of growth and development. This thesis examines the biosynthesis of ethylene in sunflower roots and the involvement of ethylene in regulating root growth. A novel aeroponic germination system, the Germ-a-Tron, was developed. Germ-a-Tron seedlings grew faster and were better suited for study than conventionally grown seedlings. Treating seedling roots with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) inhibited root elongation and elevated ethylene production by roots. Inhibition of root elongation was maximal at 50 μM ACC 24 h after treatment. The metabolism and transport of exogenously fed 2,3-^{14}C ACC was followed. ACC was rapidly taken-up and transported in the xylem to shoots, and was metabolized to ethylene and *n*-malonyl ACC (MACC) in roots and shoots. Another gas, possibly CO_2, was produced from carbon(s) 2 and/or 3 of ACC, suggesting an alternate pathway of ACC metabolism. The localization of ACC, MACC levels and ethylene production from different parts of the root was investigated. Ethylene production was highest in young, rapidly elongating parts of the root (tips and laterals), and ACC levels reflected this ethylene production. Treating roots with ACC increased ethylene production by all root parts, but a disproportionately greater amount of ethylene was produced by middles and bases. ACC oxidase activity from root parts assayed *in vitro* suggested that the middles and bases had greater ACC oxidase activity than the tips and laterals. ACC oxidase activity from roots was examined *in vitro* with ambient and elevated CO_2. High CO_2 levels changed parameters of enzyme activity including the V_{max}, substrate K_ms and the pH optimum. CO_2 was shown to inhibit ethylene production in excised roots, by reducing the synthesis of ACC. Conversely, CO_2 had no effect on ethylene production by roots of intact seedlings but did transiently inhibit root elongation.

Acknowledgements

First, I acknowledge that I am an idiot. Second, I admit to everything.

With these declarations made I would like to thank everyone for everything. Thanks.

Especially I would like to thank Albert Camus for changing my life in a most profound way. In the uncompromising words of Meursault,

> From the dark horizon of my future a sort of slow, persistent breeze had been blowing toward me, all my life long, from the years that were to come. And on its way that breeze had leveled out all the ideas that people had tried to foist on me in the equally unreal years I then was living through. What difference could they make to me, the deaths of others, or a mother's love, or his God; or the way a man decides to live, the fate he thinks he chooses, since one and the same fate was bound to "choose" not only me but thousands of millions of priveleged people...(Camus, 1942)

Just as The Stranger acknowledged and even revelled in the indifference of the universe, so do I also acknowledge and embrace the futility of my craft. For the past six years of my life to be validated, for me to feel less empty, all I ask is ..."that on the day of my execution there should be a huge crowd of spectators and that they should greet me with howls of execration." (Camus, 1942).

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Chapter 1.

General Introduction.

Although plant physiology is a large and diverse field, comparatively little work is performed on roots. The relative scarcity of research performed on roots is surprising considering that roots often make up 50% or more of the total plant biomass, and considering the many vital functions that roots perform. In reality the paucity of research reflects the complications and inconvenience of working with an organ that normally is invisible as it grows beneath the surface of the soil. From a practical standpoint, roots are important in crop production, especially in the field of forestry where the establishment of a vigorous root system figures prominently in seedling survival (Kozlowski *et al.*, 1991; Reid, 1983). Roots not only serve a structural function anchoring the shoot to the earth, but also provide the plant with water and nutrients from the soil (Drew, 1987). Roots are metabolically important, especially in nitrogen metabolism (Oaks, 1985; Schubert, 1986), and also play a role in the metabolism of endogenous plant growth regulators or hormones (see below).

Direct and indirect evidence has been presented suggesting that roots synthesize many of the plant hormones including abscisic acid (ABA), cytokinins, gibberellins (GAs) and ethylene. ABA levels have been observed to increase 20 to 30 times in roots and root apices of sunflower in reponse to drought (Hubick *et al.*; 1986, Robertson *et al.*, 1985), and ABA synthesis was found to increase in pea and *Commelina* with partial dehydration of the excised root tips (Zhang and Davies, 1987). It has been postulated that in many species, soil drying causes the root to increase ABA synthesis and that subsequent transport of ABA to the shoot signals a drought condition (Davies and Zhang, 1991). Roots of sunflower have been shown to contain several different

cytokinins (Hubick *et al.*, 1986), and circumstantial evidence for cytokinin biosynthesis in roots has been provided using flooded sunflower plants (Burrows and Carr, 1969). More recently Chen *et al.* (1985) have demonstrated the capacity of cultured carrot and pea roots to synthesize several different cytokinins. Comparison of the ability of roots to synthesize these chemicals with the capacities of other organs (leaves and stems) led the authors to conclude that the root is the major site of cytokinin biosynthesis. Like ABA, cytokinins have also been implicated in signalling drought stress, although it is suggested that a reduction in the supply of cytokinins to the shoot acts as a so-called negative signal (Blackman and Davies, 1985; Davies and Zhang, 1991). Whereas root biosynthesis of ABA and cytokinins has received a great deal of attention due to their possible involvement in stress signalling, the metabolism of GA's in roots remains more obscure. Root tips of pea have been shown to synthesize GA_3 precursors by the normal GA pathway operating in higher plants (Wylie *et al.*, 1970). Weak evidence that roots could be sites of GA biosynthesis has been presented (Jones and Phillips, 1966), however the unequivocal demonstration of *de novo* GA synthesis is lacking. On the other hand, convincing evidence for the interconversion of GA's (GA_{19} to GA_1) in root tips of *Phaseolus* has been presented (Crozier and Reid, 1971). Along with studies on hormone biosynthesis in roots, roots have also been shown to be able to transport virtually every hormone as evidenced by translocation of exogenous radioactive chemicals (Astle and Rubery, 1983; Davies *et al.*, 1976; Mozes and Altman, 1977; Prochazka and Jacobs, 1984).

The biosynthetic pathway for ethylene production in higher plants, as shown in fig. 1 has been worked out more completely than for any other plant hormone (Van Der Straeten and Van Montagu, 1991; Yang and Hoffman, 1984). 1-aminocyclopropane-1-carboxylic acid (ACC) is the immediate biosynthetic precursor of ethylene (Adams and

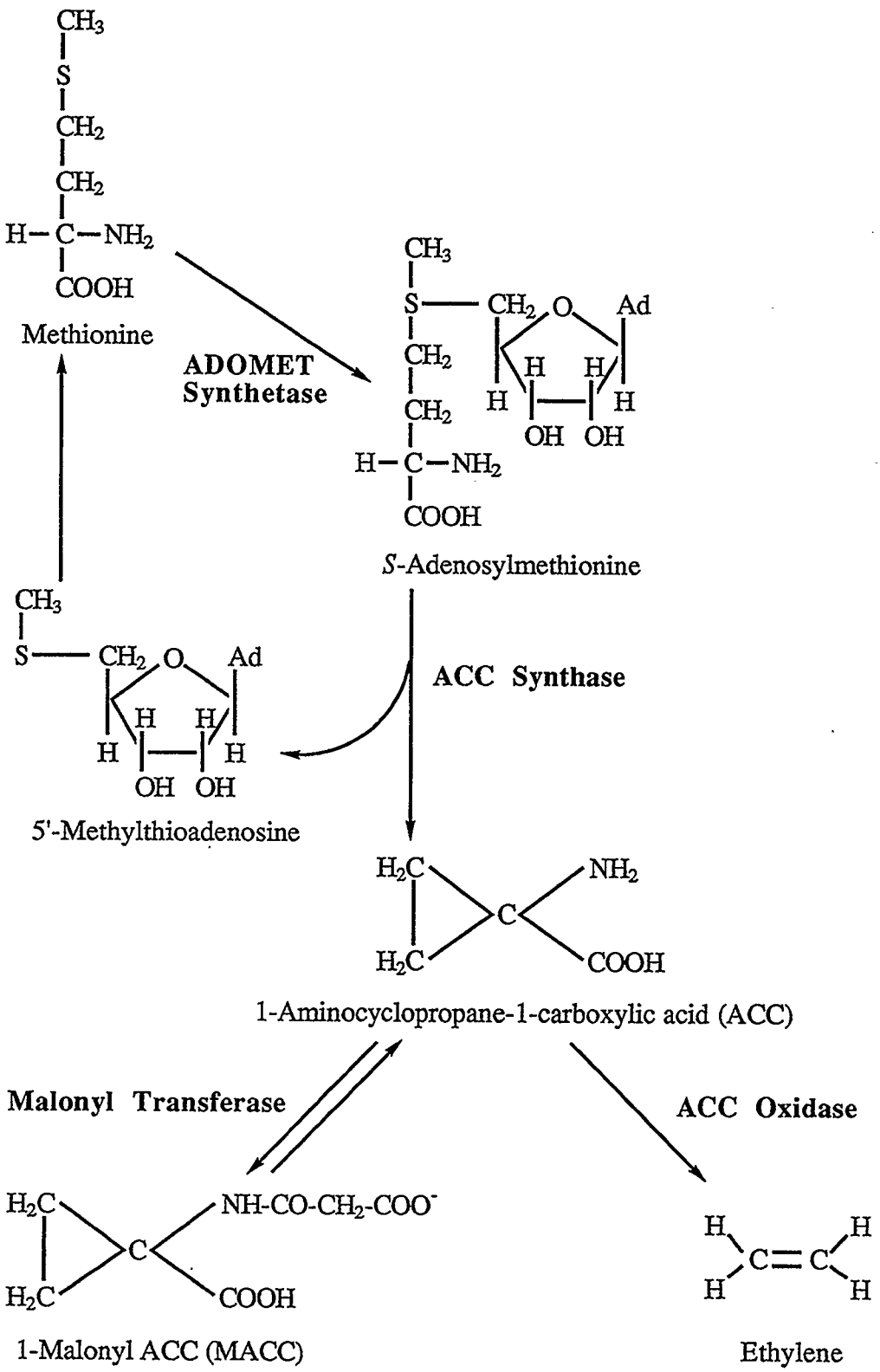


Fig. 1- Biosynthesis of ethylene in higher plants

Yang, 1979; Lurssen *et al.*, 1979), and is produced from *s*-adenosyl methionine (ADOMET) in a reaction catalyzed by ACC synthase. ACC synthase is a pyridoxal phosphate-dependent enzyme (Adams and Yang, 1979), with a MW of 45-50 kDa reported from several species (Van Der Straeten and Van Montagu, 1991). ACC synthase has been shown to exhibit extremely low *in vivo* activities, and additionally shows a high turnover rate, probably due to suicide-substrate inactivation (Kim and Yang, 1992). ACC can be malonylated to *n*-malonyl-ACC [MACC] (Hoffman *et al.*, 1982) by malonyl transferase (Kionka and Amrhein, 1984), or converted to ethylene by the ethylene forming enzyme ACC oxidase. The ACC-conjugate MACC is generally thought of as a stable end-product of ACC conversion, however several studies have shown that MACC may be hydrolyzed back to ACC and from there may be converted to ethylene (Hanley *et al.*, 1989; Jiao *et al.*, 1986). ACC oxidase utilizes both oxygen (McGarvey and Christofferson, 1992; Yip *et al.*, 1988) and ascorbate (Ververidis and John, 1991) as co-substrates (along with ACC), and requires ferrous iron for catalysis (Bouzayen *et al.*, 1991; Vereveridis and John, 1991). Molecular weights ranging from 34 KDa (McGarvey *et al.*, 1992) to 41 KDa (Smith *et al.*, 1992) have been reported for the enzyme.

Current thinking, supported by many observations suggest that the conversion of *s*-adenosyl methionine to ACC is the rate limiting step in the biosynthesis of ethylene (Yang and Hoffman, 1984). ACC synthase activity increases many fold with wounding (Kende and Boller, 1981) and it's *de novo* synthesis has been suggested to control wound-induced ethylene production (Konze and Kwiatkowski, 1981). Drought induced ethylene production has also been observed to be regulated by ACC synthase activity (McKeon *et al.*, 1982). Sarquis *et al.* (1992) have presented evidence suggesting that release of ACC from MACC by *n*-malonyltransferase in concert with

ACC synthase and ACC oxidase may regulate ethylene production by mechanically impeded maize. In general, although ACC oxidase has been observed to be induced in some systems (McKeon *et al.*, 1982; Ververidis and John, 1991), it is not thought to actually limit ethylene production in most tissues.

Recently, molecular approaches to ethylene biosynthesis have yielded exciting results. The gene encoding ACC synthase has been cloned from several species (Nakajima *et al.*, 1990; Sato and Theologis, 1989; Van Der Straeten *et al.*, 1990). Molecular analyses have shown that ACC synthase is encoded by up to 4 genes (Yip *et al.*, 1992). Van Der Straeten *et al.* (1990) showed that tomato possessed two different ACC synthase genes, which differed in 18% of their bases, while Yip *et al.* (1992) showed the existence of four different clones. These genes have been shown to be differentially expressed by wounding and the ripening climacteric (Olson *et al.*, 1991), and also by auxin (Nakagawa *et al.*, 1991; Yip *et al.*, 1992). While there is divergence between genes, even from the same tissue (Kim *et al.* report only 46% identity between two apple genes [1992]), a high level of identity is retained at the peptide's active site, even between species (Olson *et al.*, 1991; Yip *et al.*, 1990). The divergence at the nucleotide and peptide level manifests itself in the inability of antibodies raised against the peptide of one ACC synthase to react with another ACC synthase, even from the same tissue (Nakagawa *et al.*, 1988). While an *in vitro* assay for ACC synthase is available (Satoh and Yang, 1988), it is complicated by the fact that the enzyme shows extremely low abundance (especially in vegetative tissues), is unstable and ACC itself is the measured product requiring a time consuming assay to quantify properly.

The gene encoding ACC oxidase has also been cloned (Hamilton *et al.*, 1990; Jayasekera pers. comm.; McGarvey *et al.*, 1992). This gene is highly conserved, with 74% identity at the amino acid level between *B. napus* and tomato (Jayasekera, pers.

comm.). Furthermore, this gene shares homology with other 2-oxoglutarate dependent dioxygenases, and even with other Fe^{2+}, ascorbate oxygenases (Prescott, 1993). Sequence analysis of an ACC oxidase from pea revealed a putative leucine zipper that could possibly be involved in protein-protein interactions (Peck *et al.*, 1993). This potential leucine zipper is also highly conserved among all other known ACC oxidase sequences, though the functional significance remains unclear (Jayasekera, pers. comm.). The cloning and sequencing of ACC oxidase allowed an *in vitro* enzyme assay to be developed which had long eluded researchers. Early investigations on *in vitro* EFE activity from plants were stymied by complete loss of free EFE activity upon tissue homogenization. Researchers focused on membranes as the only observed activity was associated with these fractions. Authentic EFE activity (based on K_m values and substrate stereospecificity) was recovered from intact vacuoles, but with extremely low activity rates (Guy and Kende, 1984). Based on ACC oxidase homology to other 2-oxoglutarate requiring dioxygenases, Ververidis and John elucidated an *in vitro* protocol including Fe^{2+} and vitamin C (Ververidis and John, 1991). This assay is relatively easy to perform as the enzyme shows high activity in most tissues, is stable under the extraction and assay conditions used, and the measured product ethylene is easy to quantify. Evidence for an alternative EFE, catalyzed by cytochrome P-450 has been presented, although it is largely circumstantial (Kraus *et al.*, 1992). Until there is more detailed molecular evidence for the involvement of an alternative EFE, the conclusions reached by these authors must be regarded with some reservations.

As is the case for all plant hormones, no convincing receptor for ethylene has been found. Ethylene binding on the other hand has been well documented and is associated primarily with the endoplasmic reticulum and protein-body membranes (Sisler, 1991; Smith and Hall, 1985). Because no connection between binding site

concentration and tissue sensitivity has been established, the significance of this binding remains obscure. Quite possibly the localization of an ethylene receptor will be the most significant contribution to the physiology of ethylene in the near future.

While most of the ethylene produced by the plant simply diffuses out into the atmosphere, a small proportion of ethylene is metabolized to ethylene oxide, and may then be incorporated as ethylene glycol (Smith and Hall, 1984). Different species exhibit different capacities to metabolize ethylene, and it has been suggested that ethylene metabolism is required for ethylene action (Beyer, 1981).

The effect of ethylene on roots is pleiotropic (Feldman, 1984; Jackson, 1983). Typically roots show a reduction in elongation rate and a sub-apical swelling often accompanied by curling or coiling of the tip (Harvey and Rose, 1915; Woods *et al.*, 1984). Root branching may also be enhanced by ethylene treatment, although the elongation of new laterals is often inhibited (Crossett and Campbell, 1975). Working on cultured radish roots, Radin and Loomis provided evidence that ethylene inhibited elongation and reduced cambial activity (Radin and Loomis, 1969). Root extension in cereals has been shown to be reduced by as little as 0.1 ppm ethylene, with variations in the response observed between different species (Smith and Robertson, 1971). The authors postulated that under natural environmental conditions, ethylene levels in the soil might regulate root growth. More recently the effects of ethylene on root elongation were studied in detail in corn (Whalen and Feldman, 1988). It was found that applied ethylene reduced root elongation within 20 min. and that the inhibition observed was due to inhibition of cortical cell elongation, not numbers of cells produced. Some authors have reported a promotion of root growth with ethylene treatment (Krishnamoorthy, 1970; Roy *et al.*, 1972; Zimmerman and Hitchcock, 1933), specifically by promoting root numbers rather than elongation of the axis. Other

research has been conducted suggesting that in some species ethylene has the capacity to act as a promoter of primary root elongation, albeit at very low levels (Konings and Jackson, 1979; Woods *et al.*, 1984). The growth response of a plant to exogenous ethylene may actually be dependent on the plant's endogenous ethylene level, due to variations in the sensitivity of a particular species to ethylene (Konings and Jackson, 1979).

Ethylene also exhibits a strong influence on adventitious rooting. Work by Drew *et al.* (1979) implicates ethylene in the formation of adventitious roots from corn under flooded, anoxic conditions. The authors found that anoxic flooded conditions stimulated root ethylene production and that this endogenous ethylene stimulates intial emergence of adventitious roots, but appears to actually inhibit their further elongation. Similar work on sunflower plants in this lab has also suggested that flood induced ethylene is a promoter of adventitious root initiation, but an inhibitor of elongation (Fabijan *et al.*, 1981). Wample and Reid (1979) using sunflower hypocotyls have shown ethylene to be a promoter of root primordia, and have presented evidence suggesting that ethylene mediates this effect through regulation of IAA levels. Soffer *et al.* (1989) demonstrated the ability of ethylene to induce adventitious rooting in chrysanthemum cuttings, and Liu *et al.*, (1990) have implicated ethylene as the key stimulatory factor in the formation of root primordia.

Ethylene has been shown to play a central role in the formation of cortical air spaces, known as aerenchyma, in response to flooding. These air spaces are believed to allow increased gas exchange within the root in situations where gas flow outside the root is impeded by water. Drew *et al.* (1979), found that non-aerated nutrient solution promoted ethylene evolution and the formation of aerenchyma in roots. The authors postulated that ethylene levels might build up in flooded roots due to the reduced

capacity for diffusion and that high ethylene levels regulate aerenchyma formation. Subsequent investigations using inhibitors of ethylene action and synthesis have lent further credence to ethylene as a regulator of aerenchyma development (Drew *et al.*, 1981; Konings, 1982). Ethylene was also shown to stimulate the production of lysigenous enzymes responsible for the degradation of cortical cells leading to the formation of aerenchyma (Kawase, 1979).

Ethylene may be important in transducing certain environmental stimuli perceived by the root, and as a possible signal the possibility of transport was considered. Jackson and Campbell (1975) proposed that ethylene moved from roots to shoots of tomato plants in response to waterlogged soil. The directed transport of ethylene itself was refuted (Jerie *et al.*, 1978), however the classic paper by Bradford and Yang (1980b) demonstrated the ability of the ethylene precursor ACC to move from roots to shoots in response to anaerobic soil conditions. Presumably the resultant ethylene produced in the shoot could signal waterlogging at the roots, and in some way ameliorate the detrimental effects. In an elegant study on orange trees, it was shown that drought stress increased the ACC content of roots, and that subsequent rewatering allowed the transport of this ACC to the shoots where it was converted to ethylene (Tudela and Primo-Millo, 1992). The ethylene produced was shown to induce leaf abscission, nicely illustrating a perception-signal-response cascade demonstrated only rarely in the field of plant hormones.

Roots are obviously in intimate contact with their environment, and therefore are constantly subject to stimulation by the soil substrate. Soil characteristics, such as bulk density, compaction and pore size may exert an effect on the growth and morphology of roots inhabiting the soil. How the root perceives and responds (by altering growth and morphology) to variations in soil charcteristics is not known, however the possibility

that ethylene may play a role in the perception of thigmostimulation has garnered some attention (Dawkins *et al.*, 1983). Root restriction has been correlated with a slight increase in root ethylene production (Peterson *et al.*, 1991). Using pea seedlings, Kays *et al.* (1974) reported that pea roots increased ethylene evolution up to 6 times in response to mechanical impedence. Furthermore, these authors found that ethylene slowed root extension and promoted radial expansion. The authors postulated that mechanical stimulation increases ethylene production which results in a root morphology that is better able to penetrate the substrate. The role of ethylene in regulating root growth in response to mechanical impedence has been challenged by Moss *et al.* (1988). These authors demonstrated that although ethylene levels do increase in response to mechanical impedence, and although ethylene treatment mimics the morphological effect of this impedence, inhibitors of ethylene action and synthesis were not able to reverse the effects of mechanical impedence. Additionally, these authors found that ethylene evolution does not increase until well after mechanical impedence manifests an effect on root extension, and they therefore concluded that ethylene does not mediate the mechanical impedence stimulus. A more recent, and more rigorous study on mechanical impedence of maize roots was found to promote ACC accumulation in both roots and shoots, and the evolution of ethylene from these tissues was also enhanced rapidly, within 1 hour after stimulation (Sarquis *et al.*, 1991, 1992). The authors provided evidence that the increase in ethylene observed was due to increased ACC production, and an increase in EFE activity. Additionally, inhibitors of ethylene synthesis and action were shown to reverse the effects of mechanical impedence on growth. The response of root ethylene biosynthesis to mechanical impedence implicates ethylene as a possible agent involved in increasing the root's ability to penetrate it's substrate. Zacarias and Reid (1992) have demonstrated that ethylene is required for tomato roots to penetrate an

agar substrate. Seedlings germinated with silver thiosulphate could not penetrate 2% agar while untreated seedlings could, and compared to untreated seedlings, silver thiosulphate treated plants showed increased root length. Presumably endogenous ethylene might cause thicker, more sturdy roots to grow which are better able to penetrate their substrate.

While most of the work dealing with the involvement of ethylene in the response of roots to stimuli has centered on thigmostimulation, other stimuli have been considered. Ethylene has been suggested to play a role in the gravitropic response of roots, possibly by modifying the distribution of auxin. Roots of *Zea* were observed to be more responsive to gravistimulation in the presence of ethylene (Lee *et al.*, 1990). Inhibitors of ethylene biosynthesis and action reduced the maximum curvature of the root in response to gravistimulation, while ethylene treatment extended the duration of auxin assymetry in the root and increased the degree of curvature obtained. Roots of most species are not generally exposed to light, and irradiance of the root with light may result in a decrease in the rate of elongation (Eliasson and Bollmark, 1988; Pilet and Ney, 1978; Whalen and Feldman, 1988). When irradiated with white light, pea roots were observed to reduce their rate of elongation 50% and increase ethylene production 4 to 10 times (Eliasson and Bollmark, 1988). These observations, along with the ability of inhibitors of ethylene synthesis and action to overcome the growth inhibiting effects of light, led the authors to postulate that ethylene may mediate the growth inhibiting response of roots to light.

The mode of action of ethylene on root growth has not been dealt with in a comprehensive manner. Work by Barlow (1976) and more recently Rost and Sammut (1982) has suggested that ethylene may reduce cell division in the root tip, thereby slowing growth. On the other hand, Whalen and Feldman (1988) have observed that

ethylene retards cell expansion in the root tip, rather than reducing the number of cells. These authors also determined that root cells of the cortex were redirecting longitudinal expansion into expansion in the radial direction. This fits well with a plethora of data illustrating the ability of ethylene to alter microtubule and microfibril orientation to a direction favoring lateral expansion over longitudinal expansion in shoot tissues (Eisinger, 1983). Calcium has been shown to be required for the pathogen-response cascade of excised tobacco leaves. Using calcium blockers and ionophores to regulate intracellular calcium levels, evidence was provided suggesting that calcium is involved in the signal transduction of the ethylene pathogen response (Raz and Fluhr, 1992). Plants deficient in calcium were unable to exhibit the pathogen response (as evidenced by induction of pathogenesis-related proteins), however this response was restored when the plants were given calcium. Furthermore, the same authors have also shown that ethylene induces the phosphorylation of a variety of proteins and have suggested that this phosphorylation transduces the ethylene signal involved in pathogenesis (Raz and Fluhr, 1993). Using a dissimilar system in which xylogenesis of tuber explants was being studied, ethylene was previously observed to increase protein kinase activity resulting in increased protein phosphorylation (Koritsas, 1988). Although carefully investigated, the documentation of the phosphorylation effect is vague evidence at best since the function of none of the proteins is known.

My general interest has been directed towards the role of ethylene in root growth, especially the influence of ethylene on root elongation. With this in mind the purpose of this thesis was to examine and characterize the biosynthesis of ethylene and metabolism of its precursor ACC in roots of sunflower seedlings, and to elucidate some of the controls on this system. Sunflower (*Helianthus annuus* cv. Dahlgren 131) seedlings were chosen to model root growth in general because of the rapid development

of these seedlings, and the large size of the root system. Additionally, this lab and others have used this plant extensively, acquiring a considerable amount of data on sunflower physiology.

The approach taken to elucidate the biosynthesis and control of ethylene production in roots involved first developing a system for producing roots that were easy to work on and developmentally uniform. Initial experiments performed on plants germinated in trays of Terra-Green proved fruitless due to complications arising from growth in this medium. Roots of seedlings grown in Terra-Green showed a complex tortuous morphology due to the interaction of the root with Terra-Green particles, a condition that is common in nature but complicating to studies in the laboratory. The effect of ethylene on root elongation could not be documented in these plants as their convoluted growth pattern made it difficult to measure elongation rates. Additionally, roots were often damaged when they were removed from the Terra-Green, with loss of the root tip being very common. To circumvent the problems associated with growing seedlings by the conventional Terra-Green method, a novel aeroponic germination system was devised. Chapter 2 details the development of this system, the Germ-a-Tron, and describes the differences between plants grown in the Germ-a-Tron vs. those grown in Terra-Green. This chapter also documents the effect of ethylene (via ACC treatment) on root elongation of Germ-a-Tron plants.

With a viable root growth system at hand, a logical progression was to gain an overall picture of ethylene biosynthesis and ACC metabolism in whole seedlings. Studying the biosynthesis of ethylene and ACC metabolism in the whole plant was important, because the plant is an integrated whole, not a collection of separate parts operating independently. When considering a specialized project for study it is necessary to keep in mind the larger picture into which a small investigation must fit.

Therefore, even though the roots were the central point of interest, the metabolism of ACC (ethylene) in the whole plant had to be considered before one organ could be focused in on. Chapter 3 documents a collaborative project with Dr. Ken Foster, in which the transport and metabolism of ACC in sunflower seedlings was investigated. This investigation gave an overall picture of ethylene biosynthesis in the whole plant and provided the basis for the following more detailed examination of root ethylene biosynthesis and ACC metabolism.

Most studies on hormone levels within organs consider each organ as a homogenous unit, while in reality each organ is a very heterogenous mixture of tissues each with diverse functions. The variety of functions and diversity of growth and developmental responses exhibited by different parts of the root led to the hypothesis that the biosynthesis of ethylene and ACC metabolism might also be dissimilar in these different parts. Chapter four documents a detailed analysis of ACC, MACC and ethylene levels from different parts of the root based on functional and morphological divisions. Additionally this study provided some evidence implicating ACC oxidase in the control of ethylene biosynthesis.

The recent development of an *in vitro* ACC oxidase assay with strong suggestions of the involvement of ACC oxidase in regulating ethylene biosynthesis led to an analysis of ACC oxidase activity from roots. As in previous studies (Dong *et al.*, 1992; McGarvey and Christoffersen, 1992), CO_2 was found to strongly promote ACC oxidase activity *in vitro*. Preliminary assays suggested that CO_2 alters some of the parameters of ACC oxidase activity, and therefore a detailed analysis was performed. CO_2 was found to alter virtually every parameter of ACC oxidase activity studied, and shed some light on the enzyme's mode of action. These results are presented in chapter five.

Because CO_2 was observed to greatly stimulate ACC oxidase activity *in vitro*, and because of a history of literature illustrating the promotion of ACC oxidase activity *in vivo*, CO_2 was hypothesized to control ethylene biosynthesis in roots. This hypothesis was tested in chapter six by measuring ethylene production from excised and intact roots exposed to elevated concentrations of CO_2 for various times. Additionally, root elongation rates were measured to determine whether CO_2, through ethylene, might regulate the rate of root growth.

Chapter 2.

Construction and Operation of a Novel Aeroponic Germination System (Germ-a-Tron), and the Effect of Exogenous 1-Aminocyclopropane-1-carboxylic Acid on the Elongation Rate of Roots of Germ-a-Tron-Germinated Sunflower (*Helianthus annuus* L.) Seedlings.

Summary

The design and application of a novel aeroponic germination system (Germ-a-Tron) is described. The Germ-a-Tron is capable of producing completely undamaged roots which are not contaminated by the fungus prevalent in the conventional (tray) germination system. Germ-a-Tron plants are observed to develop significantly faster than tray plants, and roots of Germ-a-Tron plants exhibit an elongate, straight root morphology dissimilar to tray grown plants, possibly due to the lack of thigmostimulation. Roots of Germ-a-Tron seedlings evolve more ethylene than their tray-grown counterparts. Root elongation of Germ-a-Tron seedlings was inhibited in response to ACC treatment. This treatment was maximally effective in inhibiting elongation at 10-100 μM, and also resulted in swollen and curled primary roots with an abundance of laterals. Inhibition of root elongation could be observed 12 h after 100 μM ACC treatment, with recovery from the effects of ACC beginning at 48 h. 200 μM silver thiosulphate was effective in completely reversing the inhibitory effects of 50 μM ACC on root elongation, and also ameliorated the characteristic swelling and branching observed with this treatment. Roots treated with 50 μM ACC evolved approximately ten times as much ethylene as control roots. Ethylene production by 50 μM ACC-treated roots was highest 1 hour after treatment, and declined to near untreated levels at 24 hours.

Introduction

Sunflower seedlings represent an ideal model system for studies on the regulation of root growth. The large size of the seed and rapid germination fascilitates the production of plants with easily manipulable root systems in a relatively short period of time. Unfortunately the germination systems commonly used often produce plants with roots that are not well suited for growth analyses. Germination of sunflower seeds in root pouches almost always results in intense fungal infection, whether the seeds are surface sterilized or not, and this infection spoils the plants for further study. The conventional method of germination in trays of Terra-Green, while reducing fungal infection to a more tolerable level, does not produce seedlings conducive to analyses of root elongation. Roots of tray-germinated seedlings show a tortuous morphology, probably as a result of interaction with the clay particles, which makes measurements of elongation rates difficult. The twists and turns of these roots in conjunction with the adherent properties of the clay medium also makes removing undamaged roots problematic. Roots removed from trays of Terra-Green are often damaged and may even have the apical portion of the root broken off.

Root growth in many species of plants has been shown to be influenced by ethylene (Feldman, 1984; Jackson, 1983). Exogenous application of ethylene to roots has been shown to inhibit root elongation in many species including radish (Radin and Loomis, 1969), cereals (Smith and Robertson, 1971) and corn (Whalen and Feldman, 1988), while inhibitors of ethylene action, such as silver thiosulphate and 2,5-norbornadiene, restore normal root growth (Abeles and Wydoski, 1987; Whalen and Feldman, 1988). Endogenous ethylene production has been suggested to limit root growth *in vivo* (Whalen and Feldman, 1988), and soil ethylene has also been postulated to regulate root elongation *in situ* (Jackson, 1991). Ethylene also affects the gross

morphology of the root, inducing swelling and curling of the sub-apical root tip and often inducing lateral formation and in some cases root hair production (Crossett and Campbell, 1975; Jackson, 1983; Woods *et al.*, 1984). Studies have indicated that ethylene inhibits root elongation by redirecting cell expansion from the longitudinal to the radial orientation (Whalen and Feldman, 1988), although some studies suggest that ethylene may also reduce cell divisions in the tip (Barlow, 1976; Rost and Sammut, 1982).

Studies investigating ethylene effects on growth and development utilize a variety of treatment systems for elevating ethylene levels, including direct gassing and application of 2-chloroethylphosphonic acid. Treatment of roots with ethylene is cumbersome, and does not necessarily mimic increased endogenous ethylene production, while treatment with 2-chloroethylphosphonic acid is complicated by the low pH that the chemical must be applied at and the toxicity of associated breakdown products (chloride and phosphonic acid[Warner and Leopold, 1969]). ACC is the immediate biosynthetic precursor of ethylene in higher plants (Adams and Yang, 1979; Lurssen *et al.*, 1979), producing ethylene, CO_2, and cyanide in a reaction catalyzed by EFE (ACC oxidase) (Yang and Hoffman, 1984). Treatment of plant tissues with this compound results in increased levels of ethylene production as ACC oxidase activity is generally in excess of the endogenous requirements of plant cells (Adams and Yang, 1979; Cameron *et al.*, 1979). ACC treatment represents an effective method of increasing ethylene production by tissues *in vivo*.

In an effort to produce intact seedlings with roots suitable for analysis of growth parameters such as elongation rates, an aeroponic germination system was developed to produce seedlings with straight, undamaged roots. This chapter details the construction and operation of this sytem, known as the Germ-a-Tron, and also details some of the

differences between conventionally tray-germinated seedlings and those grown in the Germ-a-Tron. Additionally, the effects of increasing ethylene production of Germ-a-Tron seedling roots by ACC treatment on root growth, and reversal of these effects by silver thiosulphate are decribed.

Materials and Methods

The Germ-a-Tron.

An aeroponic germination system was designed and constructed as given in figures 2 through 8. The aeroponic germinator or Germ-a--Tron is composed of five main parts, the chamber, the rotor, the tank, the seed tray, and the screen, and two accessories, the motor and the pump. Nutrient solution in a reservoir tank is replenished from the large chamber reservoir below by means of an air-lift pump. A rotor is in contact with the nutrient solution in the reservoir tank, and is spun at approximately 2000 rpm by a constant duty motor. The rotor sprays a fine mist of nutrient solution in all directions, with some travelling upwards to the seed tray. Seeds are placed on a support of glass rods which are fitted into notches in the seed tray. A thin layer (1-2 cm) of Terra-Green is placed over the seeds to absorb and retain nutrient solution and act as a supporting medium for the developing seedlings. During germination, the root penetrates the spaces between the glass rods and grows downward, encountering the nutrient mist. To remove the seedlings, the glass rods are gently lifted, and the seedling root is freed, allowing undamaged plants to be harvested.

Because germination is poor when the rotor is driven constantly (probably as a result of over-watering), a system was developed whereby the rotor spins only 2 out of every 5 minutes. Additionally, the rotor is shut down for two 3 hour periods, 12 and 24 hours after sowing, to increase the level of germination. Sunflower (*Helianthus annuus*

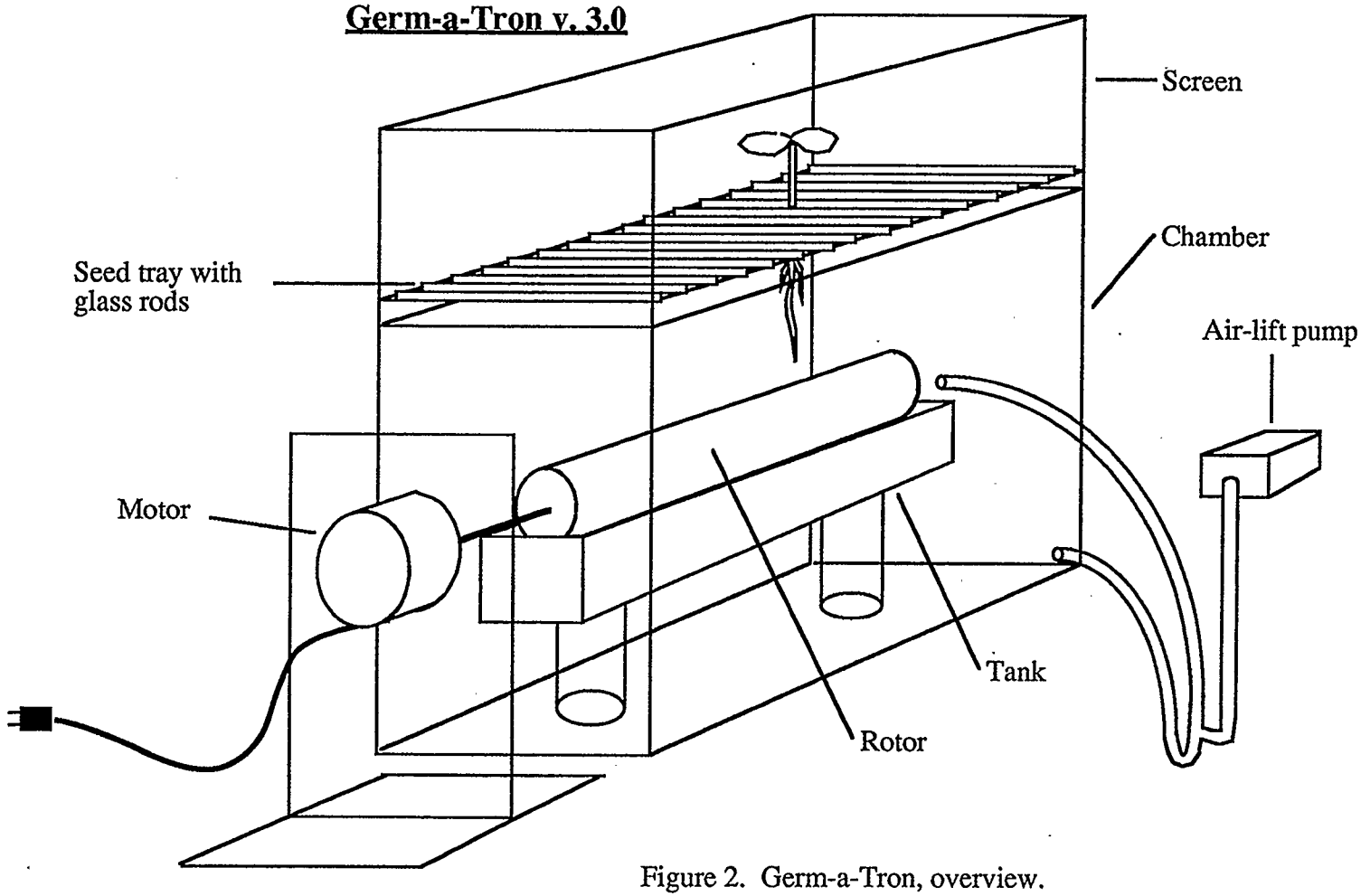


Figure 2. Germ-a-Tron, overview.

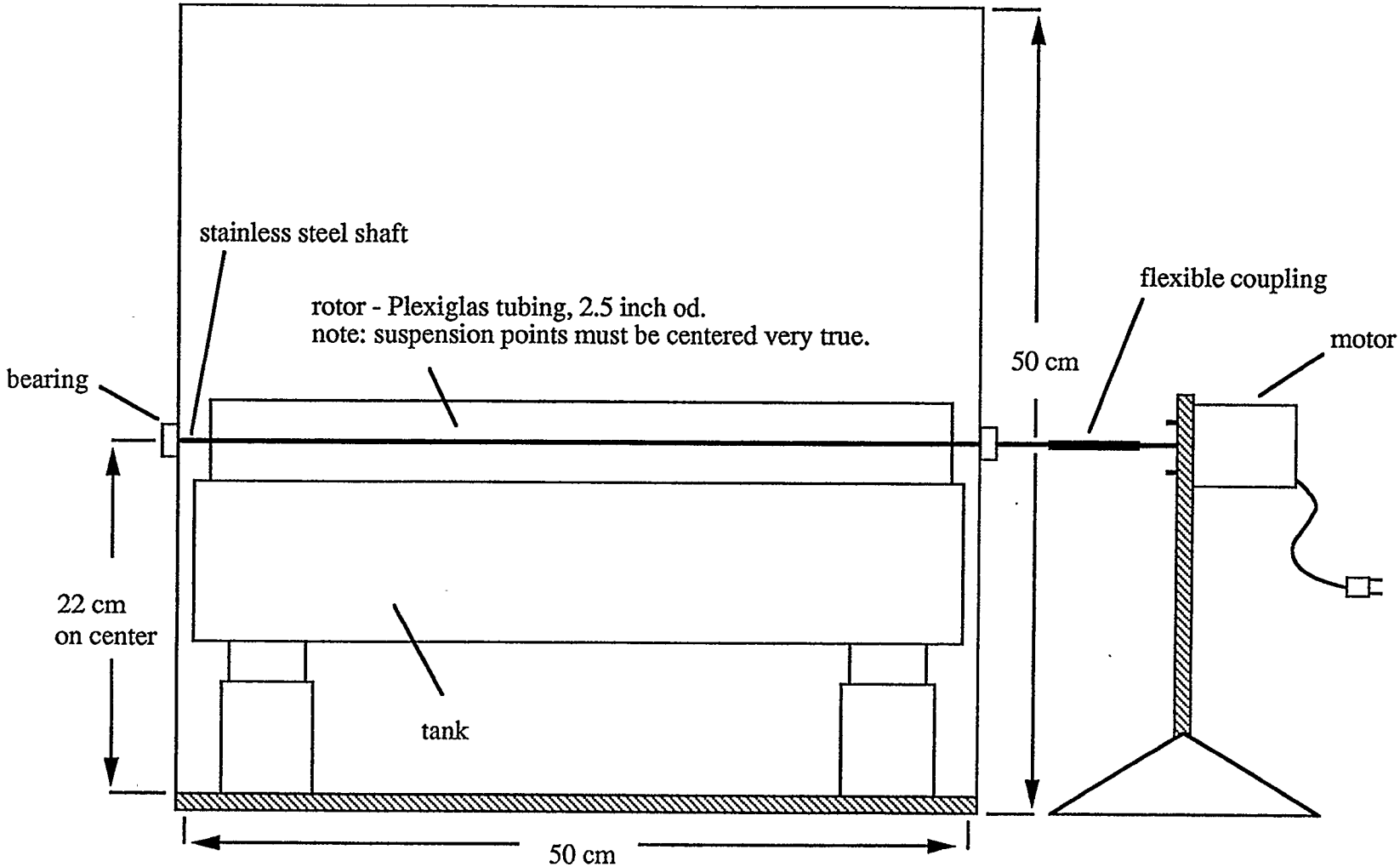


Figure 3. Germ-a-Tron chamber, 3/8 inch Plexiglas, flat black outside.
Side view, scale 1:4.

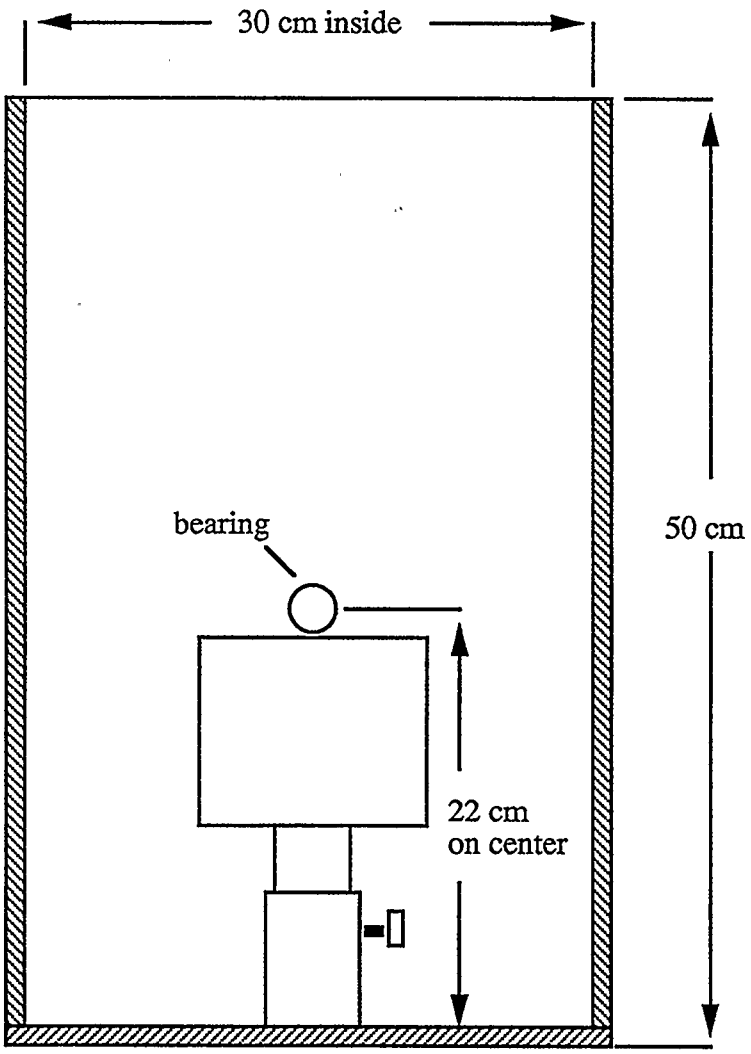


Figure 4. Germ-a-Tron chamber, 3/8 inch Plexiglas, flat black outside. End view, scale 1:4.

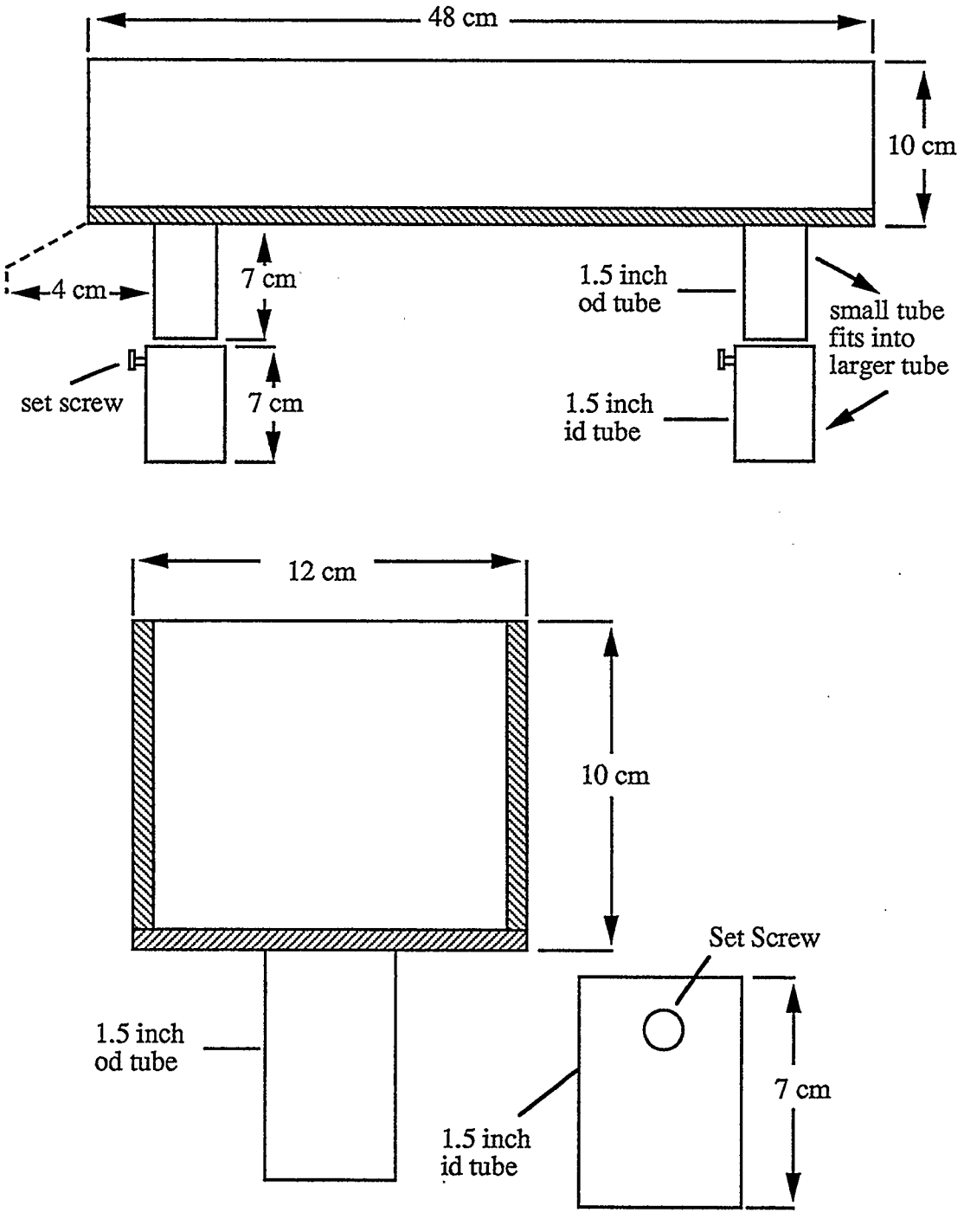


Figure 5. Germ-a-Tron tank, 3/8 inch Plexiglas, clear.
Side view (above) and end view, scale 1:2.

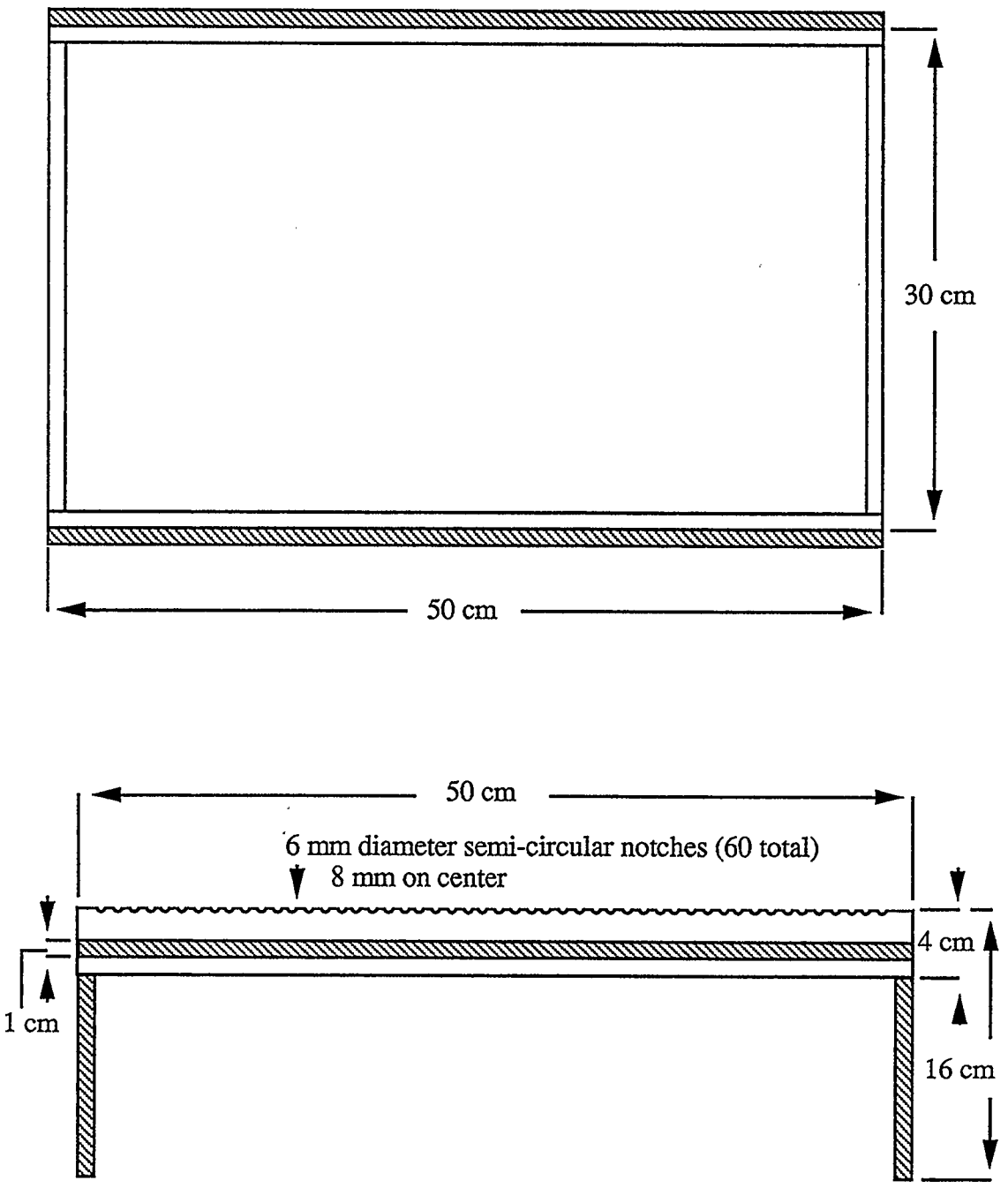


Figure 6. Germ-a-Tron seed tray, 3/8 inch Plexiglas, flat black outside. Top view (above) and side view, scale 1:4.

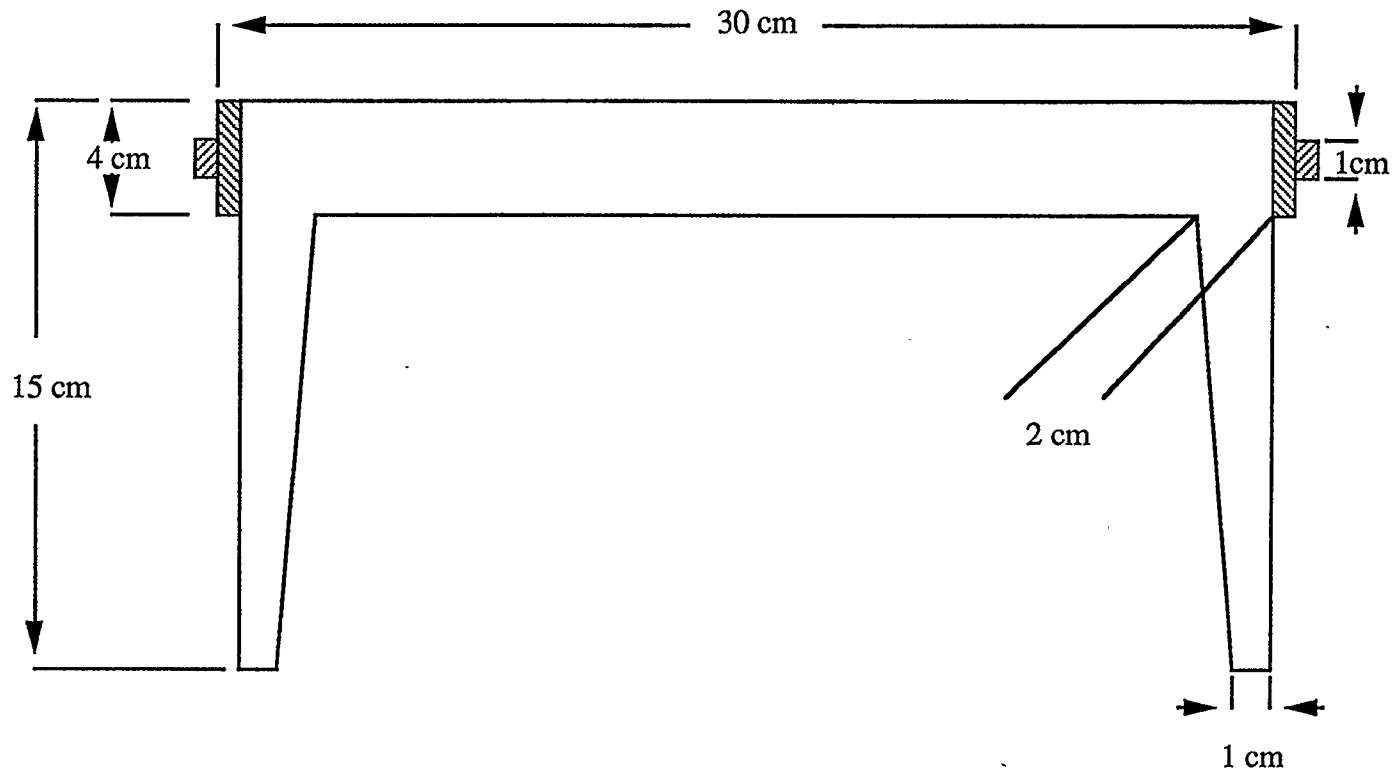
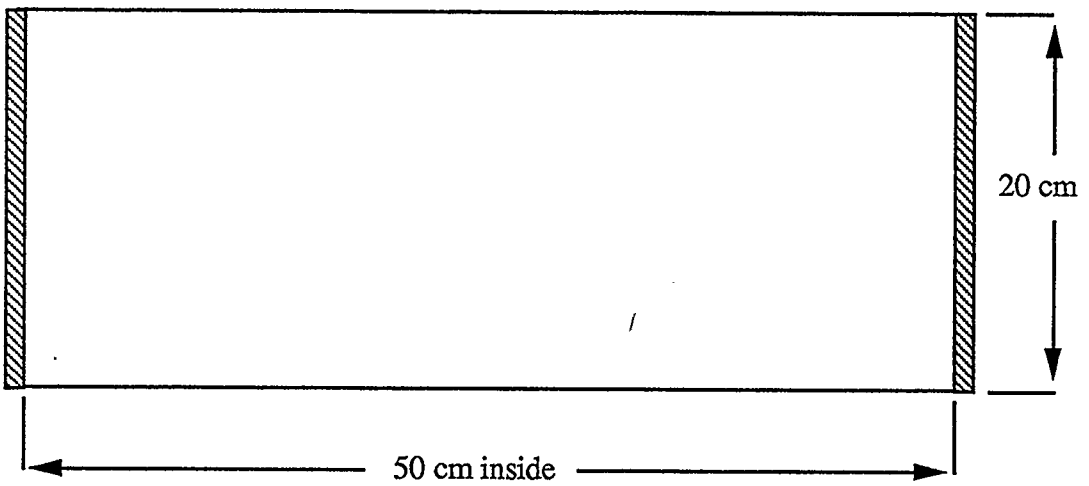


Figure 7. Germ-a-Tron seed tray, 3/8 inch Plexiglas, flat black outside. End view, scale 1:2.



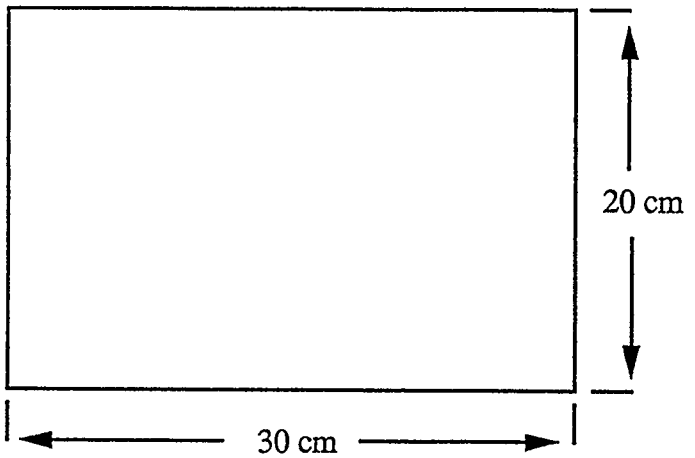


Figure 8. Germ-a-Tron screen, 3/8 inch Plexiglas, clear. Side view (above) and end view, scale 1:4.

L. var Dahlgren 131) seeds were germinated aeroponically with a 16 h photoperiod of 120 μMoles m^{-2} s^{-1} PAR (LI-COR inc., LI-185B with a quantum sensor) under a 24/18°C day/night temperature regime. Seedling roots were misted with modified one-quarter strength Hoagland solution (double amount of iron chelate). The aeroponic germination system produced plants with completely undamaged roots. At 96 hours after sowing, the plants had stems 5 to 7 cm tall and roots 7 to 10 cm long. The cotyledons had emerged from the testa but were not fully expanded, and the lateral roots were beginning to emerge.

Comparison of Germ-a-Tron and tray-grown seedlings

Seedlings were germinated in the Germ-a-Tron as given above, or grown by the conventional method in trays of Terra-Green (Oil Dri Corp., Chicago, IL, USA). Tray-germinated plants were soaked in deionized water for 15 minutes, and then planted in trays of wet Terra-Green at a depth of approximately 2 cm. These seeds were germinated under identical environmental conditions as Germ-a-Tron seedlings, and were also watered (twice daily) with the same nutrient solution used in the Germ-a-Tron.

At 96 hours after sowing, seedlings from both germination systems were gently removed, and 10 or 20 plants from each system were harvested for root and shoot weights. Means for root and shoot weight were calculated and one-tailed unpaired t-tests were performed on the means. Roots were also harvested for ethylene measurements. Seedlings were gently removed from the germination systems and roots were excised and placed in 10 mL tubes. The tubes were capped with rubber septa, and incubated for 10 min when a 1 mL gas sample was withdrawn. This sample was analyzed on a Photovac 10S10 gas chromatograph (Photovac Inc., Markham, Ont.)

equipped with a 3.2 mm X 2.45 m 60/80 Carbopak B column (1.5% XE-60/1% H_3PO_4; Supelco Canada, Oakville, Ontario, Canada) and a photoionization detector, and fresh weights of the roots were determined immediately after sampling. Carrier gas flow was 15 mL min^{-1} ultra-zero air, and peak areas were determined using a Hewlett-Packard 3390a integrator (Hewlett-Packard Co., Avondale, PA, USA).

Germ-a-Tron seedling response to ACC treatment

Seedlings were germinated in the Germ-a-Tron by the method given above for 96 hours.

Ten seedlings for each treatment, and 10 controls were removed from the Germ-a-Tron and an ink mark was made at the root-hypocotyl junction. The total length of the root was measured, and then the roots were placed in 30 mL Corex® tubes with 25 mL of the nutrient solution (controls) or nutrient solution with varying concentrations of ACC (Sigma). Alternatively, the roots were pretreated for 1 hour with 200 µM STS (prepared by mixing 800 µM silver nitrate [Fisher, A.C.S.] and 3.2 mM sodium thiosulphate [Chemonics, reagent grade] in a 1:1 ratio) and then treated with 50 µM ACC or plain nutrient solution for 1 hour. In all cases the treatment solutions were aerated just prior to use and treatments were conducted under a Sylvania Spot-Gro light producing 160 µMoles m^{-2} s^{-1} PAR. After treatment, the plants were transferred to large aeroponics chambers (Hubick *et al.*, 1982) [environmental regime as for Germ-a-Tron], and measurements of root length were made at various time intervals. Controls were handled and measured in the exact same way as treated plants. Ethylene evolution from control and ACC treated roots was monitored at various times by placing excised roots in a 3 mL syringe equipped with a three-way valve. A 1 mL gas sample was withdrawn through the valve into another syringe and analyzed for ethylene content on a

Varian 3700 gas chromatograph (Varian Instrument Division, Walnut Creek, CA, USA) equipped with a flameionization detector and a Porapak R column (Waters Associates, Inc., Milford, MA, USA). The parameters used were as follows: 2 mm X 1.5 m column, column and injector temp. 40 °C, detector temp. 120 °C, nitrogen carrier flow at 30 mL min^{-1}. Fresh weights of the roots were determined immediately after sampling, and peak areas were determined as given above.

Results

The Germ-a-Tron produced healthy seedlings with completely undamaged roots. These seedlings exhibited a different root morphology from their tray grown counterparts, possibly due to the reduction in thigmostimulation (plate 1). Germ-a-Tron plants were also not infected by the fungus that invariably afflicts seedlings grown in trays (as evidenced by mycelial growth). Germ-a-Tron plants developed significantly faster than tray grown seedlings with root fresh weights nearly double those of tray grown plants, and significantly higher shoot weights (plate 2, table 1).

Table 1: Root and shoot weights of 96 hour old Germ-a-Tron and tray-germinated sunflower seedlings.

	Germ-a-Tron	Tray	t-value	confidence
root weight (g)	.1140	.059	7	> 99%
shoot weight (g)	.554	.417	4.997	> 99%

Roots of plants grown in the Germ-a-Tron were observed to produce more ethylene than tray grown seedlings, giving 240.9 ± 16.7 pmol/g fresh weight/h compared to 145.8 ± 13.1 pmol/g fresh weight/h for tray plants.

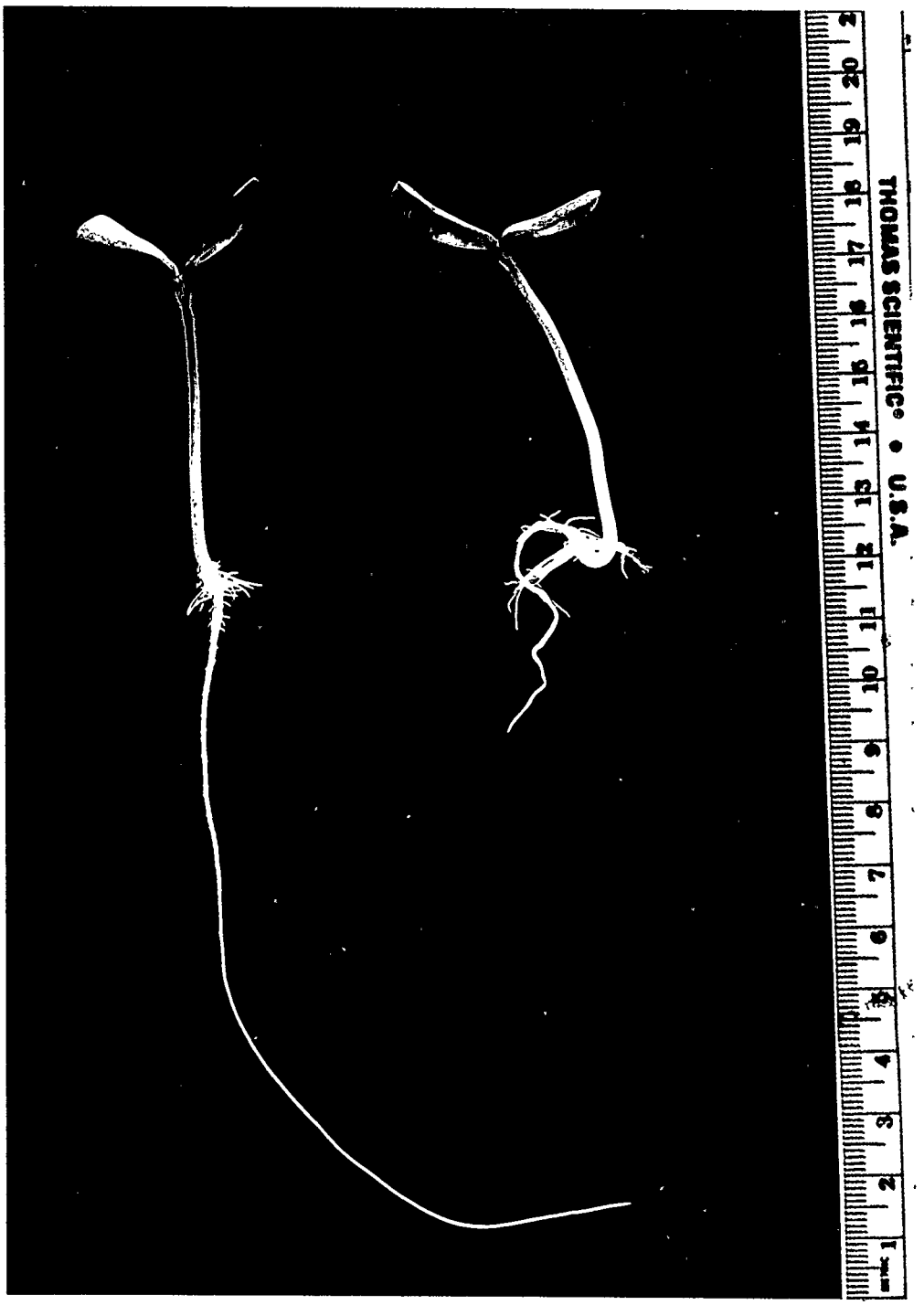


Plate 1. Morphological variation between 4 day old Germ-a-Tron grown (left) and 5 day old tray grown sunflower seedlings.

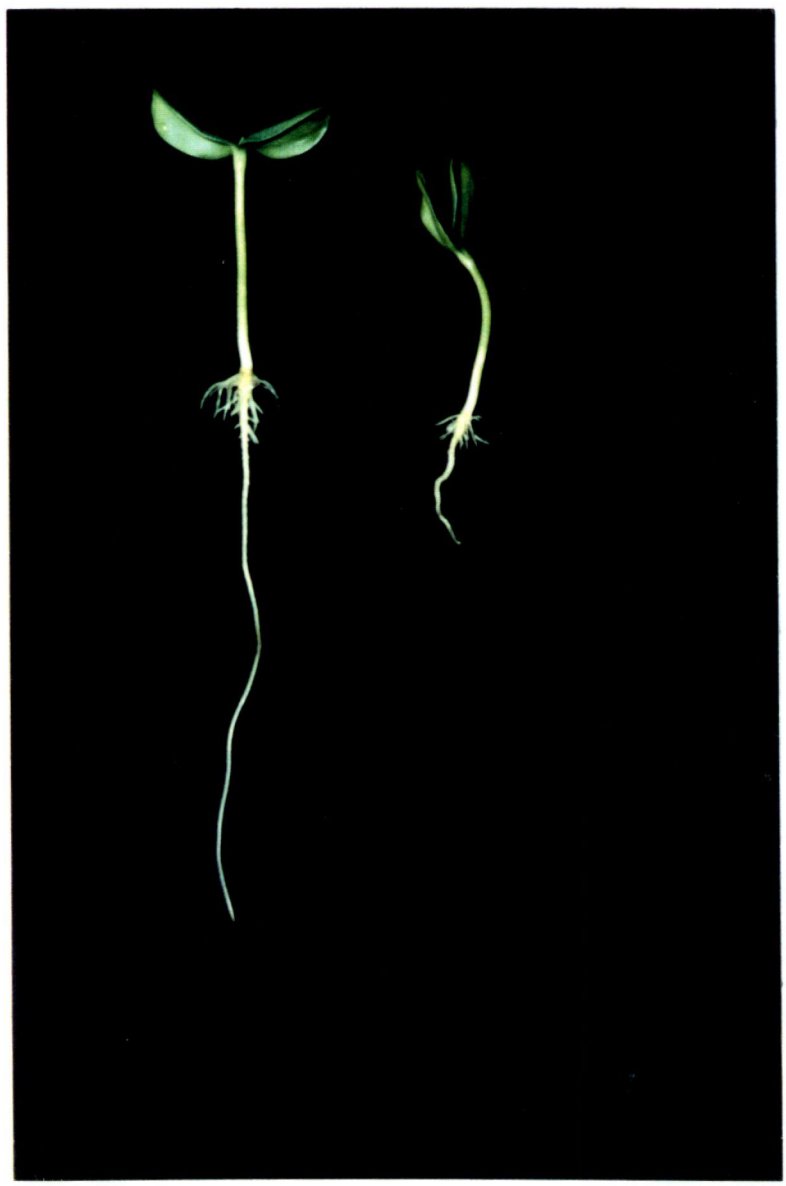


Plate 2. Developmental difference between 4 day old Germ-a-Tron grown (left) and 4 day old tray grown sunflower seedlings.

The dose response of inhibiton of root elongation in Germ-a-Tron grown seedlings to a 1 hour ACC treatment is given in fig. 9. The inhibition of root elongation after 24 hours shows a maximum near 50 μM ACC. One μM does not affect root elongation, however 10 μM strongly inhibits root growth, and at 100 μM root elongation is less than 10% of control values. Treatment of roots with 1000 or 4000 μM ACC does not further inhibit elongation over 100 μM. Plates 3 and 4 show the effect of 50 μM ACC on root morphology after 24 h. The root tips of ACC treated seedlings were swollen sub-apically and often showed a curled appearance. These roots also exhibited an increased number of lateral roots, some of which were also swollen. Ethylene effects on root morphology were observed at ACC concentrations of 10 μM and higher, usually after 12 h, and ethylene-induced epinasty in shoots was barely detectable at 100 μM ACC, but readily observed at 1000 and 4000 μM ACC.

Figure 10 illustrates the response of root elongation to various levels of ACC with time. A slight reduction in the elongation rate is observed at 12 hours in roots treated with 1 μM ACC, thereafter roots elongate normally. Ten μM ACC inhibits root elongation strongly at 12 hours, but by 48 hours normal elongation rates are observed, and by 72 hours roots elongate almost 20% faster than controls. Roots treated with 100 and 1000 μM ACC show similar responses, with the exception that inhibition of elongation occurs earlier, by 6 hours, with 1000 μM ACC. Inhibition of elongation with both 100 and 1000 μM ACC is greatest at 24 hours and elongation rates slowly increase at 48 and 72 hours. From this data, and the previous dose response curve (fig. 9) it was calculated that the maximum inhibition of root elongation would occur near 50 μM ACC.

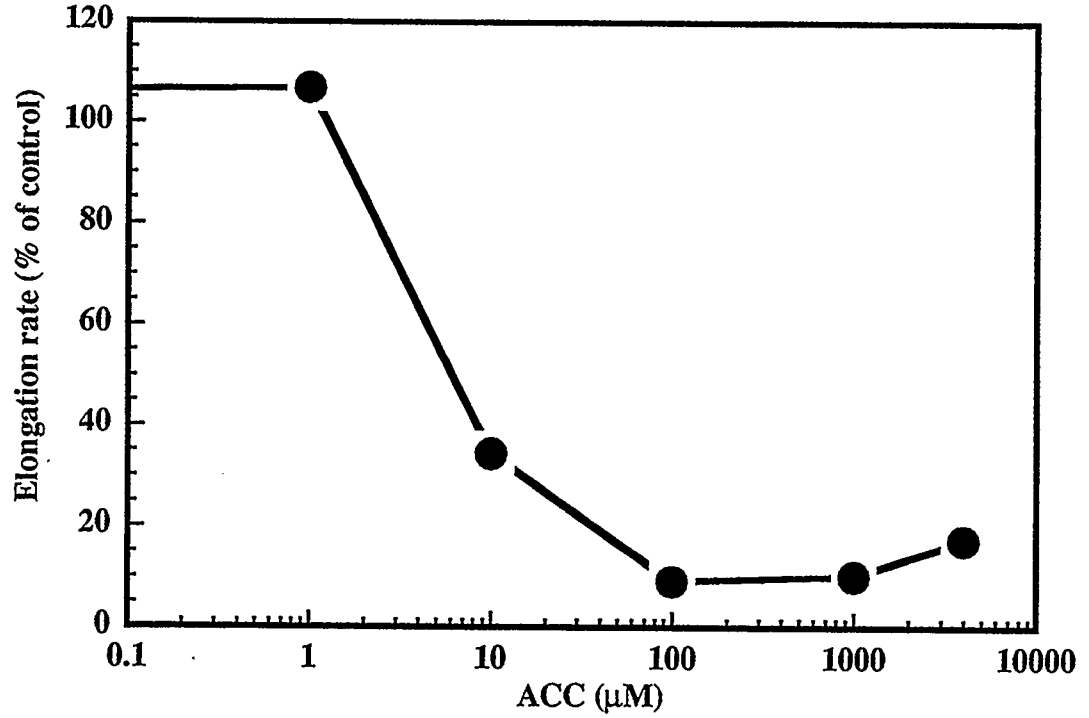


Figure 9. ACC dose response of elongation rate of roots of 4 day old sunflower seedlings.

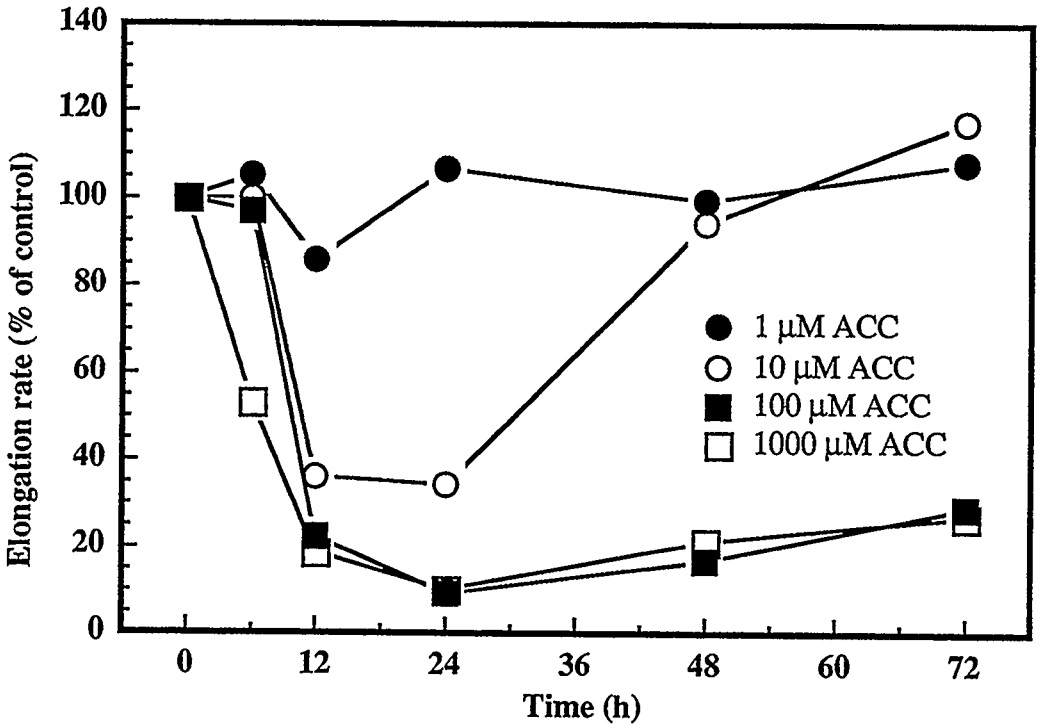


Figure 10. Elongation rate of roots of 4 day old sunflower seedlings with time, at different ACC concentrations.

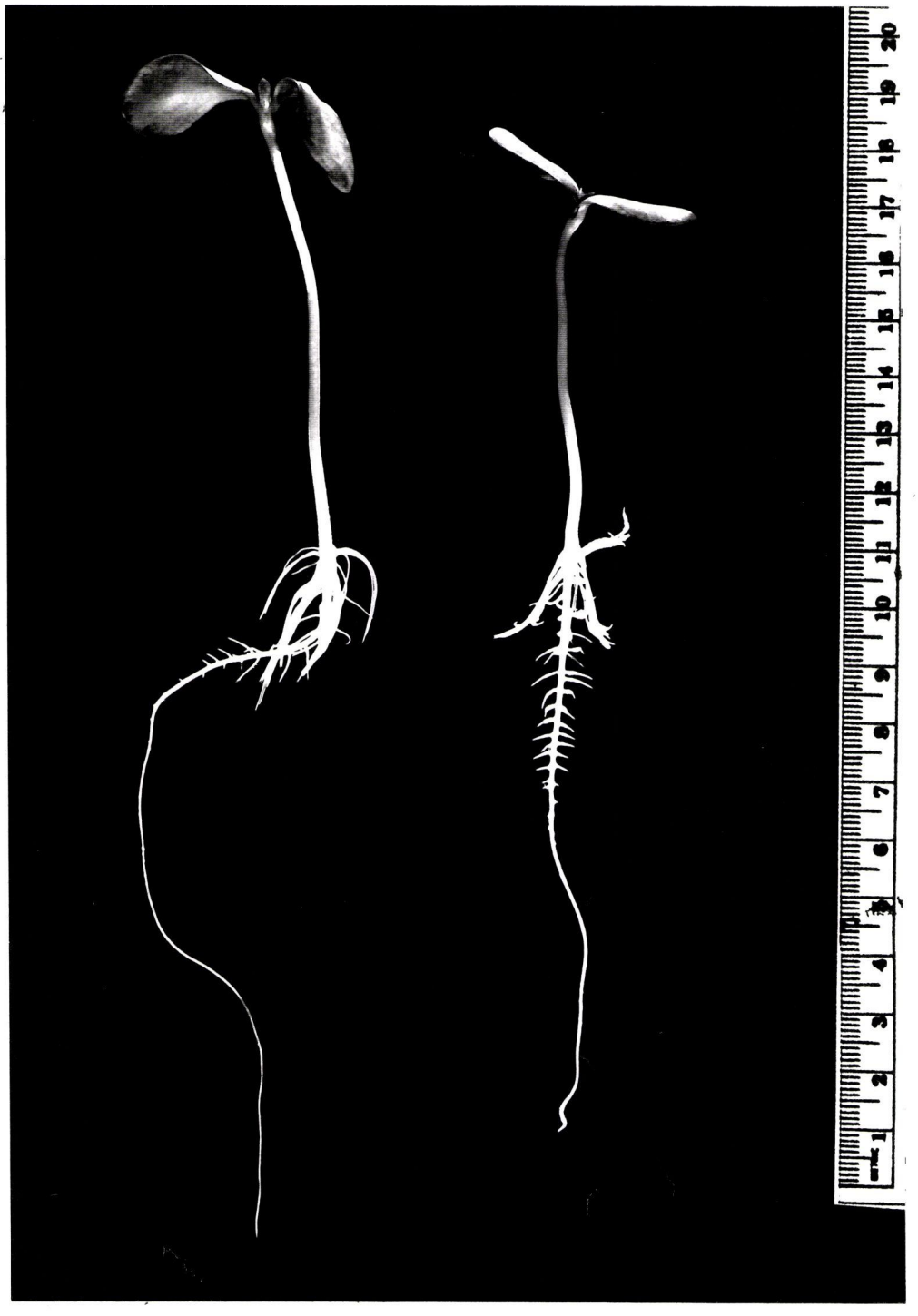


Plate 3. Morphology of untreated (left) and 1 h - 50 μM ACC-treated sunflower seedlings after 24 h.

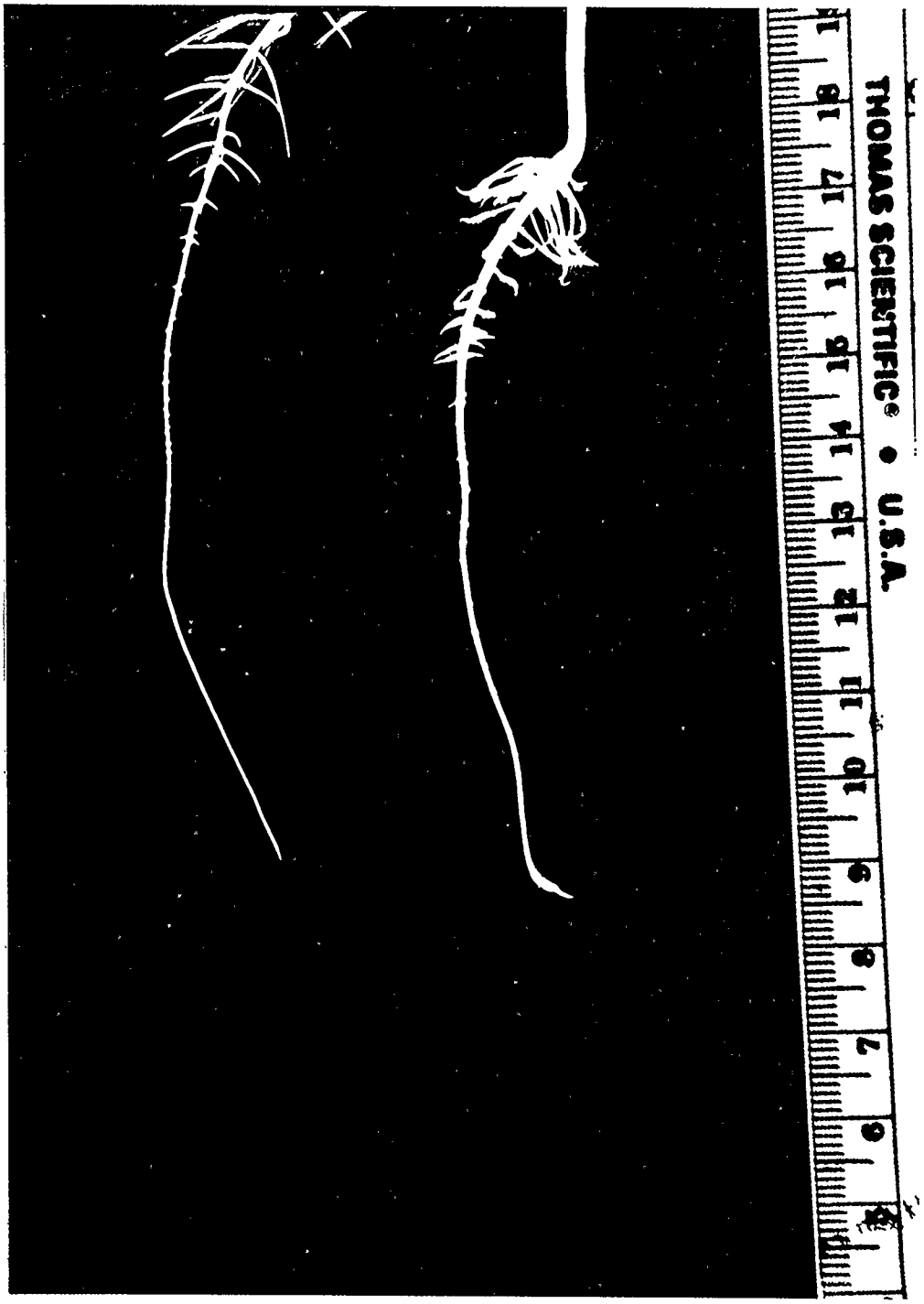


Plate 4. Root morphology of untreated (left) and 1 h - 50 µM ACC-treated sunflower seedlings after 24 h.

Figure 11 demonstrates the effect of silver thiosulphate on root elongation with and without 50 μM ACC treatment. Roots treated with 50 μM ACC showed a 83% inhibition of root elongation over 24 h, while roots treated with 200 μM silver thiosulphate showed a slight increase in elongation. Roots pre-treated with 200 μM STS and then treated with 50 μM ACC elongated at the same rate as controls, and did not exhibit any of the typical ethylene effects on root morphology.

The effect of 50 μM ACC treatment on ethylene production of Germ-a-Tron seedling roots is shown in fig. 12. Ethylene production by ACC-treated roots was approximately 10 times greater than untreated roots at 1 hour after treatment, and decreased to near control levels by 24 h after treatment.

Discussion

The aeroponic germination system described in this chapter was proven to be an effective system for producing sunflower seedlings for use in examining the regulation of root growth. The Germ-a-Tron produced seedlings with undamaged roots, which were easy to handle and measure due to their straight growth habit as compared to roots of seedlings from conventional tray-germination methods which were often damaged, and difficult to measure. Germ-a-Tron plants developed faster than tray-germinated plants, and this effect was more prominent in the roots, however the reason for this is not clear. It is possible that Germ-a-Tron plants received a more optimal watering pattern than tray-germinated plants, even though tray-germinated plants were watered twice-daily. Alternatively the thigmostimulation encountered by tray-grown plants might have restricted root growth over the Germ-a-Tron seedlings. Thimostimulation has been observed to inhibit root elongation in several systems (Kays *et al.*, 1974; Moss *et al.*,1988; Sarquis *et al.*, 1991), possibly through enhanced endogenous ethylene

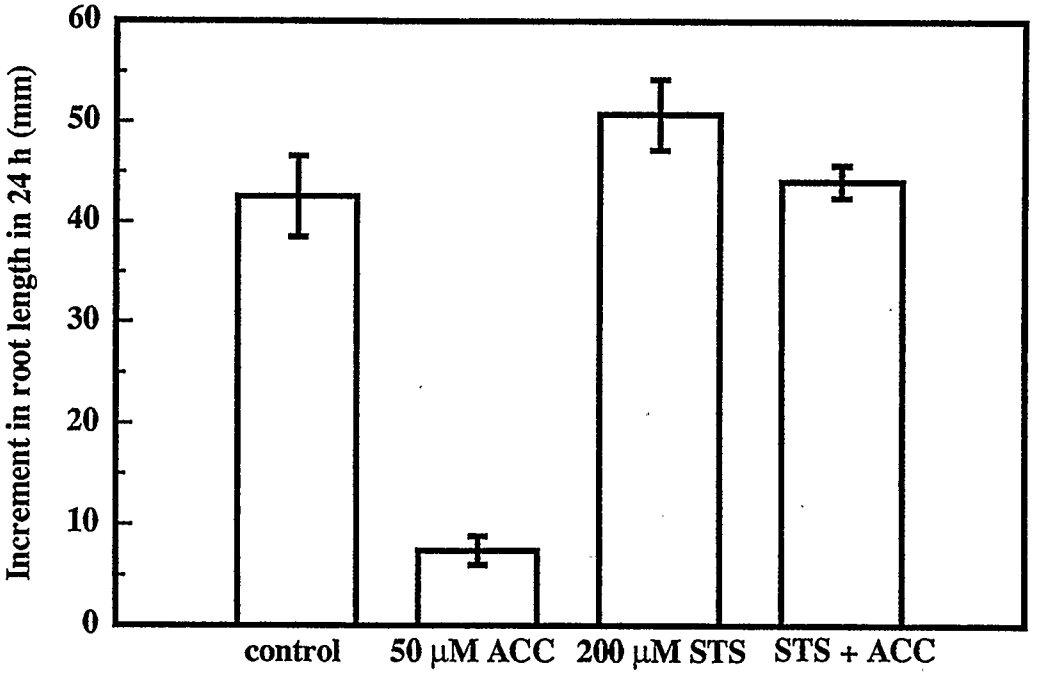


Figure 11. Increment in root length during 24 h of 4 day old sunflower seedlings treated with ACC and/or silver thiosulphate. n= 10, mean ± standard error.

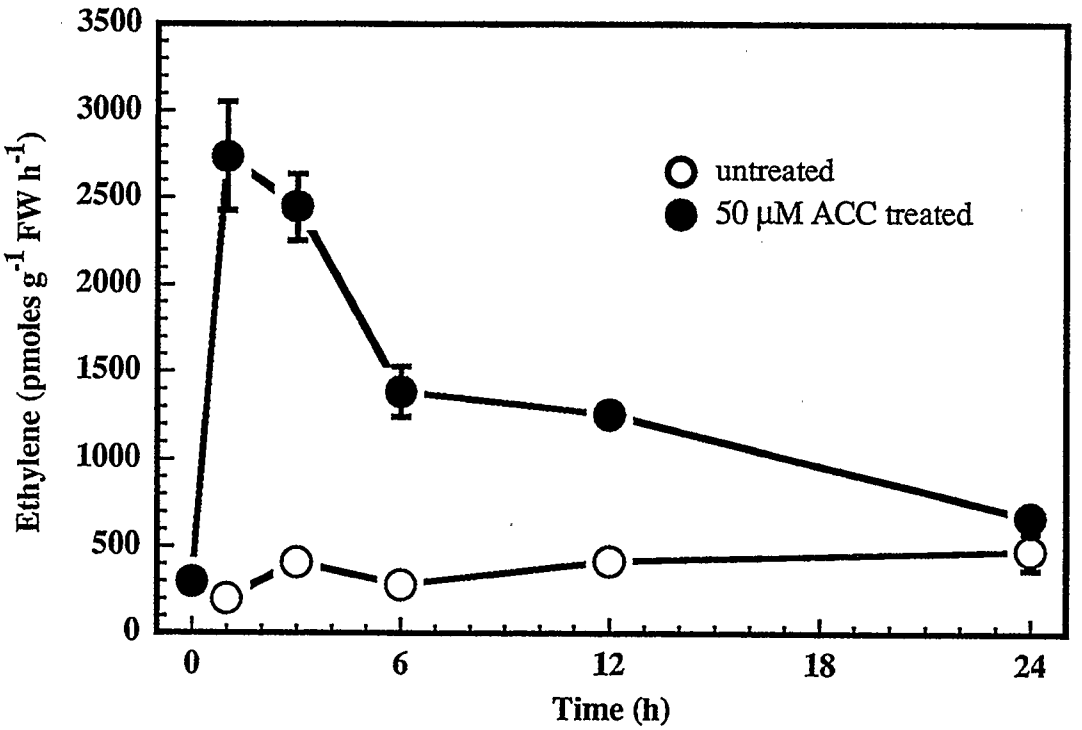


Figure 12. Ethylene production by roots of 4 day old sunflower seedlings with and without 50 µM ACC treatment. n= 5, mean ± standard error.

production (Kays *et al.*, 1974; Sarquis *et al.*, 1991). Compaction of the soil surrounding roots has been shown to inhibit root growth (Sands and Bowen, 1978; Taylor and Gardner, 1963), but in this case the Terra-Green is only loosely packed and substrate density should not be a problem.

Fungal infection which occured often in other germination systems was not a problem here which is possibly due to the greater vigor of the Germ-a-Tron plants, or the watering pattern applied.

Roots of Germ-a-Tron plants were also observed to produce more ethylene than tray-germinated seedlings. A common observation in many tissues is that more rapidly growing tissues produce more ethylene than slower growing tissues (Huxter *et al.*, 1979; Jayasekera, pers. comm.). The reasons for this are not known. It is possible that some of this increase in ethylene is merely a result of faster growth, or alternatively increased ethylene production may be actively involved in regulating rapid growth (Huxter *et al.*, 1979).

Roots of Germ-a-Tron plants were shown to respond in a characteristic manner to elevated ethylene production by exogenous application of ACC. A maximal inhibition of root elongation was observed between 10 and 50 μM of ACC treatment. A time course analysis showed a maximum inhibition occured 24 h after treatment. Pre-treatment of roots with 200 μM silver thiosulphate completely reversed the effects of 50 μM ACC suggesting that the effect was indeed attributable to ethylene, which was readily produced by roots in response to 50 μM ACC. These results confirm those of other studies (Radin and Loomis, 1969; Smith and Robertson, 1971; Whalen and Feldman, 1988) and suggest that endogenous ethylene may play a role in regulating root elongation under situations of high external ethylene levels, or under environmental conditions elevating endogenous ethylene production.

Chapter 3.

Transport and Metabolism of 1-Aminocyclopropane-1-carboxylic Acid in Sunflower (*Helianthus annuus* L.) Seedlings.¥

Summary

Transport and metabolism of [2,3-^{14}C] 1-aminocyclopropane-1-carboxylic acid (ACC) from roots to shoots in 4 day old sunflower (*Helianthus annuus* L.) seedlings were studied. [^{14}C]ACC was detected in, and $^{14}C_2H_4$ was evolved from, shoots 0.5 hours after [^{14}C]ACC was supplied to roots. Ethylene emanation from the shoots returned to normal levels after 6 hours. The roots showed a similar pattern, although at 24 hours ethylene emanation was still slightly higher than in those plants that did not receive ACC. [^{14}C]*N*-malonyl-ACC (MACC) was detected in both tissues at all times sampled. [^{14}C]MACC levels surpassed [^{14}C]ACC levels in the shoot at 2 hours, whereas [^{14}C]MACC levels in the root remained below [^{14}C]ACC levels until 6 hours, after which they were higher. Thin-layer chromatography analysis identified [^{14}C]ACC in 1-hour shoot extracts, and [^{14}C]MACC was identified in root tissues at 1 and 12 hours after treatment. [^{14}C]ACC and [^{14}C]MACC in the xylem sap of treated seedlings were identified by thin-layer chromatography. Xylem transport of [^{14}C]ACC in treated seedlings, and transport of ACC in untreated seedlings, was confirmed by gas chromatography-mass spectrometry. Evidence for the presence of [^{14}C]MACC in xylem sap in [^{14}C]ACC-treated seedlings is presented. A substantial amount of radioactivity in both ACC and MACC fractions was detected leaking from the roots over 24 hours. A

¥ This chapter has been published under the same title with Dr. Ken R. Foster and Dr. David M. Reid as co-authors in Plant Physiology (1991) 96: 1360-1367. Ken assisted with the treatments and harvest and performed the TLC and GC-MS.

second radiolabelled volatile compound was trapped in a CO_2-trapping solution but not in mercuric perchlorate. Levels of this compound were highest after the peak of ACC levels and before peak MACC levels in both tissues, suggesting that an alternate pathway of ACC metabolism was operating in this system.

Introduction

Transport of phytohormones from one plant part to another is one method of communication among plant tissues and organs. Such a system may be useful when a signal is perceived by one organ and a response is required by another distant from the site of perception. Before we can fully understand the physiology of a phytohormone and how it might be involved in the integration of activities within the whole plant, we must understand the patterns of transport of the hormone, its precursors and conjugates, and the metabolic fate of these compounds.

Ethylene differs from the other plant hormones in that it is a gas that easily diffuses out of the plant. Although transport of ethylene does occur (Jackson and Campbell, 1975; Woltering, 1990), the directed longitudinal movement of this hormone is limited (Zeroni *et al.*, 1977). The immediate ethylene precursor, ACC (Adams and Yang, 1979), can be transported in the vascular system (Bradford and Yang, 1980a, b). ACC is present in relatively low amounts; however, exposure to some types of stress can increase ACC and ethylene synthesis (Bradford and Yang, 1980a, b; Hoffman *et al.*, 1983b).

ACC may be transported among flower parts signalling the initiation of flower senescence following pollination in *Cymbidium* and carnation (Nichols *et al.*, 1983; Reid *et al.*, 1984; Woltering, 1990). Increased ACC levels in organs distal to leaves treated with ethephon were found in *Cucurbita pepo* (Hume and Lovell, 1983),

prompting the proposal that ACC is a mode of interorgan transport of an ethylene signal in this species, although the transport of ACC between plant organs was not measured directly. ACC synthesis increases in flooded roots; this ACC is transported in the xylem to the shoots where it causes an increase in ethylene production in leaves and petioles, which then exhibit a number of changes in growth and development (Bradford and Yang, 1980a, b). Basipetal transport of ACC in decapitated pea plants has also been demonstrated (Fuhrer and Fuhrer-Fries, 1985).

ACC can be conjugated to form MACC (Hoffman *et al.*, 1982, 1983a). Because large amounts of MACC are synthesized under conditions that induce ACC synthesis, the formation of MACC may be a means of removing ACC, thus controlling ethylene formation (Bouzayen *et al.*, 1988; Hoffman *et al.*, 1983b). Although MACC is a poor ethylene precursor compared to ACC, treatment of plant tissues with MACC does increase ethylene production (Hoffman *et al.*, 1983b; Satoh and Esashi, 1983), possibly through enzymatic hydrolysis of MACC to ACC (Jiao *et al.*, 1986). However, either when tissues are treated with MACC or during *in vivo* synthesis, the majority of MACC is transported into the vacuole (Bouzayen *et al.*, 1989), where it remains (Bouzayen *et al.*, 1988; Hoffman *et al.*, 1983b). Because conjugates of other phytohormones can be a source of active hormone after deconjugation, the metabolism and transport of MACC must also be considered in investigations of ethylene precursor transport. Transport of MACC from shoot to root in peas has been shown by Fuhrer and Fuhrer-Fries (Fuhrer and Fuhrer-Fries, 1985), whereas MACC is not transported out of tobacco leaves (Van Loon and Fontaine, 1984). Efflux of MACC from *Acer* protoplasts preloaded with MACC was shown by Bouzayen *et al.* (1988), although the majority of MACC fed to these protoplasts was recovered from the vacuole.

Although ACC has been shown to be transported in the few systems mentioned above, the translocation of this ethylene precursor within a whole, unstressed plant has been studied rarely. Compared to the transport of auxins, gibberellins, cytokinins and ABA, there are few data concerning the transport of ACC. This is surprising considering the large body of data showing the involvement of ethylene in many aspects of plant growth and development. In this chapter we report our study of ACC translocation from roots to shoots in young sunflower plants. We also carried out a limited investigation of the metabolic fate of ACC in these two organs.

Materials and Methods

Plant Material.

Sunflower seeds were germinated aeroponically as given in chapter 2. At the 4-d stage, plants with stems 5 to 7 cm tall and roots 8 to 10 cm long were selected.

ACC Treatment.

The seedlings were treated by immersing roots of two plants in 5-mL tubes containing 4 mL of 10 μM [2,3-^{14}C]ACC (specific activity 2.96 GBq/mmol; Commissariat á l'Energie Atomique, Gif Sur Yvette, France) in one-quarter strength Hoagland solution that was aerated before the experiment. Roots were gently coiled into the tubes, avoiding sharp bends so as to avoid wounding. The plants were treated in this solution for 1 h under Gro-Lux lights (185 μE m^{-2} s^{-1}). After the roots were fed, they were rinsed twice in distilled water, and the plants were transferred to aeroponics chambers (Hubick *et al.*, 1982) or harvested immediately. As controls, an equal number of plants were immersed in pairs in 4 ml of the above nutrient solution without ACC and were handled exactly as the [^{14}C]ACC-treated plants.

Tissue Gas Evolution.

At each harvest, the seedlings were cut at the root-shoot junction, and each portion was placed in a separate 10-mL syringe with the plungers adjusted to 6 mL. After 10 min, a 5-mL gas sample was transferred to a second gas-tight syringe through a three-way valve (we find that wound-induced ethylene can not be detected for at least 30 min). This sample (1 mL) was analyzed for ethylene content using a gas chromatograph equipped with a photoionization detector as in chapter 2. An aliquot (2 mL of the 5-mL sample) was injected through a septum into a scintillation vial containing 1.5 mL of mercuric perchlorate (Abeles, 1973). Vials were incubated overnight with shaking. Scint A XF scintillation cocktail (10 mL [Packard Instrument Co., Downers Grove, IL]) was added to each vial, and radioactivity was determined with a Packard Tri-Carb 2200CA liquid scintillation analyzer (Packard Instrument Co., Downers Grove, IL). To determine whether radiolabelled gases, other than ethylene, were produced in these experiments, a trap of $^{14}CO_2$ cocktail (Carbon 14 cocktail; R. J. Harvey Instrument Corp., Hillsdale, N.J.) was used. This cocktail contains a scintillant and is a highly efficient CO_2 trap. The final 2 mL were injected through a septum into a vial containing 10 mL of $^{14}CO_2$ cocktail and incubated with shaking overnight, after which the amount of trapped radioactivity was determined. The fresh weights of the roots and shoots used for gas analysis were determined immediately after sampling. Each experiment was performed at least twice, with four replicates.

Trapping efficiencies.

To determine the efficiency of the $^{14}CO_2$ cocktail for trapping ethylene, 100 pmol C_2H_4 were injected into vials containing 10 mL $^{14}CO_2$-trapping cocktail and incubated as above. Ethylene remaining in the headspace was determined using a gas

chromatograph equipped with a Porapak R column (2 m X 2mm I.D., 80 to 100 mesh; Waters Associates Inc., Milford, MA) and a flame ionization detector. The ethylene-trapping efficiency of the mercuric perchlorate was determined in a like manner. Each solution was also tested for trapping efficiency of $^{14}CO_2$, which was derived from acid breakdown of $NaH^{14}CO_3$ (Amersham Corp., Arlington Heights, IL).

Mercuric perchlorate trapped C_2H_4 with 99% efficiency. Efficiency of ethylene trapping by the $^{14}CO_2$ cocktail was found to be 35%. Each trapping solution was tested for efficiency in trapping CO_2, because it was possible that $^{14}CO_2$ was released in these experiments. The $^{14}CO_2$ cocktail trapped $^{14}CO_2$ with 98% efficiency. Mercuric perchlorate did not trap $^{14}CO_2$. These efficiencies were taken into account when determining the amounts of radioactive gases produced.

ACC and MACC Extraction and Identification.

During each harvest, four [^{14}C]ACC-treated plants were cut at the root-shoot junction. Each root and shoot was weighed separately, frozen in liquid N_2, and stored at -70°C. The extraction of ACC and MACC was based on previously described methods (Nieder *et al.*, 1986; Sitrit *et al.*, 1988). The tissue was powdered in liquid N_2 and extracted three times in 80% ethanol at 70°C for 15 min, each followed by filtration through a Whatman No. 1 filter. The combined extract was reduced to dryness *in vacuo* at 40°C and redissolved in 5 mL of water plus 1.5 mL of $CHCl_3$. The $H_2O/CHCl_3$ extract was centrifuged at 2500g for 15 min to minimize emulsion formation. The aqueous phase was passed in tandem through columns containing polyvinylpolypyrrolidone (4.5 X 0.8 cm i.d., 80 mesh; Sigma Chemical Co., St. Louis, MO) and cation exchange resin (AG 50W X8, H^+ form, 4.5 X 0.8 cm i.d., 100 to 200 mesh; Bio-Rad Laboratories, Richmond CA). The columns were washed with water;

this wash contained MACC and was collected. ACC was eluted from the cation exchange resin with 25 mL 2 N NH_4OH. Radioactivity in each fraction was determined. ACC and MACC fractions from tissues harvested at 1 and 12 h were reduced *in vacuo* at 40°C to approximately 1 mL and an aliquot of this was subjected to two-dimensional TLC on either microcellulose (250 μm) or silica gel (250 μm) plates (Whatman Inc., Clifton, NJ). These were developed in the first dimension in 1-butanol:acetic acid:water (12:3:5 v/v/v) and in the second dimension in 1-propanol:NH_4OH (7:3 v/v). The plates were autoradiographed and compared to authentic [^{14}C]ACC and [^{14}C]MACC standards for identification purposes. Standard [^{14}C]MACC was prepared using the protocol of Satoh and Esashi (1984b), except that the quantity of ACC precursor used was substantially lower (40.9 μg, 32.4 μCi), and instead of recrystalization, purification of the [^{14}C]MACC was done by preparative TLC on 1-mm silica gel plates (Whatman) in the first dimension solvent described above. The [^{14}C]MACC was located by autoradiography, scraped, and eluted from the silica gel. Confirmation of [^{14}C]MACC identity was accomplished by GC-MS as described below.

ACC and MACC Leakage.

Estimates of radioactive leakage from the roots were determined at 1, 6 12 and 24 h after the commencement of treatment by placing the root of one [^{14}C]ACC-treated plant in 15 mL of one-quarter strength Hoagland solution for 15 min. The radioactivity in this solution was then determined. Three replications were made at each sample time. In a separate experiment, 2 h after commencement of root treatment with [^{14}C]ACC, the Hoagland solution collecting the root leakage was fractionated into ACC and MACC

components using cation exchange resin, and radioactivity in these fractions was determined.

Analysis of Xylem Sap.

Collection of Sap.

Thirty-three seedlings were fed [^{14}C]ACC as described above and were transferred to the aeroponics chambers. The plants were decapitated with a razor blade approximately 1 cm below the cotyledons 2 h after terminating treatment, and xylem exudate was collected during a 4-h period. The sap was fractionated on cation exchange resin into ACC and MACC fractions of which aliquots were counted for radioactivity. These samples were analysed by silica gel and cellulose TLC and autoradiography as described above. To determine whether ACC and MACC were naturally present in xylem sap, 11-d-old seedlings (germinated and grown aeroponically) were decapitated approximately 1 cm below the cotyledons, and xylem exudate was collected during a 4-h period. Two samples pooled from 40 (30 mL) and 60 plants (55 mL) were collected.

Ion exchange and HPLC.

The purification and analysis of ACC was based on the protocol of McGaw *et al.* (1985). The xylem sap was dried *in vacuo* at 40°C, redissolved in 10 mL 0.1 N acetic acid and applied to a column of AG 50W X8 resin (H^+ form, 4.5 X 1.3 cm i.d.) that was washed with 0.1 N acetic acid. The washings contained MACC. The ACC was eluted with 2 N NH_4OH, dried *in vacuo* at 40°C, redissolved in 0.1 N NH_4OH, and loaded onto an anion exchange column (AG 1 X8, CH_3COO^- form, 4.5 X 1.3 cm i.d., 200 to 400 mesh; Bio-Rad) that was then washed with 0.1 N NH_4OH. The ACC was eluted with 0.1 N acetic acid, which was reduced to approximately 2 mL *in vacuo* at

40°C. This sample was transferred to a vial and freeze dried. Two milliliters of 30 mM phthalic anhydride (once resublimed) in glacial acetic acid were added to the vial, which was sealed and heated to 100°C for 1.5 h. After the addition of 2 mL of water, the sample was partitioned against 5 mL of ether. The ether phase was dried *in vacuo* at 40°C. The residue was dissolved in 300 µl methanol, after which 700 µl of 0.1 N acetic acid were added. The sample was chromatographed on a reverse-phase C_{18} Partisil ODS_4 HPLC column (110 X 4.7 mm i.d., 5-µm particle size; Whatman) in 30 to 45% methanol in a 0.1 N acetic acid gradient for 10-min. The fraction containing phthalidimo-ACC was collected and dried *in vacuo* at 40°C and methylated three times with freshly prepared diazomethane. The methylated sample was dissolved in 32% aqueous CH_3CN and chromatographed on the Partisil HPLC column isocratically in 32% aqueous CH_3CN. The fraction containing phthalidimo-ACC-methyl ester was collected and dried *in vacuo* at 40°C.

The MACC fraction was dried *in vacuo* at 40°C, dissolved in 0.1 N NH_4OH, and applied to a column of AG 1 X8 ($HCOO^-$ form, 4.5 X 1.3 cm i.d., 200 to 400 mesh; Bio-Rad). The resin was washed with 0.1 N NH_4OH, and the MACC was eluted with 4 N HCOOH. The sample was dried *in vacuo* at 40°C, and the residue was dissolved in a minimum amount of methanol, three drops of HCOOH and approximately 5 mL of ether. The resulting precipitate was removed by filtration (GF/C glass fibre filter) and the extract dried under N_2. This precipitation process was repeated, and the sample was methylated three times. Dimethyl MACC was dissolved in H_2O, and purified using the Partisil HPLC column in a gradient of 0 to 15% CH_3CN in H_2O for 15 min. The fraction containing dimethyl MACC was collected and dried *in vacuo* at 40°C. In addition, ACC derived from MACC after hydrolysis in 6 N HCl for 1.5 h (Hoffman *et al.*, 1982) was derivatized and analysed as above.

GC-MS.

A Hewlett-Packard HP-5980 Series II gas chromatograph equipped with a DB5-15m capillary column (15 m X 0.25 mm i.d., 0.25-μm film thickness; J. & W. Scientific, Folson, CA) coupled to a HP 5970A mass selective detector was used. The phthalidimo-ACC-methyl ester was dissolved in ethyl acetate, injected on-column, and run on a temperature program of 60 to 160°C (40°C min^{-1}) and then to 206°C (6°C min^{-1}). Injections of dimethyl MACC were made in ethyl acetate at an initial temperature of 60°C and temperature programming of 60 to 80°C (40°C min^{-1}), followed by 80 to 250°C (12°C min^{-1}). In both analyses, the interface temperature was maintained at 280°C and helium linear gas flow at 40 cm min^{-1}.

Results

Gas Evolution by the Tissues.

We supplied radioactive ACC to roots for 1 h and examined ethylene evolution during a 3 h period. As expected, much more ethylene was produced by both shoots and roots of ACC-treated plants than by plants that did not receive additional ACC. In shoots (fig. 13) of the ACC treated plants, ethylene production peaked at 1 h and then declined to near control levels by 3 h. The release of radioactive ethylene was also monitored by use of a mercuric perchlorate trap (Abeles, 1973). Radioactive ethylene was produced in the shoots from [^{14}C]ACC within 0.5 h of the application and increased to a maximum at 1 h. Thereafter, radioactivity declined to lower, but measurable, levels at 3 h. The pattern of radioactivity in ethylene trapped from shoots was similar to the pattern of ethylene evolution from the ACC-treated plants as measured by GLC. When the quantities of ethylene measured by GLC were plotted against the

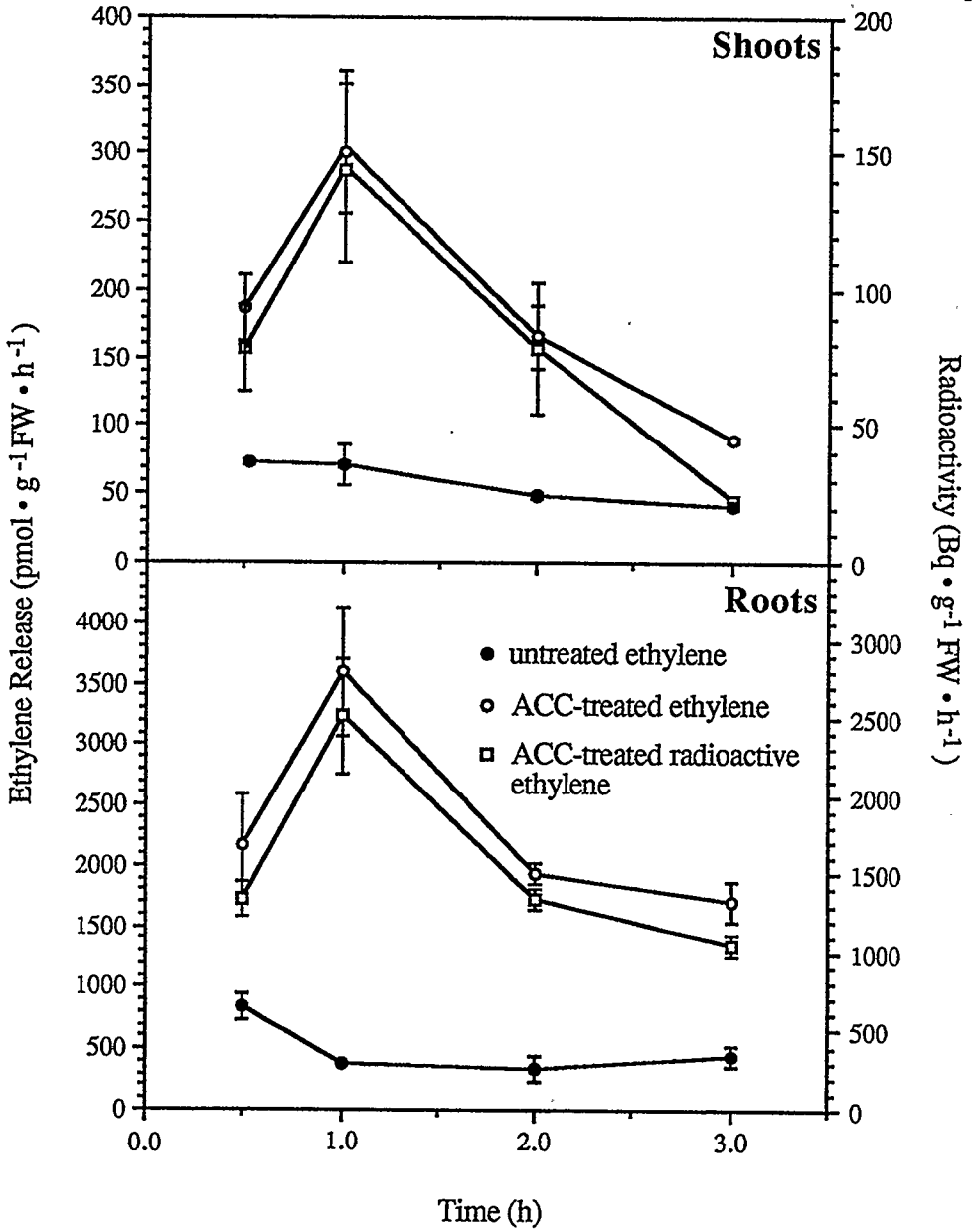


Figure 13. Ethylene release during 3 h from shoots and roots of 4 day-old sunflower seedlings. Nonradioactive ethylene was estimated by GLC. Radioactivity associated with ethylene was trapped in mercuric perchlorate and estimated by scintillation counting. n = 4, mean ± standard error.

radioactivity, there was a strong positive correlation (r^2=0.986). Therefore, much of this latter ethylene was produced from the [^{14}C]ACC that had been fed to roots.

Ethylene evolution by roots showed a similar picture except that overall ethylene production was approximately 10 times higher than in shoots, and C_2H_4 emanation from the ACC-treated roots at 3 h was approximately three times control levels (fig. 13). As seen in the shoots, the pattern of radioactivity in ethylene (measured by GLC) trapped from roots, was very similar to the quantities of radioactive ethylene trapped in mercuric perchlorate (r^2=0.984).

The fate of the labelled ACC was then evaluated during 24 h. Ethylene evolution was high at 1 h from both organs and decreased with time (fig. 14). Ethylene production by shoots decreased to control levels by 6 h; however, root ethylene production did not decrease to control values until 12 h after treatment and was slightly higher than controls at 24 hours after treatment. As before, root ethylene evolution was approximately 10-fold higher than that from the shoot. There was again a strong positive correlation between the quantity of trapped radioactive ethylene and ethylene measured by GLC from roots (r^2=0.989 roots); however, an anomalous 6-h radioactive ethylene level from shoots gave a poorer correlation coefficient for this graph (r^2=0.568).

Radioactivity released from the plant as $^{14}C_2H_4$ and a second gas (probably $^{14}CO_2$) trapped in the $^{14}CO_2$ cocktail from plants treated with [^{14}C]ACC is shown in figure 15. In the shoots, except for the 1-h time, radioactivity associated with ethylene was lower than radioactivity associated with the second volatile compound. At its peak at 6 h, production of this second gas was substantially greater than with $^{14}C_2H_4$ evolution. At the termination of the experiment, radioactivity associated with the second gas remained relatively high (136 Bq g^{-1} fresh weight h^{-1}), whereas radioactive ethylene

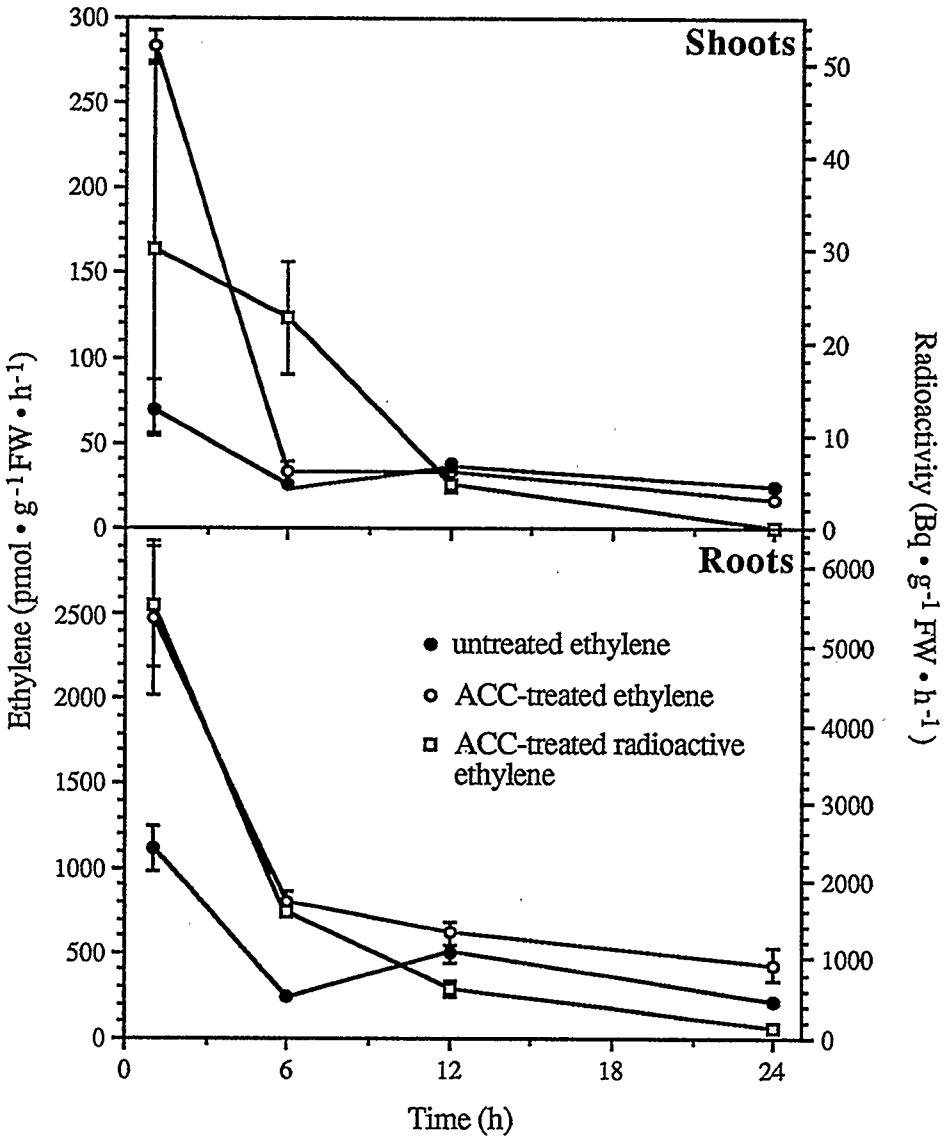


Figure 14. Ethylene release during 24 h from shoots and roots of 4 day-old sunflower seedlings. Nonradioactive ethylene was estimated by GLC. Radioactivity associated with ethylene was trapped in mercuric perchlorate and estimated by scintillation counting. n = 4, mean ± standard error.

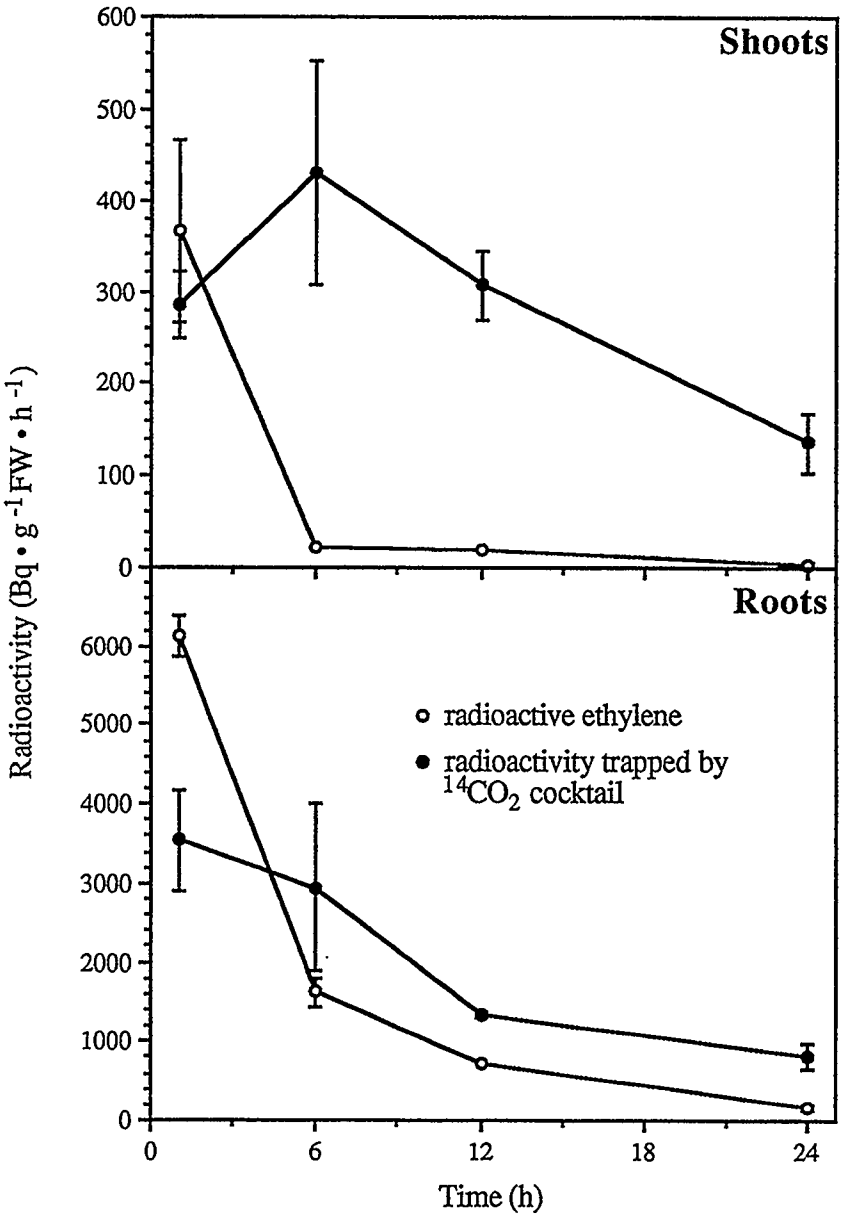


Figure 15. Release of gaseous radioactivity from shoots and roots of 4 day-old sunflower seedlings, after root treatment with 10 µM [2,3-^{14}C]ACC. n = 4, mean ± standard error.

levels were low (3 Bq g^{-1} fresh weight h^{-1}). Labeled ethylene and the unidentified second compound were also released from the roots of treated plants (fig.15). Levels of both ethylene and the second gas were highest at 1 h (6127 Bq g^{-1} fresh weight h^{-1} and 3541 Bq g^{-1} fresh weight h^{-1}, respectively) and then steadily declined. Radioactive ethylene levels decreased rapidly from 1 h to 6 h, whereas radioactivity associated with the second gas declined more slowly.

ACC and MACC Levels in Treated Seedlings.

[^{14}C]ACC was present in both roots and shoots 0.5 h after the roots had been supplied with [^{14}C]ACC, and levels peaked at 1 h (fig. 16). In shoots, [^{14}C]ACC levels decreased from 1 to 2 h and then remained constant until 3 h, and levels in roots decreased from 1 to 2 h and then increased again slightly until 3 h. MACC levels increased steadily in both organs during the 3 h period. Shoot [^{14}C]MACC levels surpassed peak [^{14}C]ACC levels at 2 h and continued to increase until 3 h, whereas root [^{14}C]MACC levels remained low relative to [^{14}C]ACC levels.

Subsequent 24-h experiments again showed the maximum amount of radioactivity in the ACC fraction from shoots 1 h after commencing treatment (fig. 17). Radioactivity in this fraction then declined rapidly from 1 to 6 h, thereafter slowly decreasing. Radioactivity in the MACC fraction increased four fold from 1 to 12 h. At 12 h there was about 10 times as much radioactivity in the MACC fraction than there was in the ACC fraction. At the termination of the experiment, MACC levels decreased from the high at 12 h but were still 2.7 times higher than at the first sampling time of 1 h. Extracts from the roots showed a similar pattern except that they had higher levels of radioactivity (fig. 17). Radioactivity in the ACC fraction of roots was highest at 1 h

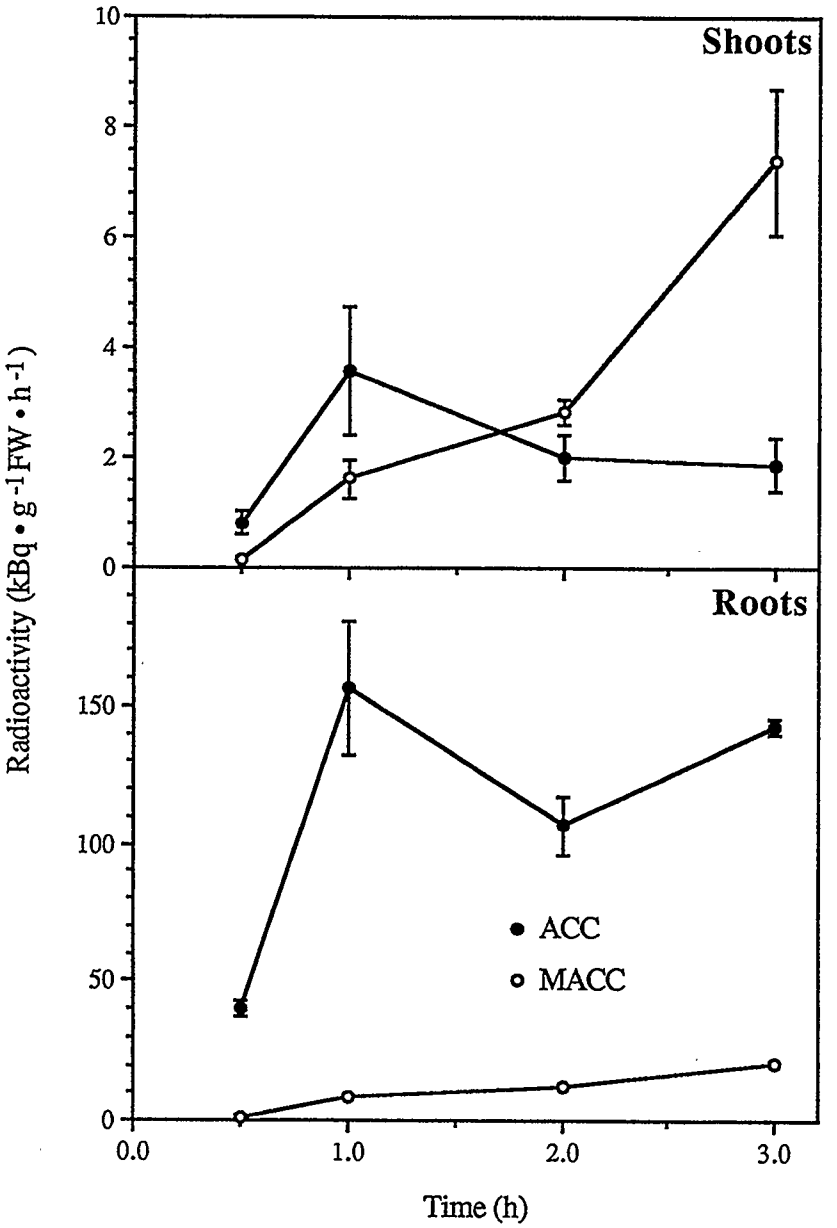


Figure 16. Radioactivity in ACC and MACC fractions from shoots and roots of 4 day-old sunflower seedlings after root treatment with 10 μM [2,3-^{14}C]ACC, measured during 3 hours. n = 4, mean ± standard error.

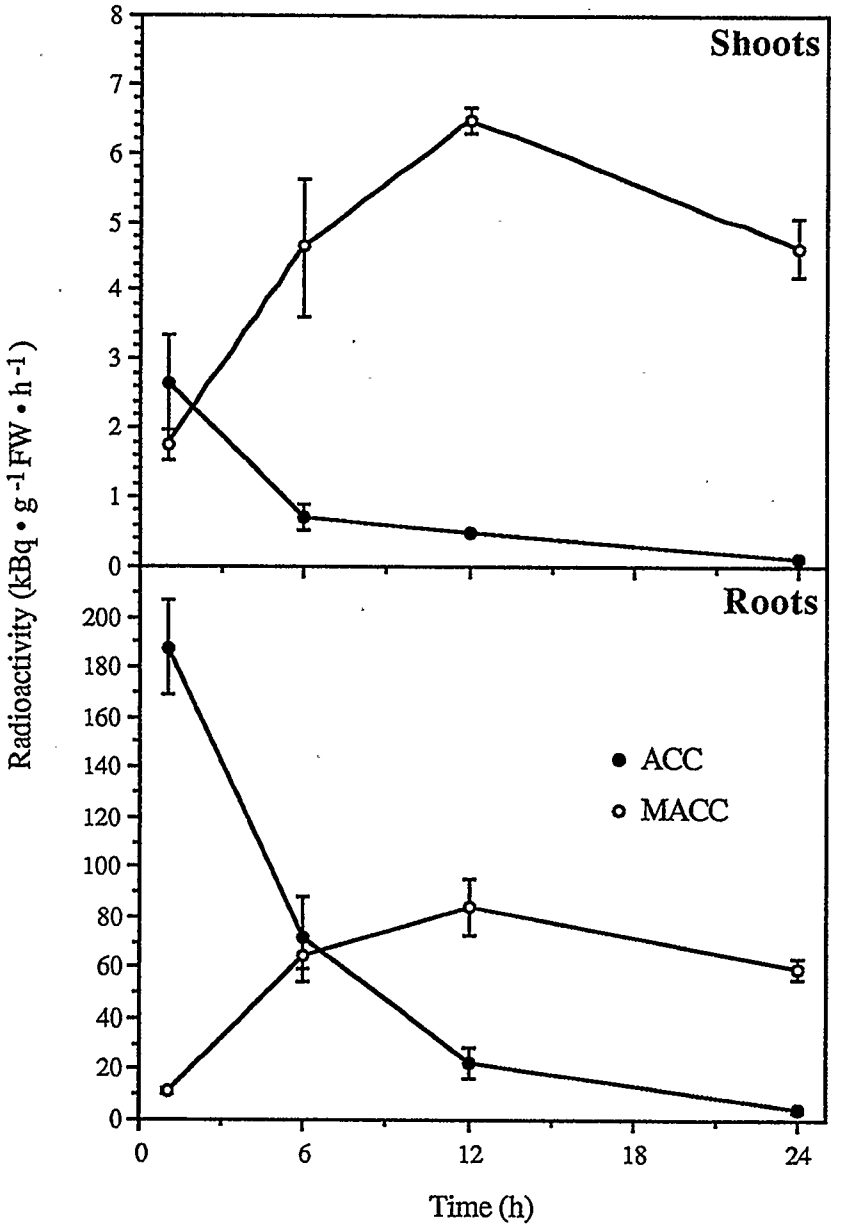


Figure 17. Radioactivity in ACC and MACC fractions from shoots and roots of 4 day-old sunflower seedlings after root treatment with 10 µM [2,3-^{14}C]ACC, measured during 24 hours. n = 4, mean ± standard error.

and declined until 24 h. Radioactivity in the MACC fraction increased from low values at 1 h to a peak at 12 h and decreased slightly from 12 to 24 h.

Samples of ACC and MACC fractions from organs harvested at 1 and 12 h were subjected to TLC and autoradiography to determine the number and identity of labelled compounds present in each fraction. The two major radioactive zones co-chromatographed with either standard [^{14}C]ACC or [^{14}C]MACC, and with only one exception, we found that the results were in full agreement with the studies shown in figures 16 and 17. The exception was that there was a strongly labelled compound on cellulose TLC of the 1-h shoot ACC fraction that did not co-chromatograph with [^{14}C]ACC or [^{14}C]MACC. In all autoradiographs, other compounds were detected in minor amounts, some of which co-chromatographed with minor impurities in the [^{14}C]ACC and [^{14}C]MACC standards.

ACC and MACC Leakage from Roots.

Leakage of radiolabelled compounds into 15 mL of one-quarter strength Hoagland solution was high at 1 h (194±53 Bq g^{-1} fresh weight h^{-1}), low at 6 h (40±15 Bq g^{-1} fresh weight h^{-1}), and stable from 12 to 24 h (93±15 and 95±36 Bq g^{-1} fresh weight h^{-1}, respectively). Fractionation on cation exchange resin of a sample collected 2 h after the start of treatment showed 45% of the radioactivity in the ACC fraction and 55% in the MACC fraction.

Analysis of xylem sap.

Xylem sap collected from seedlings treated with [^{14}C]ACC had large amounts of radioactivity in both the ACC and MACC fractions. Xylem sap collections during a 2- to 6-h period after feeding showed 1917 Bq mL^{-1} in the ACC fraction and 527 Bq mL^{-1}

in the MACC fraction. TLC and autoradiographic analysis of this xylem exudate showed six compounds. The two major components co-chromatographed with ACC and MACC standards; 64% of the radioactivity was associated with [^{14}C]ACC and 23% with [^{14}C]MACC. GC-MS analysis positively identified the presence of ACC in xylem sap from [^{14}C]ACC treated plants.

Xylem exudate obtained from untreated 11-d-old sunflower seedlings was found to contain ACC as shown by GC-MS (fig. 18). MACC was not found in the xylem sap collected from these seedlings, nor was ACC detected in the MACC fraction after acid hydrolysis of this fraction.

Discussion

Our experiments show that young sunflower seedlings readily take up ACC from a nutrient medium supplied to roots. In other ongoing studies, we find that the concentration of ACC fed to 4-d-old sunflower plants in these experiments (10 μM) is sufficient to cause a 65% inhibition of root elongation (see chapter 2). Other characteristic ethylene effects such as epinasty and hypertrophy of shoots are not seen. The levels of ACC used in these experiments, therefore, represent physiological amounts and not pharmacological quantities.

It is well established that ACC is the direct ethylene precursor (Adams and Yang, 1979). We find substantial amounts of ethylene in roots 0.5 h after commencement of treatment with 10 μM [^{14}C]ACC, and ethylene evolution peaks at 1 h (fig. 13). After the peak of ethylene synthesis, ethylene production decreases in concert with declining ACC levels (figures 13, 16, and 17) and increased ACC conjugation to MACC (figures 16 and 17). This work agrees with previous reports (Chen and Kao, 1990; Hoffman *et al.*, 1983b) in that the plants show a large capacity to malonylate ACC within a relatively

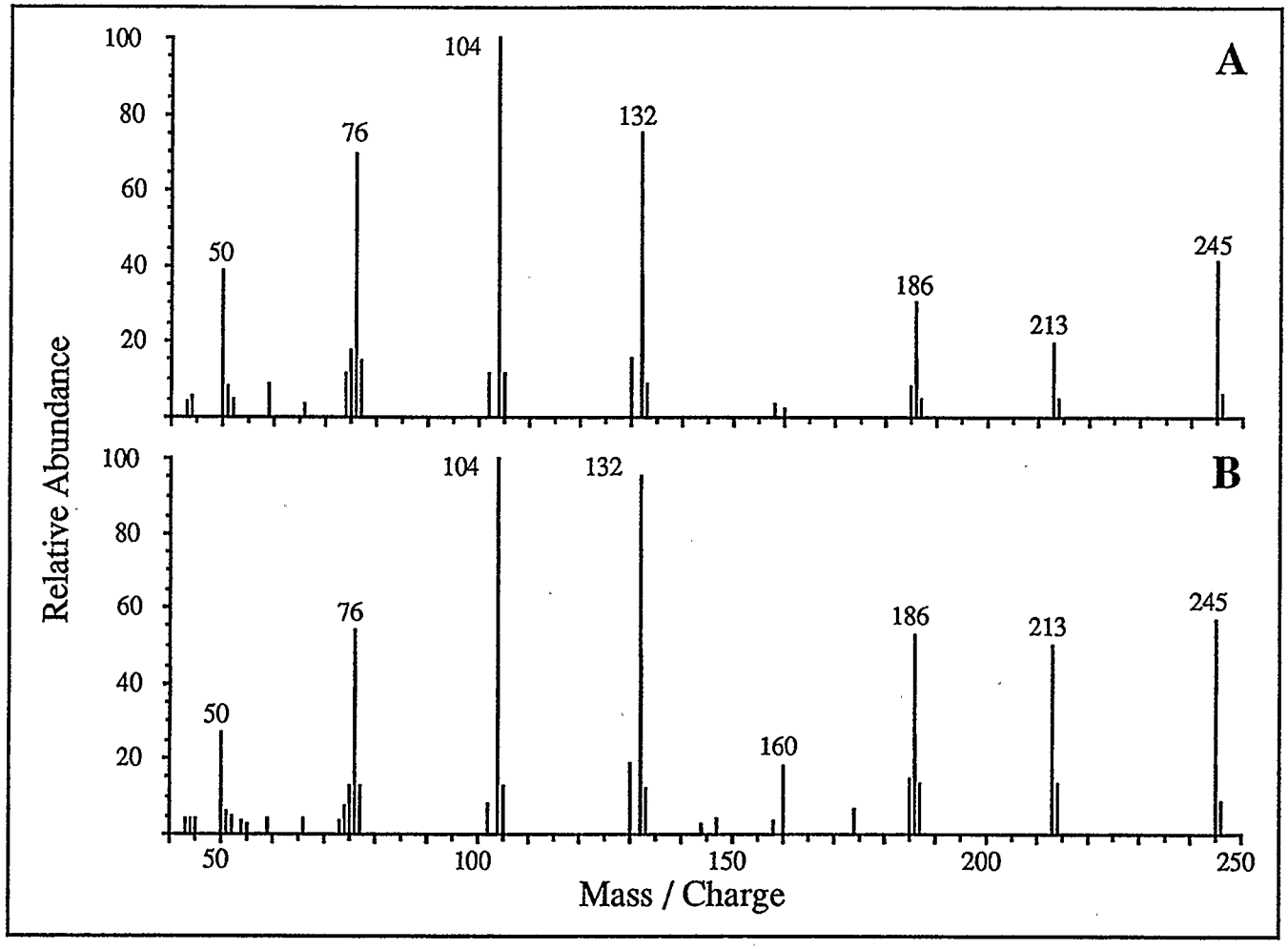


Figure 18. Mass spectra of (A) standard phthalidimo-ACC-methyl ester and (B) phthalidimo-ACC-methyl ester from ACC isolated from the xylem sap of 11 day-old sunflower seedlings.

short time (figures 16 and 17). Conjugation of ACC has previously been shown to reduce ethylene synthesis (Philosoph-Hadas *et al.*, 1985). Ethylene and MACC synthesis in the root, and transport of these compounds out of the root, reduced root ACC to low levels 12 h after treatment (figures 14 and 17).

Transport of ACC to the shoot occurs rapidly after feeding (fig. 16); however, ACC levels in the shoot remain lower than those found in the root (figures 16 and 17). Shoot ACC levels are consistently lower than shoot MACC levels (figures 16 and 17). This suggests that either ACC is metabolized immediately into ethylene and MACC upon arrival in the shoot or MACC itself is being exported from the root. TLC and autoradiographic analysis of the xylem sap of treated seedlings showed the presence of both [^{14}C]ACC and [^{14}C]MACC in the xylem sap. The presence of [^{14}C]ACC in the xylem exudate was confirmed by GC-MS analysis; however, [^{14}C]MACC content was not confirmed. We found that large quantities of MACC must be present in a sample before a clear mass spectrum can be obtained, because losses during precipitation of impurities and methylation are substantial. In untreated seedlings, MACC was not found in the xylem sap, nor was it found as ACC after acid hydrolysis of the MACC fraction. Because GC-MS analysis of ACC in plant samples is fairly sensitive (McGaw *et al.*, 1985), we must conclude that MACC is not a component of xylem sap under the growing conditions used in these experiments. However, when roots are supplied with ACC, MACC is exported in the xylem. It remains to be seen whether MACC is in fact transported in the xylem under conditions which increase root ACC production (*i.e.* drought, flooding, wounding). That MACC can be exported across the plasmalemma (Bouzayen *et al.*, 1988), is exported from a site of wounding (Fuhrer and Fuhrer-Fries, 1985), and is found by us in the xylem of [^{14}C]ACC-treated seedlings as [^{14}C]MACC support the speculation that MACC may be a natural component of xylem sap under

some conditions. MACC synthesis, long-term vacuolar MACC storage (Bouzayen *et al.*, 1988; Hoffman *et al.*, 1983b), and dilution of ACC and MACC via transport throughout the whole plant may represent a complex system of regulation of ethylene levels in plant tissues.

MACC levels decrease from 12 to 24 h in both roots and shoots (fig. 17). Possibly some of the MACC is being deconjugated to form ACC (Jiao *et al.*, 1986), although the possibility that MACC is being metabolized via an alternate route cannot be ruled out (Satoh and Esashi, 1984a). MACC has been shown to be metabolized to ACC and ethylene in other systems (Hanley *et al.*, 1989; Jiao *et al.*, 1986); however, the extent of this metabolism in sunflower seedlings has not been determined. In the roots, the leakage of [^{14}C]MACC contributed to the decreasing levels of [^{14}C]MACC found in this organ. The amount of leakage of [^{14}C]ACC and [^{14}C]MACC was substantial. If the leakage was due to residual [^{14}C]ACC in the root apoplasm, it would be expected that washing and time spent in the aeroponic mist would deplete the apoplasm of [^{14}C]ACC. Since large amounts of radioactivity are being released from the root over a long time period (24 h) and a substantial portion can be attributed to MACC (55% 1 h after treatment), this leakage is considered to arise from a symplastic source. Leakage of other compounds from roots in hydroponic and aeroponic environments has been observed (Drakeford *et al.*, 1985; Jayasekera *et al.*, 1990). Because almost every soluble compound present in plants can be found in root exudates (Stolzy and Soijka, 1984), it is not unreasonable to expect ACC and MACC exudation as well.

Treatment of roots with [^{14}C]ACC was found to give rise to a second labeled gas in both roots and shoots. Our working hypothesis is that this compound was $^{14}CO_2$, because the second labeled volatile compound has properties similar to those expected of CO_2: it is absorbed in a cocktail designed to trap CO_2 and it is not absorbed

in mercuric perchlorate (and is therefore not an olefin [Abeles, 1973]). It is well established that the carboxylic acid group of ACC is released as CO_2 during ACC conversion to ethylene (Peiser *et al.*, 1984). However, the [^{14}C]ACC used in these experiments was labelled on carbon atoms 2 and 3 of the cyclopropane ring. The possibility exists that some of the [^{14}C]ACC was labeled in the carboxylic acid position; however, the release profiles of $^{14}C_2H_4$ and this second labeled substance differ substantially (fig. 15), indicating that different precursors are involved for these compounds. It is also possible that one or more of the impurities in the [^{14}C]ACC solution (98.5% radiochemical purity) may be precursors of this unidentified gas. However, the amount of this compound released exceeds the amount of impurities supplied to the plants. Although the existence of an as yet uncharacterized pathway of MACC degradation has been proposed (Satoh and Esashi, 1984a), it is unlikely that the gas is produced from [^{14}C]MACC, because the levels of [^{14}C]MACC increase in both roots and shoots and peak (figures 16 and 17) 6 h after the peak of release of the labeled volatile compound (fig. 15). $^{14}C_2H_4$ may be metabolized to $^{14}CO_2$ (Beyer, 1984); however the amount of labeled gas produced is in excess of the amount of $^{14}CO_2$ expected from the level of $^{14}C_2H_4$ evolved in these experiments (Beyer, 1984). These results suggest that an alternative metabolic pathway for ACC degradation exists. The intermediate steps in the pathway and the significance of production of this second gas from [^{14}C]ACC were not investigated further, because production and sale of [^{14}C]ACC ceased during the course of these experiments.

Transport of [^{14}C]ACC and [^{14}C]MACC from the root to the shoot occurs rapidly in [^{14}C]ACC-treated plants. ACC is a natural component in the xylem in whole, unstressed sunflower seedlings. This work substantiates and extends that of Bradford and Yang (1980b) and gives credence to the proposal that ACC is used within the whole

plant as a means of interorgan communication. MACC transport occurs under some conditions, although the function of MACC as a signal is uncertain. It was surprising to find that carbon(s) 2 and/or 3 of ACC were metabolized into a gas other than C_2H_4, possibly CO_2.

Chapter 4.

Localization of 1-Aminocyclopropane-1-carboxylic Acid Metabolism in Roots of Sunflower (*Helianthus annuus* L.) Seedlings.

Summary

The metabolism of 1-aminocyclopropane-1-carboxylic acid (ACC) in different parts of roots of aeroponically grown *Helianthus annuus* L. seedlings was followed. Ethylene production was highest in the lateral roots and tips of the main roots and lowest in the middle and basal (part nearest the hypocotyl) portions of the main root. ACC and *N*-malonyl-ACC were monitored by gas chromatography-selected ion monitoring-mass spectrometry. ACC levels in these parts mirrored the levels of ethylene production observed. Upon gently transferring seedlings from an aeroponic system to treatment tubes, ACC content transiently increased; the greatest increase occuring in tips and laterals. This increase in ACC was not correlated with an increase in ethylene production. *N*-malonyl-ACC levels reflected the ACC content of the tissues. ACC oxidase (also known as the ethylene forming enzyme [EFE]) activity was lowest in the tip and laterals and highest in the middle and base; the opposite of the trend in ethylene production. Treatment of the seedlings with ACC produced a rapid increase in ACC and ethylene production. The increase in ethylene was disproportionately large in the middle and base parts, suggesting a greater efficiency of these parts for the conversion of ACC to ethylene. ACC oxidase activity was not induced by ACC treatment, however under the assay conditions used all root parts had a greater *in vitro* potential for ethylene production than they exhibited *in vivo*. ACC oxidase activity, and its substrate ACC, may be spatially separated. ACC oxidase activity may not limit ethylene production in untreated roots.

Introduction

In chapter 3, the transport and metabolism of the immediate ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) was studied in roots and shoots of sunflower (*Helianthus annuus* L.) seedlings. Inter-organ transport of ACC occurred and it seems reasonable to suppose that such transport of this ethylene precursor is an useful method of inter-organ communication. We found that exogenous ACC was readily taken up by the roots and that the root and shoot metabolized ACC differently. The root appeared to be a major site of ACC metabolism. We are particularly interested in a more precise localization of synthesis of ACC, *N*-malonyl-ACC and ethylene in roots, as we are presently investigating the methods by which different portions of the roots respond to various environmental perturbations.

The root is a structure made up of morphologically and physiologically distinct parts. For instance, cell division in the primary root occurs mainly in apical regions, while most of the mineral and water absorption takes place in more distal tissues. Lateral root emergence occurs some distance away from the growing tip. The dissimilar growth and developmental responses of the different parts of the root are also reflected in the varying responses of these root parts to ethylene. For example, primary root elongation is inhibited by ethylene (Whalen and Feldman, 1988), while lateral elongation and root hair production can be increased (Crossett and Campbell, 1975). Drew *et al.* (1979) have demonstrated the ability of ethylene to induce aerenchyma in a tissue-specific manner in *Zea* roots, with more aerenchyma being formed near the base of roots than near the tip.

Just as the root is developmentally diverse, it is also diverse in the way it responds to environmental stimuli. Gravitational stimulus causes the primary root to grow downwards, yet causes the laterals to grow in a positively plagiogravitropic

manner. While the mechanism of gravitational perception and responses are not fully understood, ethylene might be involved (Lee *et al.*, 1990). Roots are also influenced by texture and strength of the soil, and again ethylene may be involved in thigmomorphogenesis (Sarquis *et al.*, 1992) and in substrate penetration by the root tip (Zacarias and Reid, 1992). The primary root tip has been of interest in studies of thigmostimulation, and although the role of thigmostimulation in the development of other root parts has been less investigated, the laterals should be able to perceive and respond to this stimulus.

Since the root is a heterogeneous organ and the different parts of the root may respond differently to these stimuli, and because ethylene has been implicated in the reaction to these and other stimuli, levels of ethylene, ACC and MACC may vary within different portions of the root.

Studies on the distribution of ethylene synthesis in shoots (Schierle *et al.*, 1989) and ACC metabolism in flowers (Nichols *et al.*, 1983) shows that these processes are not the same throughout all parts of these structures. In view of the heterogeneous ACC metabolism in other organs, ACC metabolism in roots may show a similar degree of variation. Indeed, Atwell *et al.* (1988) have shown that in nodal roots of *Zea mays*, ethylene production and ACC levels are high in the tip portion of the root and lower in distal tissues.

Ethylene metabolism can be controlled through the regulation of ACC production, via ACC synthase (Kende and Boller, 1981; Konze and Kwiatkowski, 1981), conjugation of the ACC to MACC (Hoffman *et al.*, 1982, 1983b; Liu *et al.*, 1985) and ACC conversion to ethylene by regulating ACC oxidase (also called EFE) activity. Although ACC oxidase activity is generally not believed to limit ethylene production (McKeon *et al.*, 1982), ACC oxidase activity has been shown to be inducible

(McKeon *et al.*, 1982; Ververidis and John, 1991) and cultured cells can show considerable variation in EFE activity (Pengelly and Su, 1991).

The purpose of this research was to determine if morphologically different parts of the root system varied in their levels of ACC, MACC and ethylene, and also to see if they differed in their capability to metabolize exogenous ACC.

Materials and methods

Plant Material.

Sunflower seeds were germinated aeroponically as given in chapter 2. At the four day stage, plants with stems 5 to 7 cm tall and roots 8 to 10 cm long were selected. The cotyledons had emerged from the testa but were not fully expanded, and the lateral roots were 3 to 7 mm long.

ACC Treatment.

The seedlings were treated by immersing roots of pairs of plants in 30 mL Corex® tubes containing 25 mL of 50 µM ACC (Sigma) in one-quarter strength Hoagland solution (as in chapter 2) which was aerated just prior to introducing the seedlings. The plants were treated in this solution for 1 h under a Sylvania Spot-Gro lamp producing 160 µmoles m^{-2} s^{-1} PAR. After feeding, the roots were briefly rinsed in one-quarter strength Hoagland solution and the plants were transferred to aeroponics chambers (Hubick *et al.*, 1982), or harvested directly from the feeding solution. As controls, an equal number of plants were immersed in pairs in 25 mL of the above nutrient solution without ACC, and were handled exactly as the ACC treated plants.

In all cases experiments were repeated at minimum of three times.

Tissue Ethylene Evolution.

At each harvest, three untreated and three ACC treated seedlings were cut at the root-shoot junction and the roots were further cut into three sections; (i) the actively dividing and elongating 20 mm root tip ("tip") in which we have found elongation to be greatly inhibited by ethylene (chapter 2), (ii) the 20 mm nearest the root-hypocotyl junction from which most lateral roots emerge ("base") and (iii) the remaining middle portion ("middle"). In some cases we also looked at the lateral roots ("laterals"). Ethylene evolution from these tissues was determined using a gas chromatograph equipped with a photoionization detector (as in chapter 2), using the sampling methods given in chapter 3. Tissues were incubated for ten min and the headspace gas was analysed for ethylene. The fresh weights of the tissues used for gas analysis were determined immediately after sampling. Measurements represented ethylene evolution from 1 root and were replicated three times.

ACC and MACC Extraction and Identification.

At each harvest, ten untreated and ten ACC-treated plants were cut at the root-shoot junction. The roots were partitioned as above, and each group of ten root parts was weighed separately, frozen in liquid N_2, and stored at -70°C for later extraction. The extraction of ACC and MACC used ion exchange and HPLC as described earlier in chapter 3. $^2H_4$-ACC and $^2H_4$-MACC were added as internal standards. MACC was converted to ACC and ACC levels were determined by GC-SIM-MS (chapter 3) using ions 244.7 and 248.7 for quantification. For data obtained for a single time (i.e. 6 h) measurements were replicated three times.

Extraction and Assay of EFE Activity.

The enzyme assay is based on the procedure of Ververidis and John (1991), with minor modifications. Root parts from about 30 untreated and 30 ACC treated plants were harvested as for tissue gas evolution samples, weighed and then frozen in liquid nitrogen and stored at -70°C for later extraction. The root parts were ground under nitrogen in 7.5 volumes of cold buffer containing 100 mM Tricine, BTP (1,3-bis[tris(Hydroxymethyl)-methylamino]propane) pH 7.4 with 10% glycerol (w/v), 5% polyvinylpolypyrrolidone (w/v), 1 mM DTT, 50 μM $FeSO_4$ and 5 mM sodium ascorbate (all Sigma Chem. Co.). The homogenate was centrifuged at 11,000g for ten min and the supernatant was retained. One mL of the above buffer, minus the polyvinylpolypyrrolidone and DTT but with 1 mM ACC, was placed in 10 mL pyrex tubes. These tubes were pre-incubated with air (Linde, Ultra Zero) which contained 5% CO_2 for 30 minutes at 30°C on a shaking water bath. This air had been scrubbed free of all detectable ethylene by passing it through a column filled with beads of Purafil (Circul-Aire Inc., Montreal, Quebec). The supernatant of the enzyme extract was spun again at 11,000g for five min. and 25 μL of extract was added to each tube, which was then incubated for 30 min at 30°C on the shaking water bath. A 1 mL headspace gas sample was then removed and analyzed for ethylene content as given above. Each assay was replicated four times, and controls of buffer and boiled enzyme extract were also run.

Results

Gas Evolution by the Root Parts.

Figure 19 shows the ethylene evolution, on a per g fresh weight basis, from various parts of the root. A small initial decrease in ethylene evolution was observed in

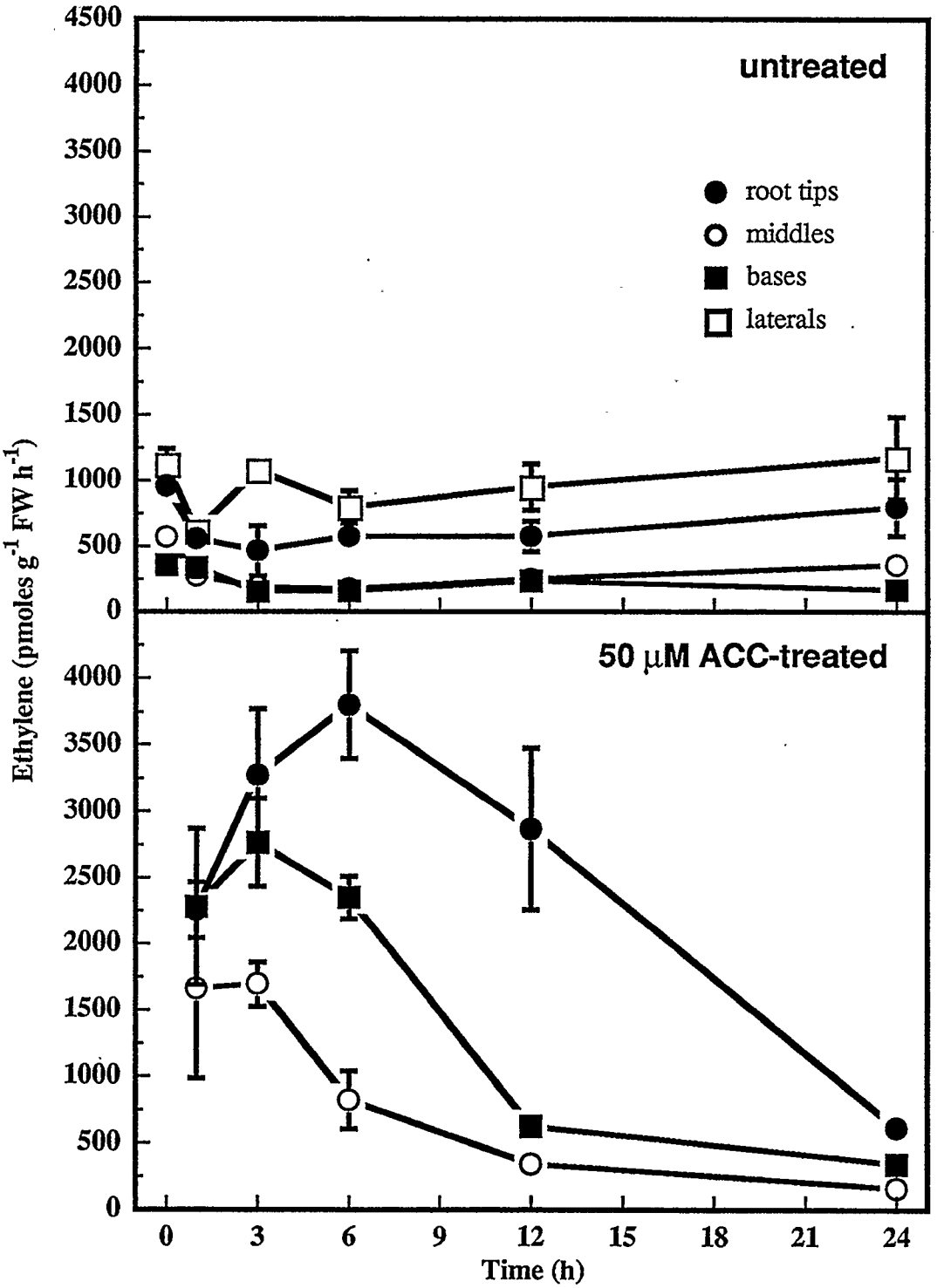


Figure. 19. Ethylene release from root parts of 4 day old sunflower seedlings. Measured over 24 hours by GC. n= 3, mean ± standard error.

all root parts for at least 1 h after transferring from the aeroponic germination system to treatment tubes. The middle and base portions evolved similar levels of ethylene. Root tips produced 2 to 3 times as much ethylene as the middle and base while the laterals evolved approximately 50% more than the tips.

We supplied ACC to roots for 1 h and then followed ethylene evolution over 24 h (fig. 19). As expected much more ethylene was produced from the ACC-treated plants than from tissues not supplied with the ethylene precursor. The largest amount of ethylene (on a fresh weight basis) was produced by the tips. The middle portions produced least and bases an intermediate amount. By 12 h ethylene evolution from the middle and base was back to the levels seen in non-ACC treated tissues, and by 24 h ethylene production from the root tip was near untreated values.

Figure 19 shows that in response to ACC treatment, the maximum ethylene evolution from the tip was approximately 6 times as high as non-ACC treated controls at 6 h, while the middle produced about 10 times at 1 to 3 h, and the base about 15 times control values at 3 h. At 6 h, laterals produced only about 1.5 times as much ethylene as controls, in response to ACC treatment (fig. 20).

While the amount of ethylene produced by the tip is very high on a fresh weight basis, the total amount of ethylene produced by the root tip is small compared to the other root parts when total ethylene evolution from the whole root is considered.

ACC and MACC Levels in Root Parts.

Figure 21 shows ACC levels in the various root parts of untreated seedlings after transfer from the aeroponic germination system to treatment tubes. In all root parts ACC levels increase greatly after transfer, reaching a maximum in 1 or 3 h and then decrease

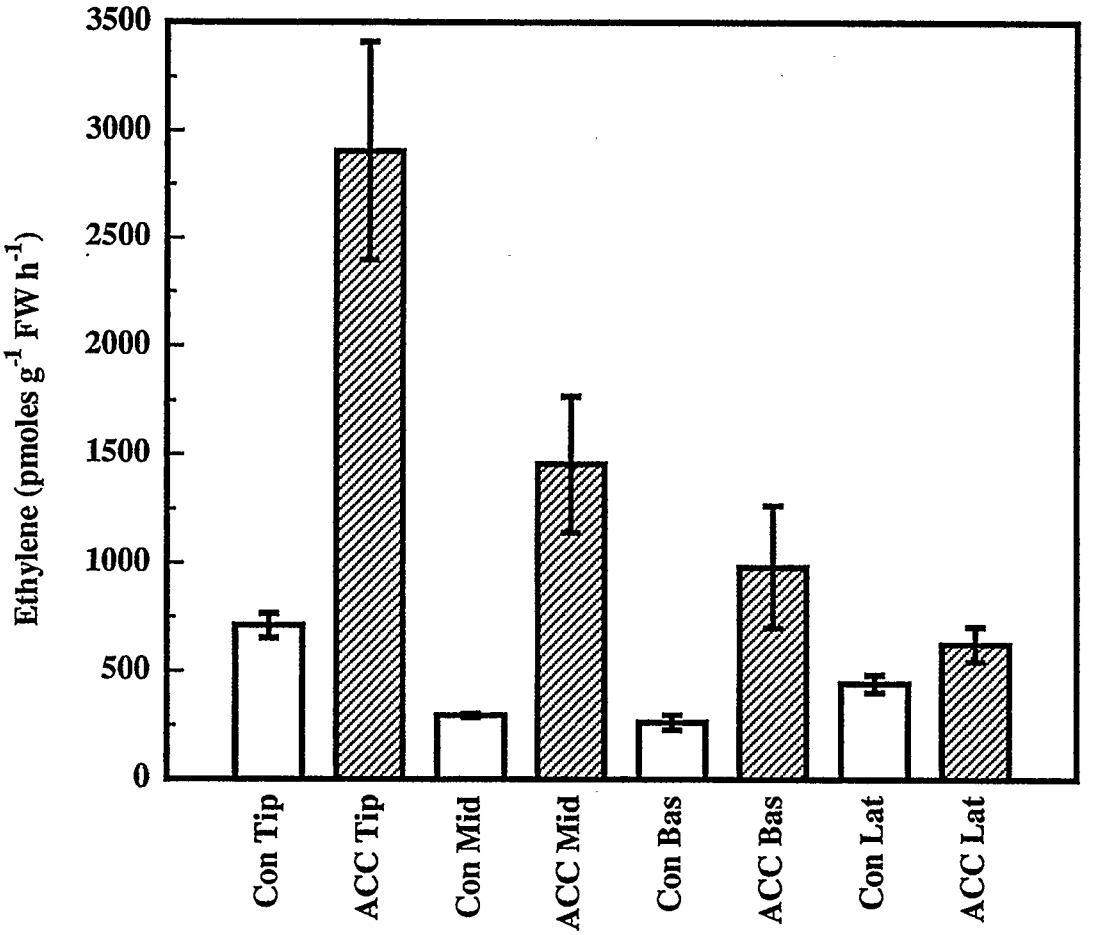


Figure 20. Ethylene release from root parts with and without 50 μM ACC treatment. Measured at 6 hours by GC. n= 3, mean ± standard error.

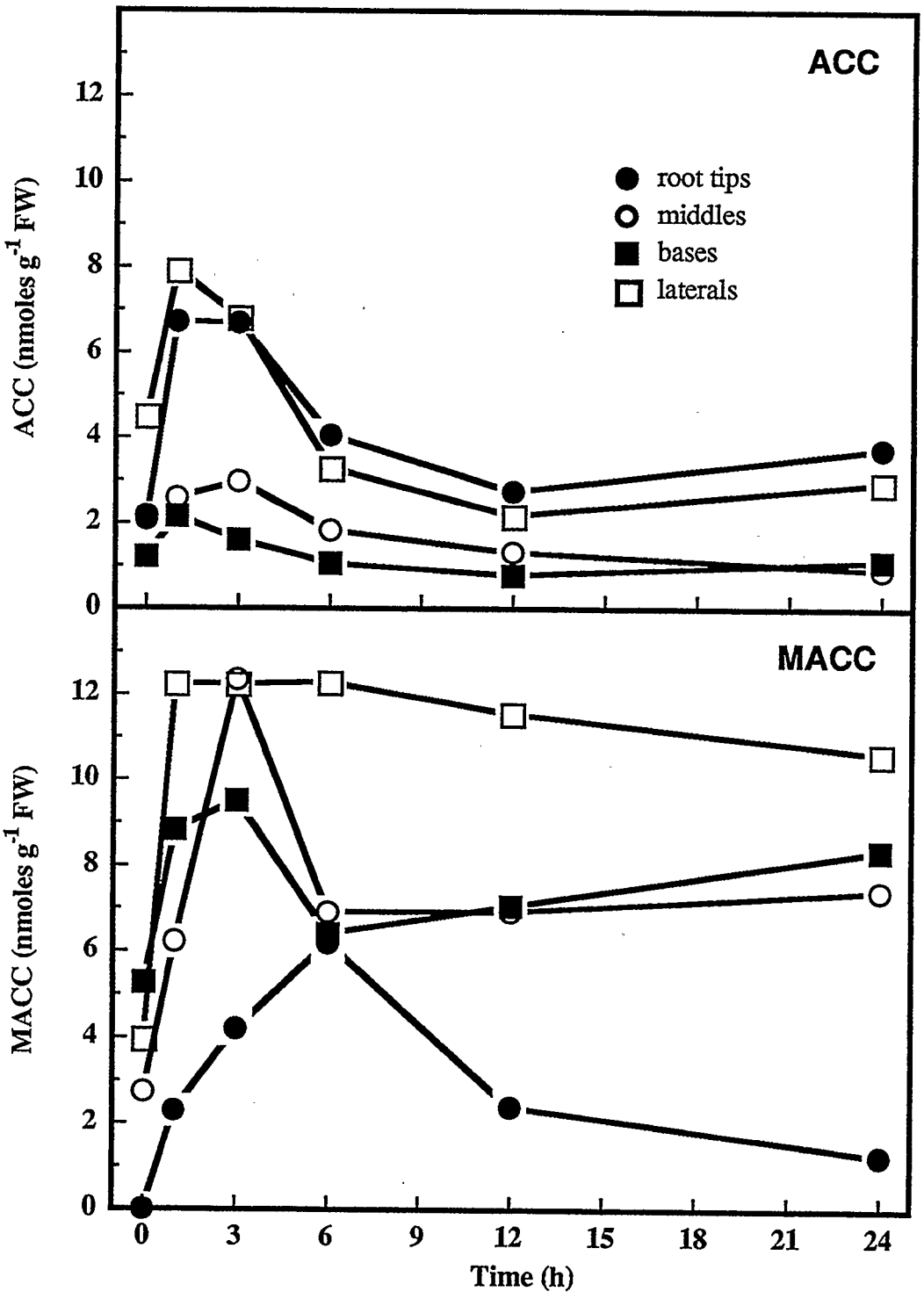


Figure 21. ACC and MACC levels in untreated root parts from 4 day old sunflower seedlings. Measured over 24 h by GC-SIM-MS.

to near initial values by 12 h. At 1 to 3 h the tip and lateral ACC levels are about 3 times as high as base and middle levels.

MACC levels in untreated roots are shown in figure 21. MACC levels in the root tips are the lowest of all tissues examined. In the tips MACC is virtually undetectable at 0 h, rises to a maximum at 6 h and decreases until 24 h. The quantity of MACC in the middle and basal parts of the roots follows a somewhat similar trend except that levels are higher, reach a maximum 3 h earlier and stabilize after 6 h. Laterals have the highest MACC levels which reach a maximum quickly by 1 h and show only a relatively small decrease by 24 h.

The ACC levels in root parts from seedlings treated with ACC (50 μM) for 1 h are shown in figure 22. The ACC level in the tip is, on a fresh weight basis, much higher than in the other root parts. In the 1 h sample the ACC concentration is already approximately 5 times the quantity found in the other tissues and it reaches a maximum by 3 h. There is then a rapid decline and at 24 h the levels are similar to those found in base and middle portions of the roots. The middle portion of the root shows the lowest ACC levels of the three parts examined and are highest by 1 h and steadily decrease over the course of the experiment. Base ACC levels are similar to those of the middle, however ACC content reaches a small maximum at 3 h.

MACC levels in ACC treated root parts are shown in figure 22. In the tip, MACC remains low from 1 to 3 h then rises steadily until 12 h by which time it is approximately 8 times higher than at 3 h. In both basal and middle root portions MACC concentrations also remain low until 3 h, however a maximum is reached at 6 h in these parts and the levels of MACC observed are lower than those seen in the tip. In all the tissues peak MACC levels persist until the end of the experiment.

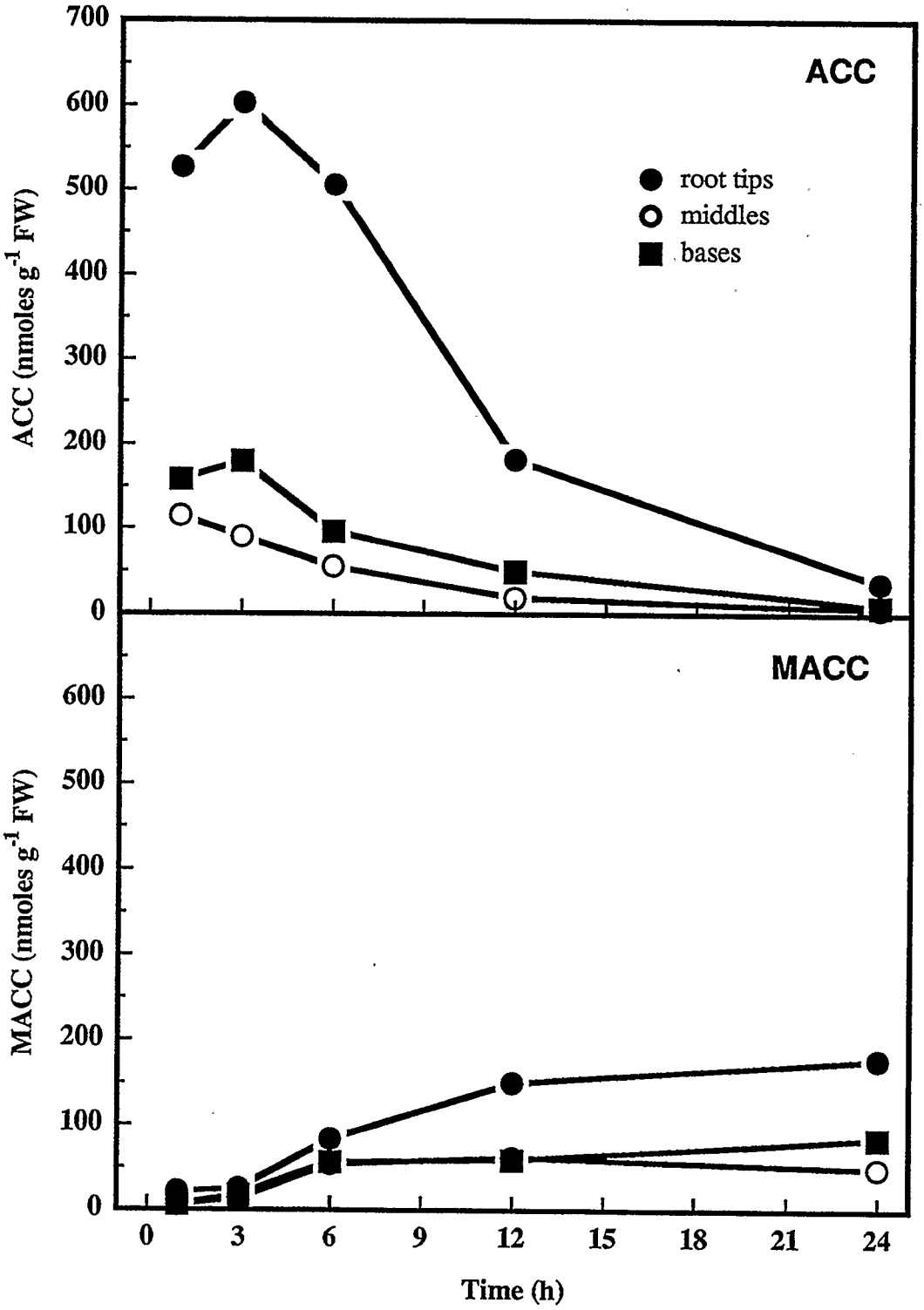


Figure 22. ACC and MACC levels in 50 µM ACC-treated root parts from 4 day old sunflower seedlings. Measured over 24 hours by GC-SIM-MS.

Figure 23 shows ACC and MACC levels in untreated and 50 μM ACC treated root tips, bases and laterals at 6 h after treatment. In all cases there is an increase in ACC and MACC with ACC treatment. Following ACC treatment, ACC levels are highest in the root tips and lowest in the bases. In the basal tissues, ACC and MACC levels are almost equal, while in the tips and laterals ACC levels are considerably higher than MACC levels.

EFE Activity in Root Parts.

The EFE activity in various root parts, with and without ACC treatment (50 μM), is shown in figure 24. High EFE activity was found in all the root parts, with greater activity in middle and base root parts than in tips or laterals. Thirty minutes of treatment with 50 μM ACC did not increase the enzyme's activity, nor was an increase in activity observed in assays performed on roots exposed for 1 or 3 h to ACC (data not shown). Although in this case a slight decrease in activity can be seen with ACC-treatment in some tissues, this effect was not consistent between replicates.

Discussion

ACC is the immediate biosynthetic ethylene precursor in higher plants (Adams and Yang, 1979). Our results show that the different parts of the root metabolize ACC differently. Ethylene evolution is low in the middle and base parts of the root. However the tip and laterals, which possess the most metabolically active cells with the greatest amount of cell division, show substantially higher ethylene production. These findings are in accord with those of Atwell *et al.* (1988) who also found the highest ethylene production from the apical regions of nodal roots of *Zea.* ACC oxidase activity in the root parts does not correlate well with the amount of ethylene evolved. In the

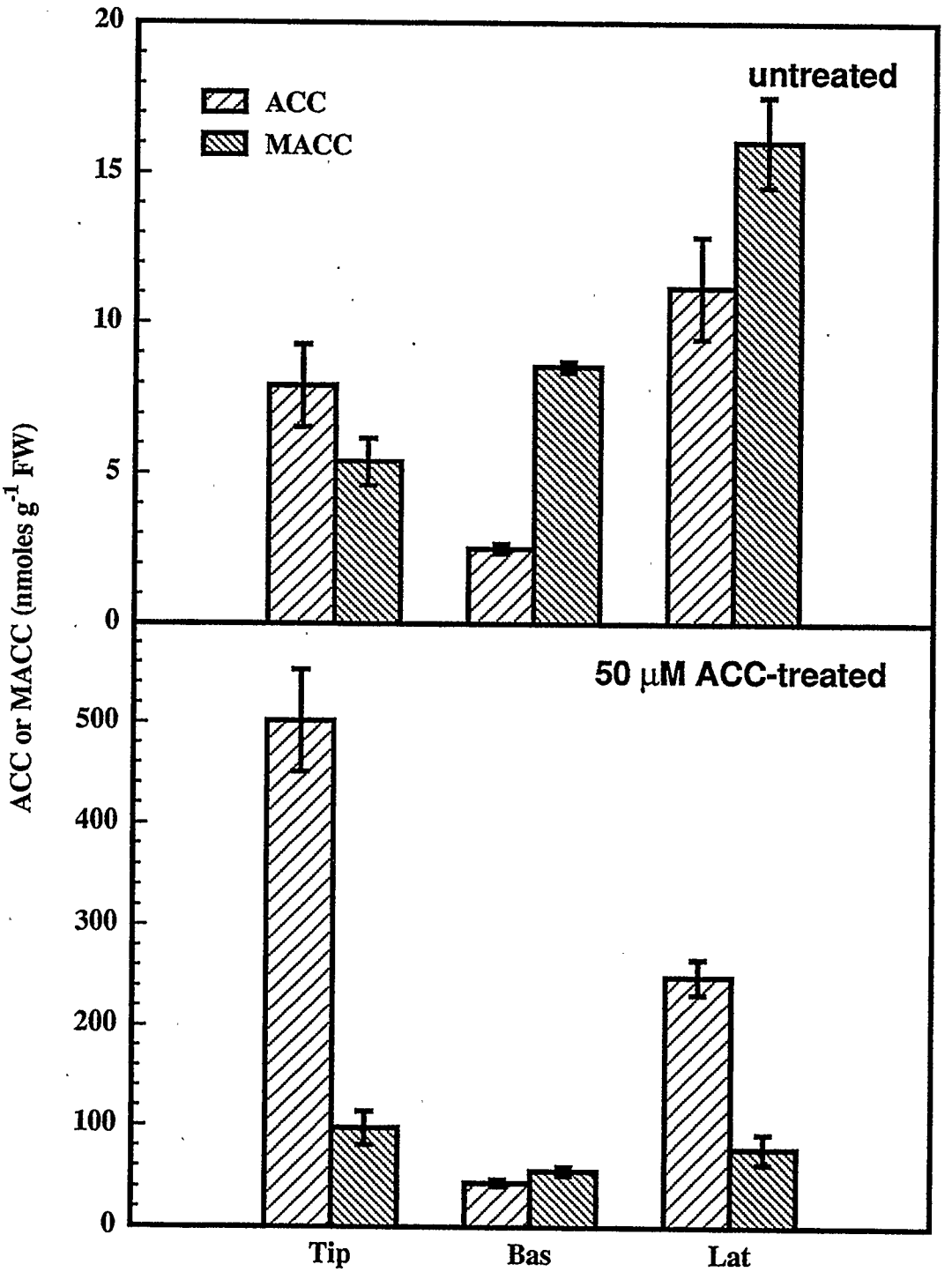


Figure 23. ACC and MACC levels in root parts from 4 day old sunflower seedlings. Measured at 6 hours by GC-SIM-MS. n= 3, mean ± standard error.

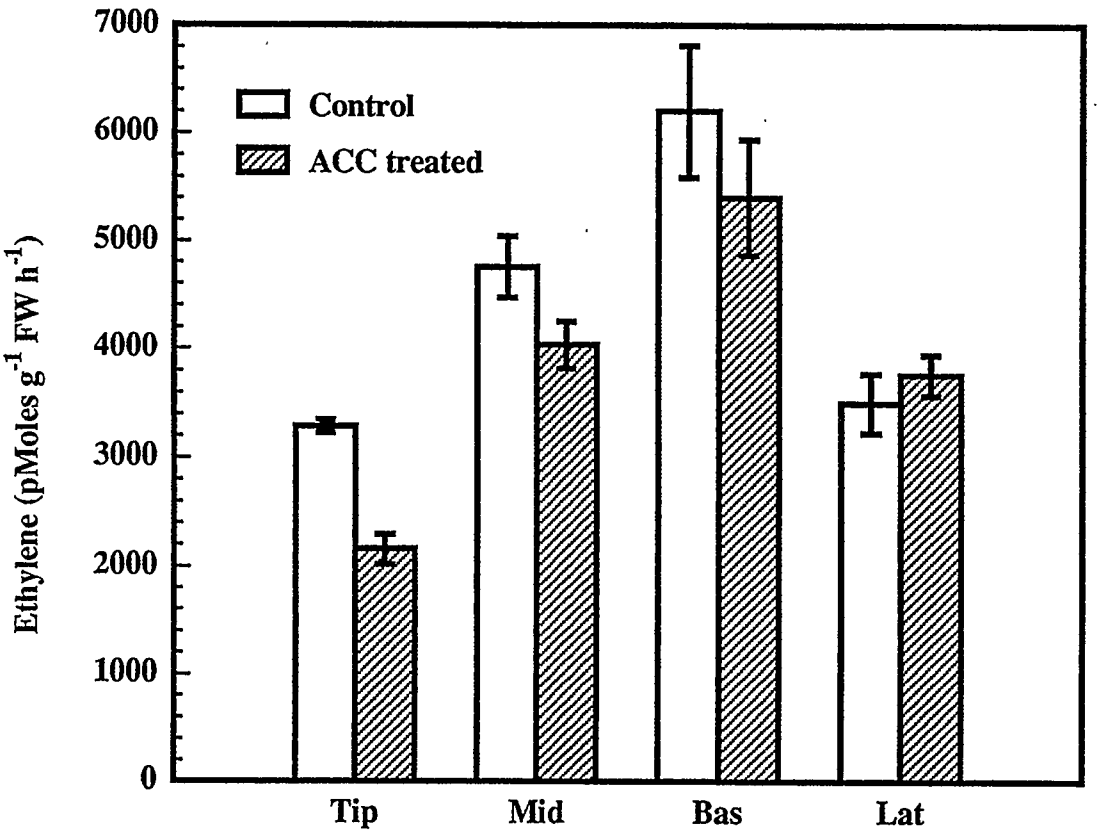


Figure 24. ACC oxidase activity in control and 50 μM ACC-treated root parts from 4 day old sunflower seedlings. Measured at 0.5 hours by GC. n= 4, mean ± standard error.

non-ACC treated tissues there is enough potential ACC oxidase activity (as measured *in vitro*) to produce from 3 to 30 times as much ethylene as is actually measured *in vivo*. The reasons for this are unclear, however it is possible that the stimulation of ACC oxidase activity by CO_2 (Dong *et al.*, 1992) may overestimate the actual activity *in vivo*, as intracellular CO_2 concentrations are not known. Alternatively, the ACC oxidase and ACC might be spatially separated in the cell or tissue. There is evidence that suggests that ACC is sequestered in the vacuole of tomato pericarp (Saftner and Baker, 1987) and also possibly in unspecified compartments in apple parenchyma (Cheverry *et al.*, 1988; Yip *et al.*, 1988). Saftner and Martin (1993) have recently demonstrated the carrier-mediated transport of ACC into isolated mesophyll vacuoles of maize. Conversely, other investigators have reported that while MACC appears to be compartmentalized in the vacuole of cereal mesophyll and protoplasts, ACC is not (Tophof *et al.*, 1989). If ACC oxidase is loosely bound to the plasma membrane (Ververidis and John, 1991), or freely cytosolic (McGarvey and Christoffersen, 1992) and ACC is compartmentalized, then the possibility for regulation of ethylene synthesis through intercellular transport or intracellular release of ACC to the ACC oxidase exists. We found that ACC oxidase activity was higher in the middle and base of the roots than in the tip and lateral portions, while ethylene evolution rates show the reverse trend. This evidence seems to imply that ACC oxidase activity is either being regulated by some means, or that ACC oxidase activity is indeed separated from the ACC.

ACC levels have previously been shown to be highest near the apex of nodal *Zea* roots, where ethylene evolution is also highest (Atwell *et al.*, 1988). ACC levels measured by GC-SIM-MS in this study are also commensurate with the ethylene levels evolved from control roots, with higher levels in the tip and laterals than in the base and middle. Time-course analysis of the ACC levels show a rapid increase in ACC in all

root parts immediately after transferring to treatment tubes. This increase may be due to the slight handling stress imposed on the plants during this procedure. Although the disturbance involved in moving the plants from the aeroponic germination system to the tubes is minimal, it may be sufficient to elevate ACC levels in the root. The exact nature of this stress is not known, however thigmostimulation and/or gravistimulation are candidates. Of note is the dramatic increase in ACC content in the laterals and tip. Although the base and middle ACC levels do increase, the rise in the tip and laterals far outstrips these, suggesting that main and lateral root tips might sense and respond to stimulation by producing ACC. Previous experiments on *Zea* have shown that mechanical impedance increases both ACC and ethylene levels in roots (Sarquis *et al.*, 1992). Though not studied in roots, gravistimulation of sunflower shoots will promote ethylene production (De Wit *et al.*, 1990) and presumably this stimulation in ethylene evolution follows an increase in ACC levels. Why the increase in ACC observed here does not manifest itself in elevated ethylene production remains unknown. In view of the fact that ACC oxidase is considered to be constitutive and non-regulatory (Liu *et al.*, 1985), the higher ACC levels after transfer should manifest themselves in an increased evolution of ethylene soon after handling. As there is an increase in ACC without a concomitant rise in ethylene, the possibility that ACC oxidase may be regulating ethylene biosynthesis must be considered. Alternatively, and as discussed above, it is also possible that there is a partitioning of ACC oxidase and ACC from each other, either by intracellular or intercellular means, thereby preventing an increase in ethylene production.

MACC has been shown to be a stable conjugate of ACC (Hoffman *et al.*, 1982, 1983b) formed by the malonylation of ACC by the enzyme ACC malonyltransferase (Kionka and Amrhein, 1984). Commensurate with the pulse of ACC following

transfer to the treatment tubes is a rise in MACC content in all parts of the root, generally after a small lag, reflecting the metabolism of ACC to MACC. MACC levels in the base and middle are similar, and attain a level much higher than those of ACC. Additionally, in the untreated seedlings these MACC levels persist at a high level after the initial peak subsides. Presumably the persistence reflects the slow metabolism of MACC as has been previously reported (Jiao *et al.*, 1986). The decline in MACC levels from peak values suggests the possibility that MACC is being metabolized (Jiao *et al.*, 1986), or transported from these areas (Fuhrer and Fuhrer-Fries, 1985). In the lateral roots MACC levels reach a maximum within 1 h after transfer, illustrating the very rapid metabolism of ACC to the conjugate, however unlike the base and middle portions in which MACC levels peak and then drop to a moderate level, MACC levels in the laterals remain at or near peak levels for the duration of the experiment. MACC is not detectable in the tip before transfer suggesting that MACC is not a significant component of the tip's biochemistry in an unstressed situation, even though the tip naturally contains a substantial amount of ACC. Soon after transfer however, MACC levels in the tip rise, peaking 6 h after transfer. MACC levels in the tip are the lowest of all the parts measured, and unlike the case in the other parts, MACC levels in the tip decline substantially over the course of the experiment, approaching a very low level. As the tip is an actively growing region, it seems likely that the portion of the tip containing the MACC subsequently becomes part of the middle portion of the root, as the tip region elongates and grows away from the MACC.

In an effort to understand how the root growth might be affected by a stress that caused the production of large amounts of ethylene, exogenous ACC was supplied to the roots. The level of ACC supplied (50 μM) was able to produce an 87% inhibition of root elongation while other effects such as hypertrophy were not evident (chapter 2).

The roots readily and rapidly took up the ACC as reported earlier (chapter 3). Root tips took up the most on a fresh weight basis, and by 1 h after feeding, ACC levels were very high but did not peak until the 3 h mark. The lag in the peak of ACC in the tip was surprising and not easily explainable. Levels of ACC in the root base also peaked at the 3 h mark, however root middle levels were highest at the 1 h mark as expected. It is possible that the ACC treatment is inducing endogenous ACC synthesis in the root, or that ACC is being transported from the shoot to the root, however at this time the reason for the latent increase remains unknown. In the root tip ACC concentrations remain higher for a longer period of time than the other root parts. Unlike the endogenous system in which we expect the tip to constantly grow away from any biochemicals produced, root elongation in ACC-treated plants is inhibited by the high levels of ethylene evolved (Whalen and Feldman, 1988, chapter 2). It is possible that the inhibition of elongation prevents the tip from growing away from the ACC taken up, and therefore ACC levels remain high until metabolized to MACC or ethylene. MACC levels increase from 3 to 12 h, after a slight lag, perhaps indicating time required for synthesis of the ACC malonyl transferase enzyme.

Ethylene production from ACC treated root parts reflects ACC content in these parts in the profile of evolution, however the relative levels of ethylene released do not correspond to ACC content. ACC content in the root tip peaks 4 to 6 times higher than the levels in the middle and base, yet the tip ethylene production peaks only 1.5 to 2 times higher. Analysis of ACC oxidase activity in the root parts showed that while treatment with ACC did not induce the enzyme, the levels of ACC oxidase were higher in the middle and base of the root than in the tip or laterals. The higher ACC oxidase activity in the middle and base may confer greater efficiency in converting ACC to

ethylene, which could explain the disproportionately greater amount of ethylene evolved by these tissues.

ACC oxidase activity is not increased by ACC treatment, however ethylene evolution increases dramatically in response to exogenous ACC. Thus, it seems as if ACC oxidase activity is not a factor that limits ethylene production in untreated roots. Presumably it is the concentration of ACC, perhaps regulated by ACC synthase or through compartmentalization of ACC, that regulates ethylene production in these tissues.

Lateral roots, and root tips that are not supplied with exogenous ACC, possess similar levels of endogenous ACC. Accumulation of ACC in lateral roots of seedlings fed with ACC was only about 50% of the accumulation of ACC in the tip, suggesting that the laterals do not take up ACC as readily as the tip. Additionally, ethylene evolution from ACC-treated laterals was only about 25% of that seen in ACC-treated tips. Apparently the laterals are not as effective in converting ACC to ethylene as are the root tips.

The data supports the hypothesis that morphologically and physiologically different parts of the root differ in ethylene physiology. Even the most gentle and careful handling of the roots may result in an increase of endogenous ACC levels. Additionally, discrepancies between ACC levels within the root parts and ethylene evolution suggest that ACC oxidase activity, or more probably the accessibility of ACC to ACC oxidase, regulates ethylene production by these tissues.

Chapter 5.

The Influence of CO_2 on ACC Oxidase Activity from Roots of Sunflower (*Helianthus annuus*) Seedlings.§

Summary

The activity of the ethylene-forming enzyme ACC oxidase was assayed *in vitro* under aerobic conditions with and without high levels of exogenous CO_2, varying the parameters of reduced iron, ascorbate, pH, temperature, ACC and O_2. It was found that CO_2 had no effect on the optimal temperature of the enzyme. High CO_2 decreased the optimal reduced iron concentration, increased the optimal ascorbate level, and radically increased the V_{max} of the enzyme. Five percent CO_2 doubled the K_m for ACC and decreased the K_m for O_2 slightly. The pH optimum of the enzyme was shifted lower with high CO_2. The possibility that CO_2 may have an influence on ethylene biosynthesis by roots *in vivo* is discussed.

Introduction

Ethylene is a plant growth regulator that has been shown to regulate root growth, among many other effects. The biosynthetic pathway leading to ethylene production in higher plants has been elucidated, as have many of the control points (Adams and Yang, 1979). Ethylene metabolism can possibly be controlled through the regulation of 1-aminocyclopropane-1-carboxylic acid (ACC) production, via ACC synthase (Kende and Boller, 1981; Konze and Kwiatkowski, 1981), conjugation of the ACC to *N*-malonyl-ACC (Hoffman *et al.*, 1982, 1983b; Liu *et al.*, 1985) and ACC conversion to ethylene by regulating ACC oxidase activity. Recently an assay was developed for the last

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§ This chapter has been accepted for publication under the same title with Dr. David M. Reid as co-author in Phytochemistry.

enzymatic step in the pathway, the oxidation of ACC to ethylene by the ethylene forming enzyme ACC oxidase (Ververidis and John, 1991). ACC oxidase activity has been shown to be inducible in fruits (McKeon *et al.*, 1982; Ververidis and John, 1991), however is generally not believed to limit ethylene production (McKeon *et al.*, 1982).

ACC oxidase was shown to have properties similar to those of many 2-oxoglutarate dependent dioxygenases in its requirement for ascorbate and Fe^{2+} (Britsch and Grisebach, 1986; Smith *et al.*, 1990; Ververidis and John, 1991). Unlike the 2-oxoglutarate dioxygenases however, ACC oxidase does not require 2-oxoglutarate for activity, nor is activity enhanced by 2-oxoglutarate (Smith *et al.*, 1992). ACC oxidase also shows a stimulation of activity with CO_2 (Dong *et al.*, 1992; McGarvey and Christoffersen, 1992; Poneleit and Dilley, 1993; Smith and John, 1993), which has not been shown in the other dioxygenases. While CO_2 has been shown to stimulate ACC oxidase activity, the mechanism of its action has not been elucidated, although it has been suggested that CO_2 itself, rather than HCO_3^-, is the active species (Smith and John, 1993; Poneleit and Dilley, 1993). Recently it has been shown that CO_2 (as $NaHCO_3$) may affect ACC oxidase affinity for the substrates ACC and O_2 in extracts obtained from melon fruits (Smith and John, 1993). CO_2 has also been shown to increase the K_m for ascorbate and ACC in apple fruit preparations (Poneleit and Dilley, 1993).

The purpose of this chapter is to examine ACC oxidase from roots of sunflower. Treatments eliciting ethylene production, including IAA and wounding, are examined for their effects on extractable ACC oxidase activity. Additionally, the effect of stimulation of ACC oxidase activity by CO_2 *in vitro* on other parameters of ACC oxidase activity are examined.

Materials and Methods

Plant Material. Sunflower seeds were germinated aeroponically as given in chapter 2. At the four day stage, plants with stems 5 to 7 cm tall and roots 8 to 10 cm long were selected. The cotyledons had emerged from the testa but were not fully expanded, and the lateral roots were 3 to 7 mm long.

Treatment with Hormones or Wounding. The seedlings were treated by immersing roots of pairs of plants in 30 mL Corex® tubes containing 25 mL of 100 μM IAA or ABA (Sigma) in one-quarter strength Hoagland solution (as in chapter 2) which was aerated just prior to introducing the seedlings. The plants were treated in this solution for 1 h under a Sylvania Spot-Gro light producing 160 μMoles m^{-2} s^{-1} PAR. After feeding, the roots were briefly rinsed in distilled water and the plants were transferred to aeroponics chambers (Hubick *et al.*, 1982). As controls, an equal number of plants were immersed in pairs in 25 mL of the above nutrient solution without growth regulators, and were handled exactly as the other plants. At either 2 or 3 hours after beginning treatments, the plants were harvested for tissue samples for ACC oxidase analysis and for measurement of ethylene.

Alternatively, seedlings were wounded by placing the plants on moist paper towels and carefully rolling a weighted cylinder over the roots. The seedlings were then transferred to aeroponics chambers and tissue samples and ethylene measurements were made 2 hours later. An equal number of non-wounded control plants were also used.

In all cases experiments were repeated at least three times.

Tissue Ethylene Evolution. At each harvest 4 or 5 seedlings from each treatment were cut at the root-shoot junction and the roots were incubated in 10 mL test

tubes capped with rubber septa for 10 minutes. Ethylene evolution from these tissues was determined using a gas chromatograph equipped with a photoionization detector, using the methods given in chapter 2.

Extraction and Assay of ACC Oxidase Activity. The enzyme extraction and assay is based on the procedure of Ververidis and John (1991), with minor modifications as given in chapter 4. Sodium ascorbate and $FeSO_4$ were omitted from the extraction buffer. Each assay was replicated 3 or 4 times, and controls of buffer and boiled enzyme extract were also run. For assays without exogenous CO_2 100 µL of enzyme extract was used, and the parameters of ACC, O_2, $FeSO_4$, sodium ascorbate, pH and temperature were varied individually, keeping all other parameters constant. O_2 levels were manipulated by degassing the assay buffer, and filling the assay tubes in an oxygen free environment, under nitrogen. Different levels of oxygen (Linde) were then added back by injecting the pure gas through the septa, and the tubes were allowed to equilibrate for 30 min at 30°C on a shaking water bath.

To determine the response of the enzyme to CO_2, the assay tubes were pre-incubated with varying concentrations (of headspace volume) of Purafil scrubbed CO_2 (Linde) for 30 minutes at 30°C on a shaking water bath (Purafil removes all detectable ethylene). One set of tubes contained a well with 200 µL of 6 M KOH (Fisher) to remove CO_2 from the atmosphere.

Assays of the effect of CO_2 on parameters of ACC oxidase activity were performed with 5% CO_2 pre-incubated in the headspace of the tubes as in chapter 4. Only 25 µL of enzyme extract was used for these assays, and 10 µM $FeSO_4$ and 20 mM sodium ascorbate were used. Again the variables of ACC, $FeSO_4$, sodium ascorbate,

pH and temperature were varied individually, keeping all other parameters constant. For the assays varying ACC and O_2 concentration, a BTP-HCl buffer, pH 6.6 was used.

Assays of ACC oxidase activity from roots treated with growth regulators or wounding were performed as above, with 5% CO_2 and 25 µL of enzyme extract, using BTP-HCl pH 6.6.

Determination of oxygen concentration. Oxygen levels in the buffer of 0% added O_2 assay tubes were measured using a Clark-type oxygen electrode (Hansatech CB1; Hansatech Ltd., Kings Lynn, UK). Measurements were made before and after the assay and averaged, and compared to standards of saturated and oxygen depleted water.

Results and Discussion

Although the reaction rate for ACC oxidase is non-linear over longer time spans (Smith *et al.*, 1992; Smith and John, 1993), the reaction rate observed for sunflower root ACC oxidase was linear over the 30 min incubation period used (fig. 25). From figure 26 it can be seen that even small amounts of exogenous CO_2 increases ACC oxidase activity *in vitro*. ACC oxidase activity was stimulated approximately 8 times by 5% CO_2 in the headspace. The results presented here corroborate other studies which show that CO_2 dramatically increases ACC oxidase activity *in vitro*. (Dong *et al.*, 1992; McGarvey and Christoffersen, 1992; Poneleit and Dilley, 1993; Smith and John, 1993). While our results are similar, it became apparent during the course of the experiments that as the CO_2 level was increased, the optimal levels of ascorbate and Fe were shifted. When new optimal ascorbate and Fe concentrations were used, this in turn increased the saturation point for CO_2 stimulation of ACC oxidase activity. The graph presented in

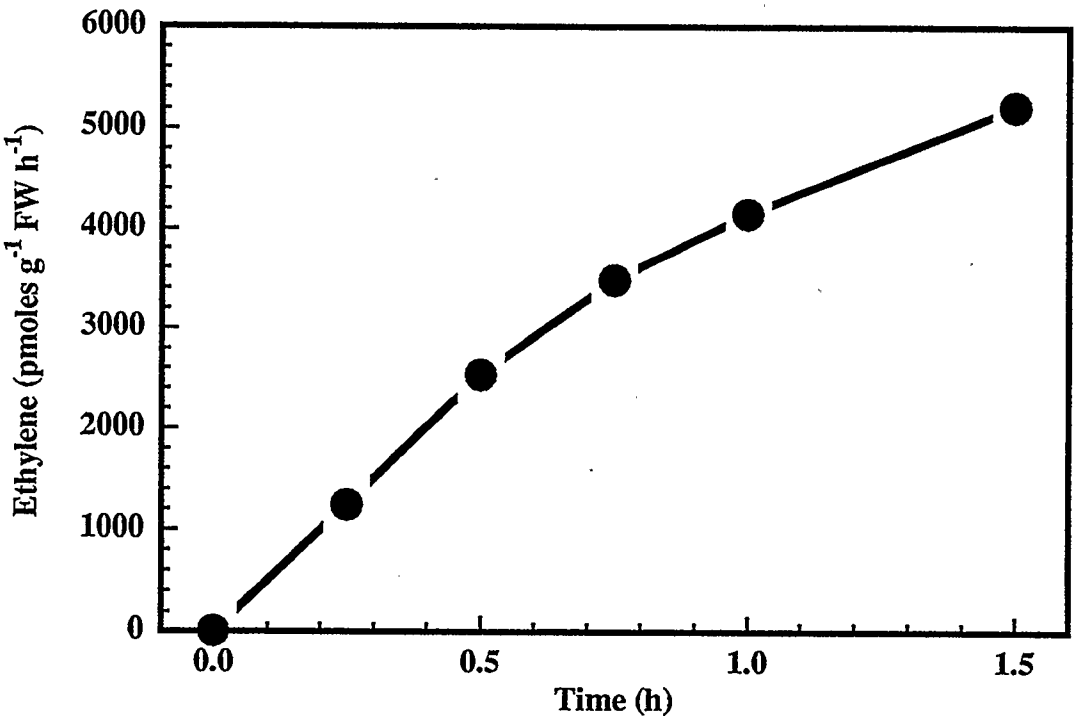

Figure 25. ACC oxidase activity with time. n= 5, mean ± standard error.

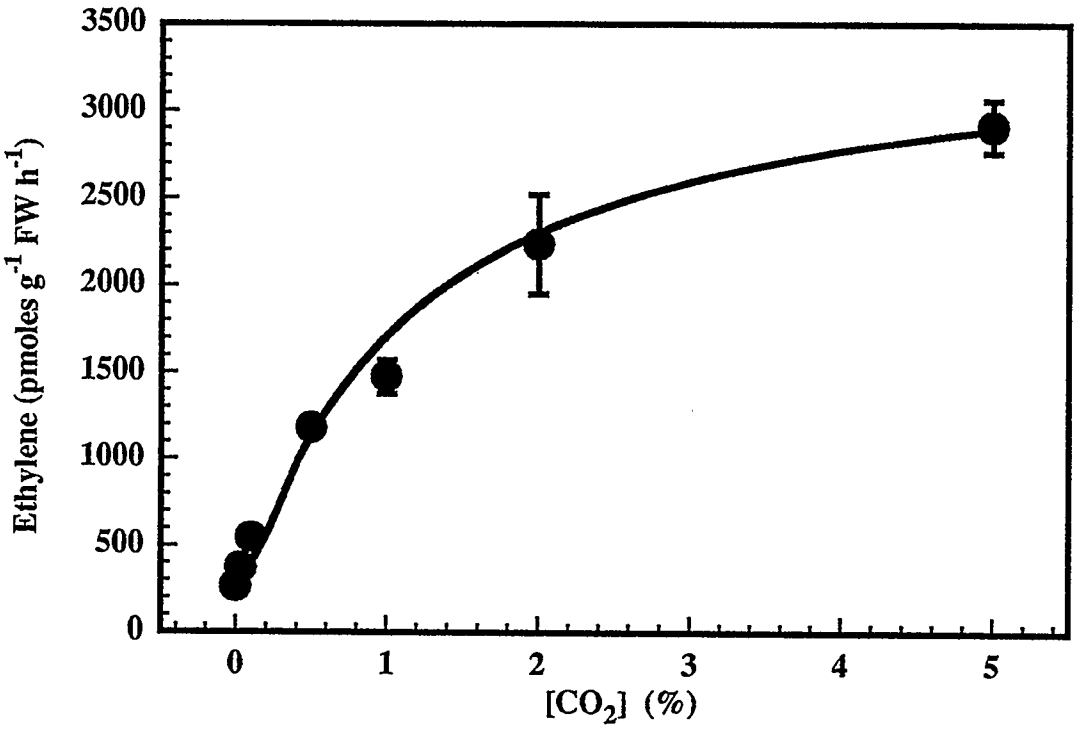

Figure 26. ACC oxidase activity dependence on CO_2 concentration. n= 3, mean ± standard error.

figure 26 was the end result of 3 repetitive assays, readjusting Fe and ascorbate levels, and shows an optimal CO_2 level greater than 5%. It was apparent that the actual optimal CO_2 level would be significantly higher than this, however at higher concentrations of CO_2 a negative peak generated by CO_2 interfered with ethylene detection by the photoionization detector used. While this problem may not be encountered using a flameionization detector, the sensitivity of this type of detector was too low to measure the small amounts of ethylene produced in the assays.

Ascorbate has been shown to be required for ACC oxidase activity, and is thought to be a substrate for the enzyme (Dong *et al.*, 1992). The effect of varying sodium ascorbate concentrations on ACC oxidase activity without exogenous CO_2 is given in figure 27. Very little activity is observed without added sodium ascorbate, however as little as 1 mM results in a dramatic increase in activity, with 2.5 to 5 mM being optimal. After reaching an optimum level, increasing concentrations of sodium ascorbate reduce enzyme activity. When CO_2 is included at 5%, the curve of activity versus ascorbate concentration is shifted (fig. 27). Again, with no ascorbate added activity is virtually absent, however higher concentrations are required to obtain maximal activity. With added CO_2, an optimum ascorbate concentration of 20 mM is obtained as compared to 5 mM without CO_2. The inhibition of enzyme activity at higher ascorbate levels precluded the determination of a K_m for this substrate.

Figure 28 illustrates ACC oxidase activity as a function of $FeSO_4$ concentration without exogenous CO_2. Low activity is observed without added Fe, and an optimum level is observed between 25 and 50 μM. As with ascorbate, ACC oxidase activity decreases with supra-optimal Fe levels. When CO_2 is added the optimal Fe concentration shifts lower (fig. 28). Activity is relatively low without added Fe, but peaks between 10 and 25 μM Fe, before decreasing with increasing Fe.

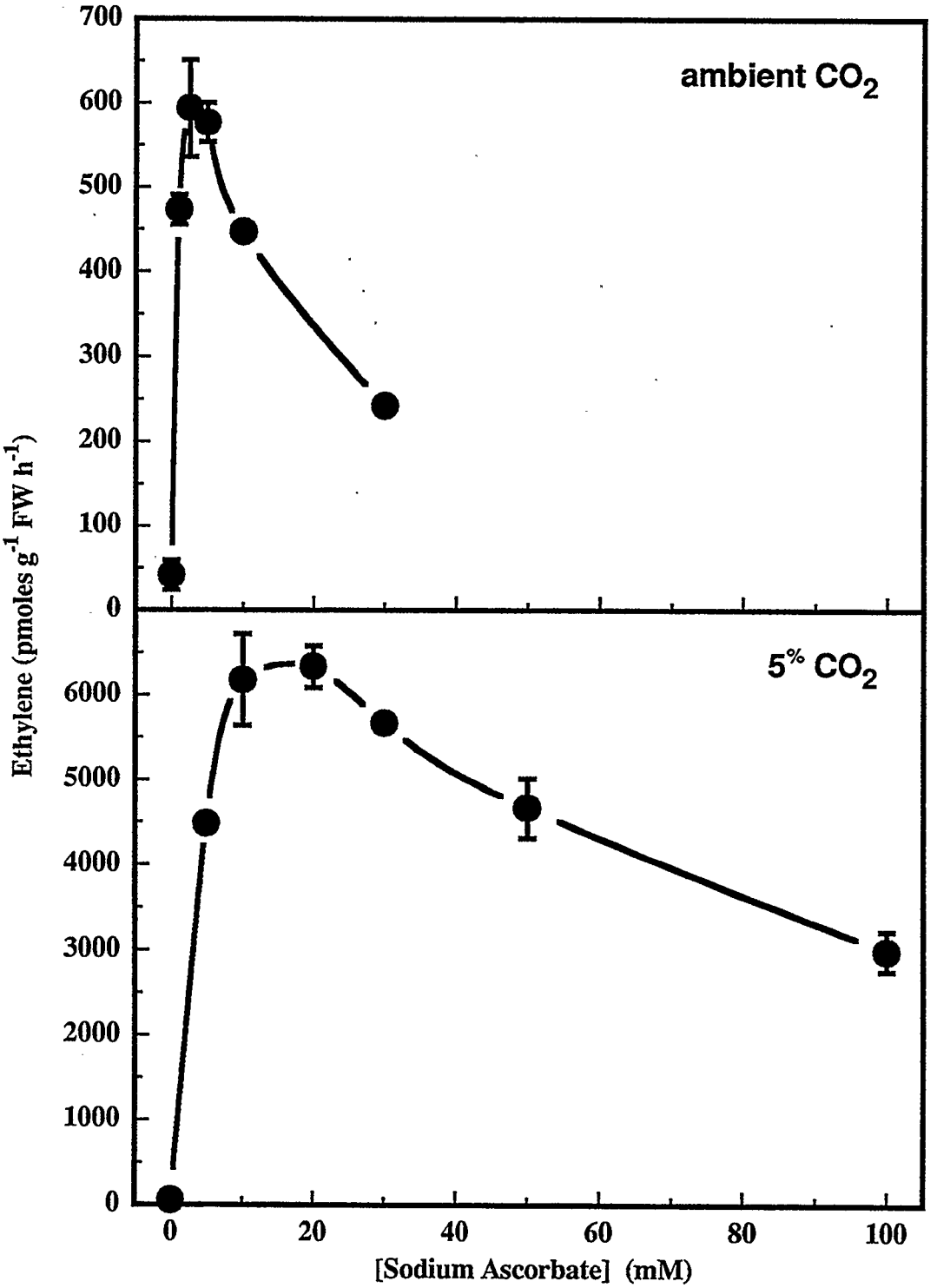

Figure 27. ACC oxidase activity dependence on ascorbate concentration with ambient or 5% CO_2. n= 3, mean ± standard error.

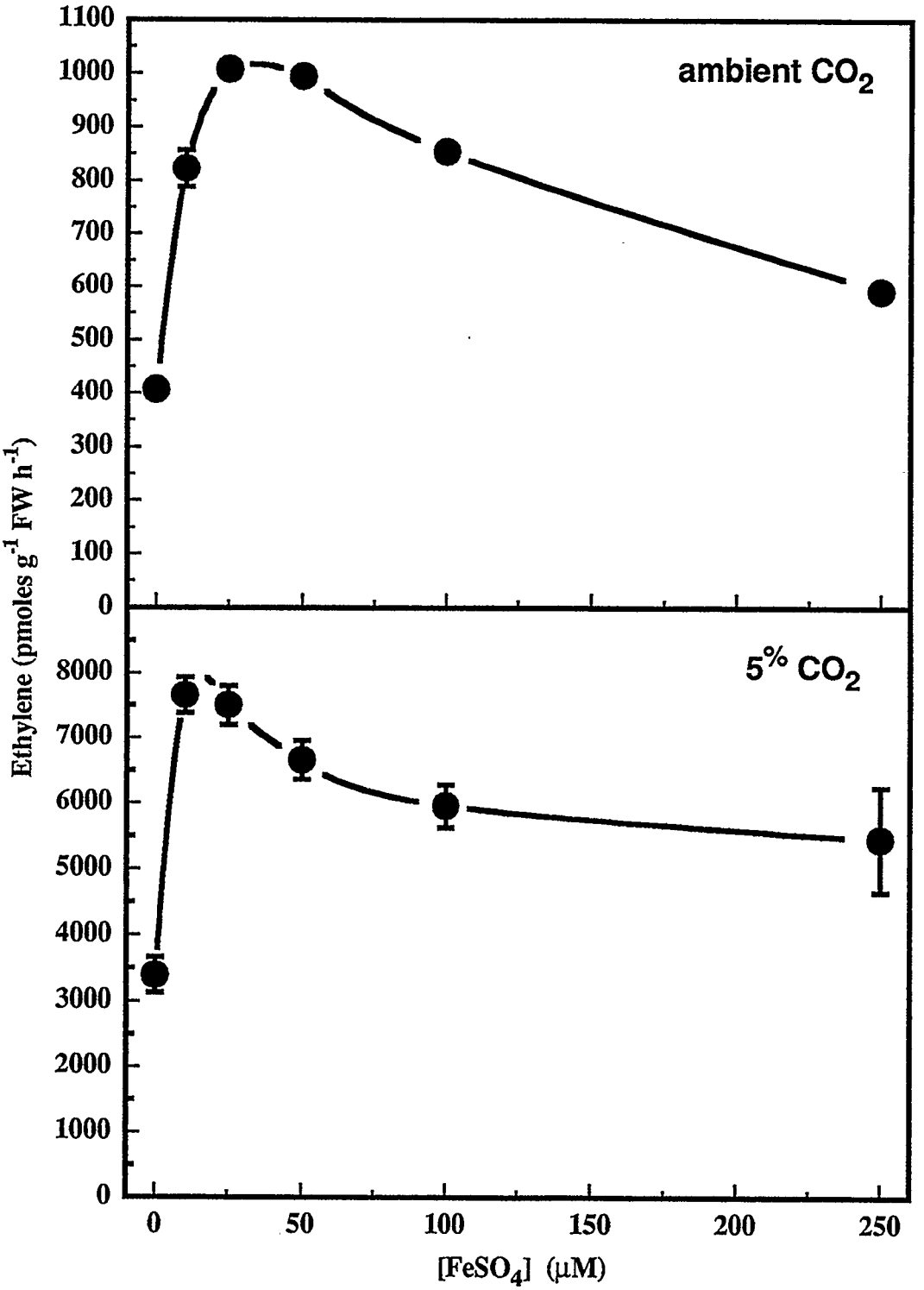

Figure 28. ACC oxidase activity dependence on $FeSO_4$ concentration with ambient or 5% CO_2. n= 3, mean ± standard error.

ACC oxidase activity as a function of pH is shown in figure 29. Without exogenous CO_2, ethylene production shows a broad optimal pH, with peak ethylene production occuring near pH 7. With 5% exogenous CO_2 however, the optimum pH curve shows a sharper, earlier peak. Both with and without CO_2, activity is virtually absent at pH 8.2 or greater. ACC oxidase activity increased gradually with increasing temperature to an optimal temperature at 30 to 35°C, and thereafter rapidly decreased. No difference in the temperature optimum was observed with or without exogenous CO_2 (fig. 30).

Figure 31 shows the response of ACC oxidase to increasing concentrations of ACC and corresponding Eadie-Hofstee plots. Without exogenous CO_2 a V_{max} of 1279 pmoles g FW^{-1} h^{-1} and a K_m of 48 μM for ACC is obtained. When exogenous CO_2 is added, the V_{max} increases to nearly 7500 pmoles g FW^{-1} h^{-1} and the K_m also increases to 98 μM (fig. 31).

Oxygen dependence is presented in figure 32. Without exogenous CO_2, the enzyme shows a typical saturation curve, and the corresponding Eadie-Hofstee gives a K_m of about 0.6%. This K_m is substantially lower than the 4.6 kPa reported for avocado fruit *in vitro* (McGarvey and Christoffersen, 1992), 1.4% for melon fruit *in vitro* (Smith and John, 1993), and 1.3% for mung bean hypocotyls *in vivo* (Yip *et al.*, 1988). When CO_2 is added the K_m for O_2 decreases slightly to about 0.5% (Fig. 32), whereas it has previously been reported that the K_m of ACC oxidase from melon fruit increased from 1.4% to 3.3% with 30 mM $NaHCO_3$ (Smith and John, 1993). Some activity was observed even with 0% added O_2, and this could be attributed to low levels of O_2 present in the buffer, which even rigorous degassing could not fully remove (at 0% added O_2, the O_2 concentration in the buffer was 0.02 mM).

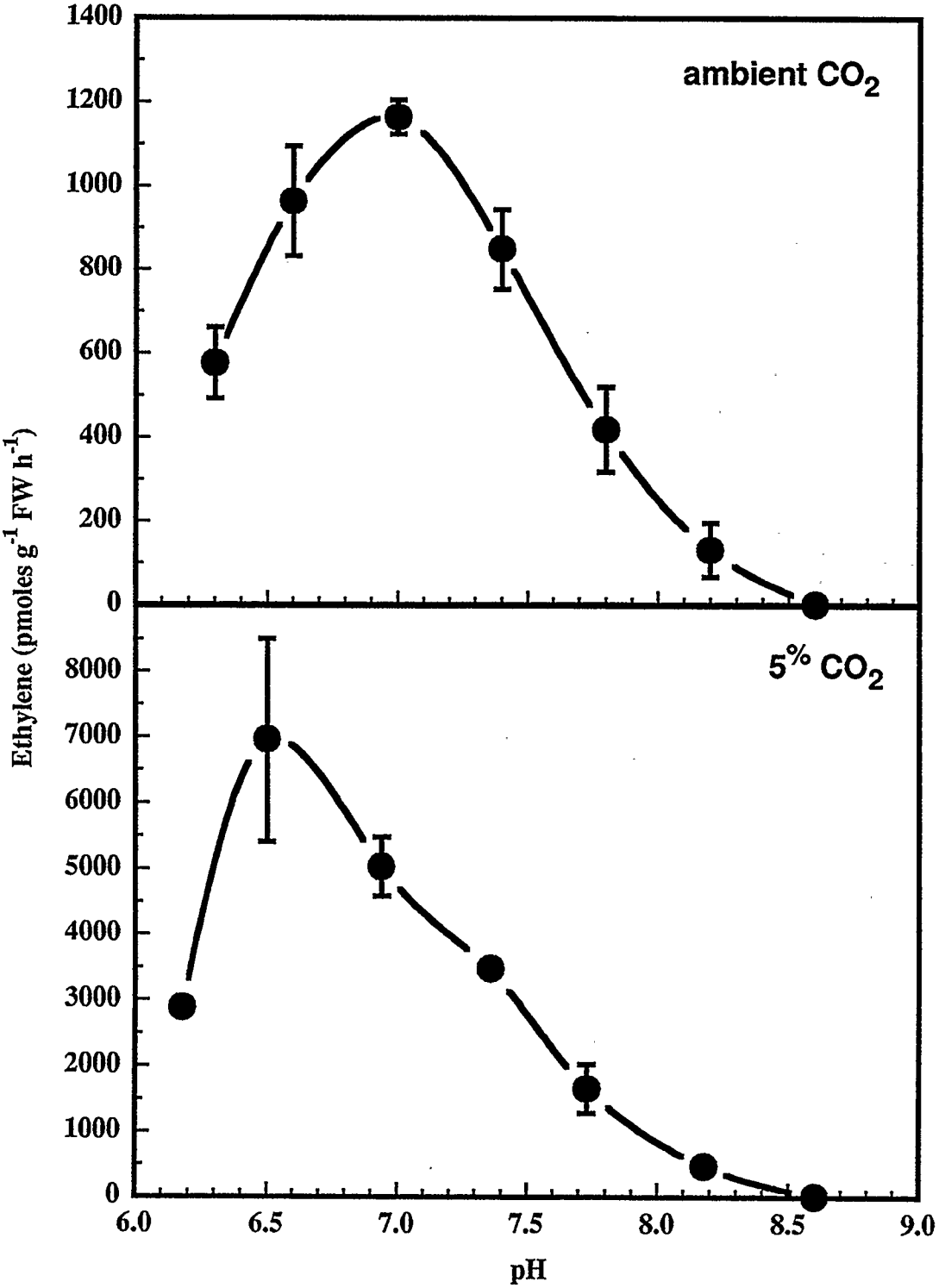

Figure 29. ACC oxidase activity dependence on pH with ambient or $5^{\%}$ CO_2. n= 3, mean ± standard error.

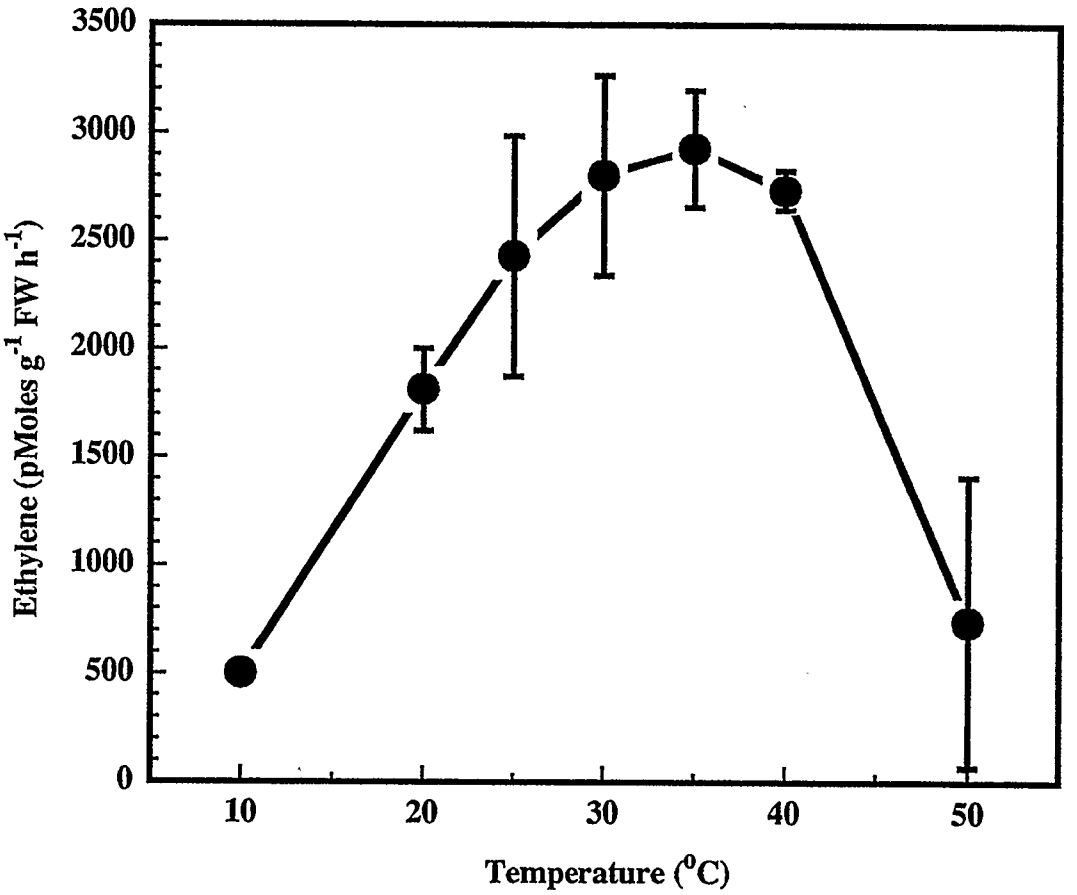

Figure 30. ACC oxidase activity dependence on temperature.
n= 3, mean ± standard error.

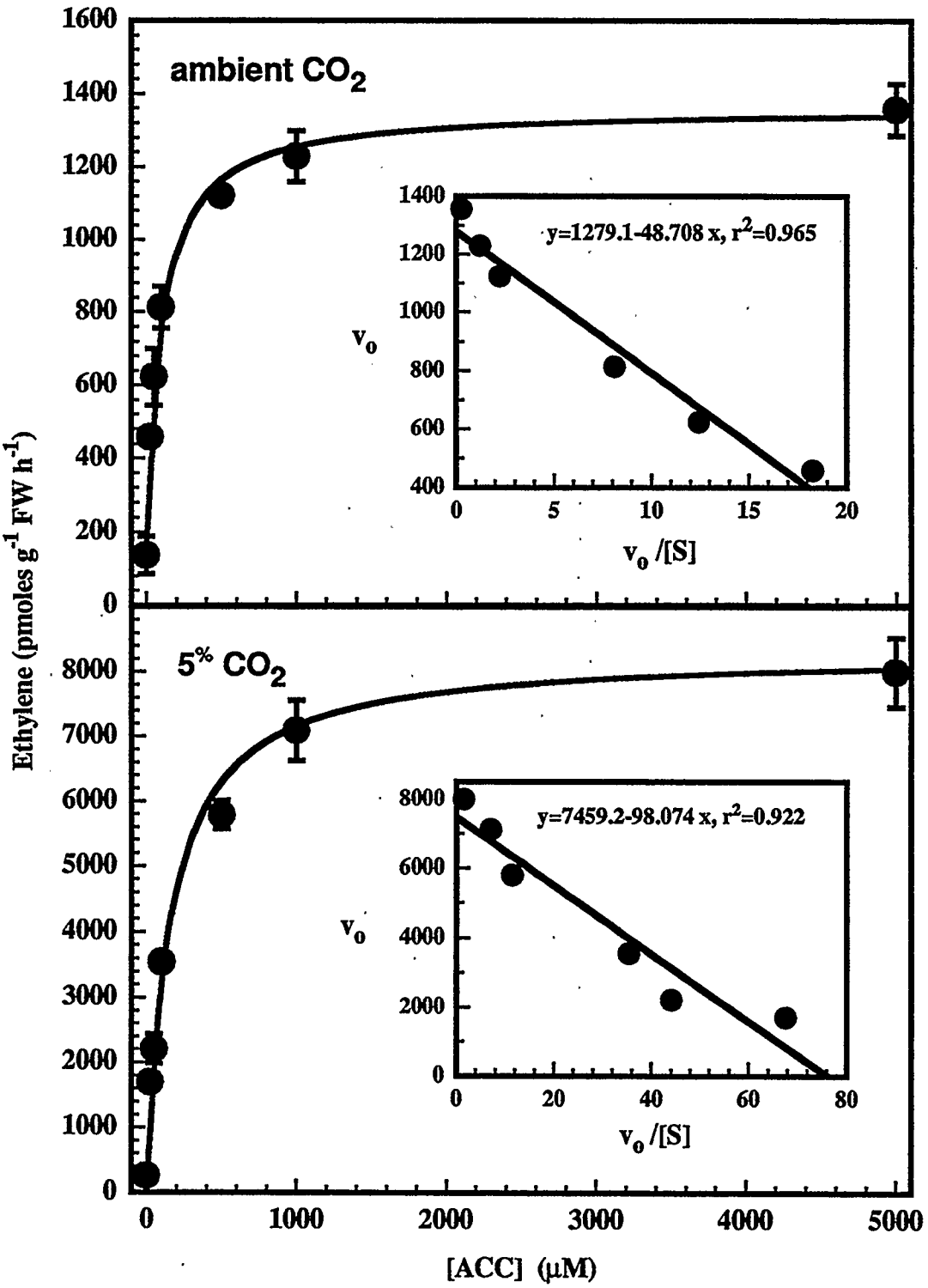

Figure 31. ACC oxidase activity dependence on ACC concentration with ambient and $5^{\%}$ CO_2. Insets are Eadie-Hofstee plots. n= 3, mean ± standard error.

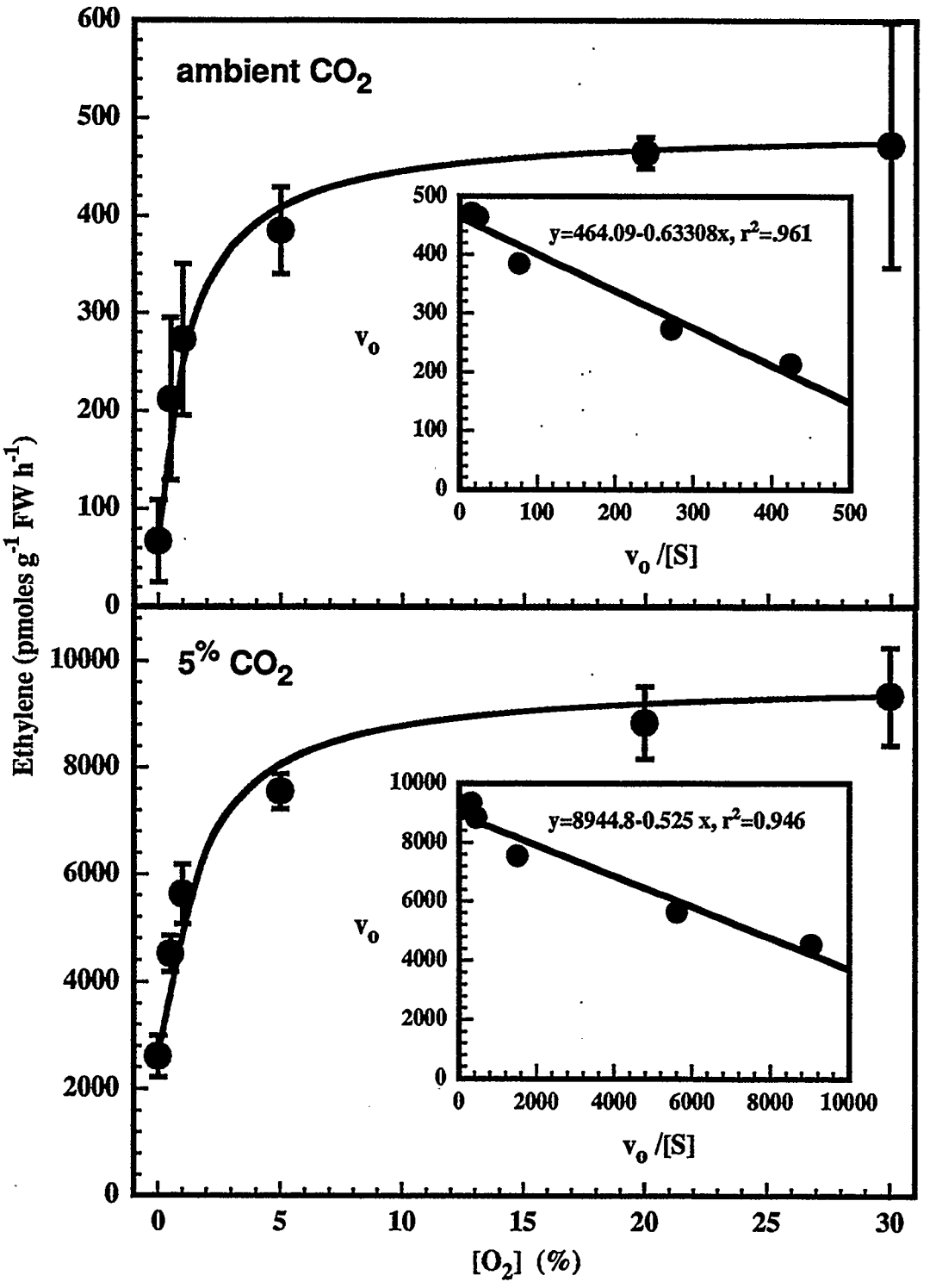

Figure 32. ACC oxidase activity dependence on O_2 concentration with ambient and 5% CO_2. Insets are Eadie-Hofstee plots. n= 3, mean ± standard error.

Wounded seedling roots exhibited a two-fold increase in *in vivo* ethylene production, and roots treated with IAA exhibited about 3 times the ethylene production of non-treated roots. ABA had no effect on ethylene production by roots. In none of the cases was ACC oxidase activity increased over non-treated counterparts (data not shown), as was expected. Apparently enough ACC oxidase activity is present to accomodate enhanced ethylene production by wounding and IAA.

High (5%) CO_2 levels shifted the optimal ascorbate concentration higher, with a broader peak, and the pH optimum to a lower pH with a narrower peak. CO_2 may be an allosteric effector which by binding changes the binding properties of the substrates and cofactors resulting in altered optima. Alternatively, CO_2 may bind to ACC oxidase by forming a carbamate with the terminal amino group which again could alter charge and conformation. High CO_2 increased both the V_{max} and K_m of the enzyme for ACC. If the rate of dissociation of the [ES] complex to product is appreciable compared to the formation of [ES] from ACC, then an increase in V_{max} would also increase the K_m for ACC as $E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E + P$ and $V_{max} = k_{+2} * [E]$ and $K_m = \frac{k_{-1} + k_{+2}}{k_{+1}}$.

Conversely, the slight decrease in the K_m value for O_2 with 5% CO_2 might suggest that the rate of dissociation to product is very small compared to the rate of formation of [ES] from O_2, and that the binding affinity for O_2 may actually be increased.

High levels of CO_2 have a dramatic effect on ACC oxidase activity *in vitro*. Elevated CO_2 increases the velocity of the enzyme catalysis, and also alters other enzyme parameters. The CO_2 levels in plant cells *in vivo* from the material studied are not known. CO_2 levels in the root environment however have been shown to vary greatly from as little as 0.1% (Palta and Nobel, 1989) to 8% (Buyanovsky and Wagner, 1983). Whether the variation in CO_2 in soils is reflected in intracellular CO_2

concentrations remains to be discovered, but certainly the possibility that environmental CO_2 levels affect ACC oxidase activity *in vivo*, and thus root growth, must be entertained.

Chapter 6.

The Effect of CO_2 on Ethylene Evolution and Elongation Rate in Roots of Sunflower (*Helianthus annuus* L.) Seedlings.

Summary

The effect of CO_2 on ethylene production by excised roots of sunflower was studied. A slight increase in ethylene production was observed at 0.25 to 0.5% CO_2, however CO_2 concentrations of 2% and higher inhibited ethylene evolution. The inhibition of ethylene production by CO_2 was not due to inhibition of ACC oxidase activity, but due to a reduction in the availability of ACC. ACC levels in excised roots were depressed by 2% CO_2 as compared to untreated roots, but MACC levels were not affected. The effect of high levels of CO_2 in the root environment on ethylene production by intact roots of sunflower, and on the elongation rate of roots was also studied in an aeroponic growth system. Treating roots with 2% CO_2 rapidly inhibited elongation, by over 50%. Ethylene evolution from roots was not affected by 2% CO_2. Maximum inhibition of elongation occured 1 hour after beginning CO_2 treatment, but gradually returned to untreated values by 6 hours. *In vitro* EFE activity extracted from the 2 cm tip portion of roots showed a 25% inhibition after 6 hours of treatment with CO_2. The possible physiological implications of high soil CO_2 levels on root growth are discussed.

Introduction

The root is the site of a large amount of the total ethylene biosynthesis in plants (chapter 3), and ethylene has long been known to influence a number of aspects of root growth and development. Ethylene induced inhibition of root growth has been reported in many species (Chadwick and Burg, 1967,1970; Smith and Robertson, 1971; Whalen

and Feldman, 1988), and it has been suggested that endogenous ethylene production may actually regulate root elongation *in vivo* (Jackson, 1991).

CO_2 has also been shown to exert a major influence on root growth. Roots of desert succulents were shown to become less viable (as quantified by the uptake of vital dyes) when grown under elevated CO_2 (2%) levels (Nobel, 1990). Elevated CO_2 at 3 and 10% inhibits lettuce seedling root growth by 20 and 35% respectively (Abeles and Wydoski, 1987), and 1% CO_2 was shown to inhibit the elongation of cultured radish roots by approximately 40% (Radin and Loomis, 1969).

CO_2 has also been shown to greatly enhance EFE activity, both *in vivo* (Dhawan *et al.*, 1981; Kao and Yang, 1982) and *in vitro* (chapter 5; Dong *et al.*, 1992; McGarvey and Christoffersen, 1992). The mechanism of activation of EFE activity is unknown at this time, however it has been shown that CO_2 modifies substrate and co-factor binding *in vitro*, as well as optimal pH (chapter 5; Poneleit and Dilley, 1993; Smith and John, 1993).

CO_2 levels vary greatly in different soils, and under different environmental regimes. Desert soils may contain as little as 0.1% CO_2 in the airspace (Palta and Nobel, 1989) compared to 8% in silt loam with rapidly decaying organic matter (Buyanovsky and Wagner, 1983). CO_2 concentrations can vary considerably with the moisture content of soil and with increasing soil depth (Yamaguchi *et al.*, 1967). Roots are exposed to varying levels of CO_2 throughout their lifespan, with levels increasing from 1.4 to 7% during the growing season of wheat having been reported (Buyanovsky and Wagner, 1983). It is possible that CO_2 concentrations in the soil could have a significant effect on ethylene production by roots, perhaps by stimulating EFE activity, and through this CO_2 could inhibit root elongation.

The purpose of this study was to examine the effect of high CO_2 levels on ethylene production and ACC/MACC levels of excised roots of sunflower, and on ethylene evolution and elongation rates of intact seedlings. The effect of exogenous CO_2 treatment of sunflower roots on *in vitro* EFE activity was also examined.

Materials and Methods

Plant Material.

Sunflower seeds were germinated aeroponically as given in chapter 2. At 96 hours after sowing, plants with stems 5 to 7 cm tall and roots 8 to 10 cm long were selected. The cotyledons had emerged from the testa but were not fully expanded, and the lateral roots were just beginning to emerge. The plants were transferred to large aerponics chambers (Hubick *et al.*, 1982), and allowed to acclimate for 4 hours. Measurements of root elongation rates were started beginning 3 hours before transferring plants to a high root CO_2 environment. The elongation rate was determined by measuring root length from the tip to the base of the root at the root-hypocotyl junction at various times.

CO_2 treatment of roots *in vivo* was performed by sealing a large aeroponic chamber and flowing a 2% mixture of CO_2 and air (Linde) through it at a rate of approximately 3 L per min. An initial 2% level of CO_2 was obtained by melting a small quantity of solid CO_2 in the chamber. A fan inside the chamber ensured thorough mixing, and CO_2 levels were measured by withdrawing samples from the chamber or from shoot level, and analyzing for CO_2 content with an infra-red gas analyzer (ADC 225 MK 3; Analytical Development Corp., Hoddesdon, UK). CO_2 treatment was begun by transferring plants from a chamber without exogenous CO_2 to the one in

which CO_2 levels had been elevated. Untreated plants were grown in an identical chamber without exogenous CO_2.

Each experiment was repeated a minimum of 3 times.

Tissue Ethylene Evolution.

At each harvest, 5 untreated and an equal number of 2% CO_2 treated seedlings were selected for ethylene determinations. The plants were removed from the chambers and the root was excised and incubated with either ambient air, or 2% CO_2. Ethylene evolution from these tissues was determined using a gas chromatograph equipped with a photoionization detector (chapter 2) employing the sampling system of chapter 3.

For analysis of ethylene evolution from CO_2-treated excised roots, plants were grown as above, and entire roots were cut and incubated in 10 mL test tubes sealed with serum caps with varying levels of CO_2 for 30 min. One mL of gas was withdrawn and analyzed for ethylene as above. Alternatively, 5 roots were placed together in 38 mL tubes and sealed with a serum caps with varying levels of CO_2 and 20% O_2. These tubes were incubated for 3 h with ethylene samples withdrawn and analyzed at various times using a gas chromatograph equipped with a flameionization detector (chapter 2). Seedlings were also pre-treated with 5 or 30 mM $NaHCO_3$ for 0.5 h, or 1 mM ACC in 1/4 strength Hoagland solution for 1 h by immersing roots in 25 mL of solution in 30 mL Corex® tubes under a Sylvania Spot-Gro light producing 160 μmoles m^{-2} s^{-1} PAR. The roots were excised and incubated in 38 mL tubes sealed with serum caps for 3 h, and gas samples were withdrawn at various times and analyzed for ethylene as above. The fresh weights of all roots were determined immediately after the termination of the sampling period.

Extraction and Determination of ACC and MACC levels.

ACC and MACC levels from excised roots treated with 2% CO_2 for 3 hours as given above were determined by GC-SIM-MS as given in chapters 3 and 4. Six replicates of 2% CO_2-treated and untreated control roots were extracted at 3 h, and 3 replicates of untreated control roots were extracted at 0 h, prior to beginning incubation.

Extraction and Assay of EFE Activity.

The enzyme assay is based on the procedure of Ververidis and John (1991), with minor modifications, as described in chapters 4 and 5. Briefly, excised root parts treated with 5% or ambient CO_2 for various times were ground under nitrogen in extraction buffer (pH 7.4) and assayed in assay buffer (pH 6.6) with 5% CO_2 with a 30 min incubation at 30°C. A 1 mL headspace gas sample was then removed and analyzed for ethylene content using a gas chromatograph equipped with a photoionization detector (chapter 2). Three replicates of each treatment were run, and each assay was repeated 4 times.

Results

The effect of CO_2 on ethylene production by excised roots.

Figure 33 shows ethylene production by excised roots of sunflower seedlings with increasing concentrations of CO_2 after 30 min of incubation. Ethylene production was observed to increase from ambient CO_2 levels to 0.25% CO_2. Ethylene production at 0.5% was lower than at 0.25%, but still slightly higher than that observed at ambient CO_2. Ethylene production decreased with increasing CO_2 concentrations, and was lower than ambient levels at 2% CO_2. Ethylene production was further inhibited by higher CO_2 levels (fig. 34).

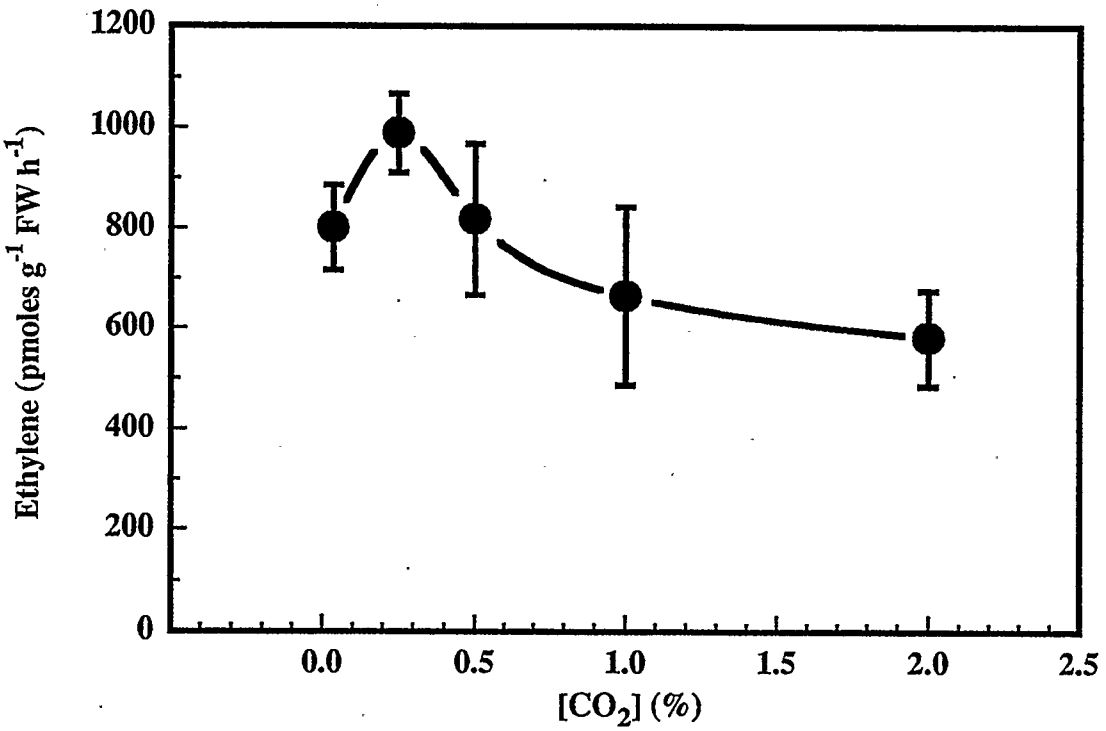

Figure 33. The effect of CO_2 on ethylene production of excised roots of 4 day old sunflower seedlings after 30 min incubation. n= 5, mean ± standard error.

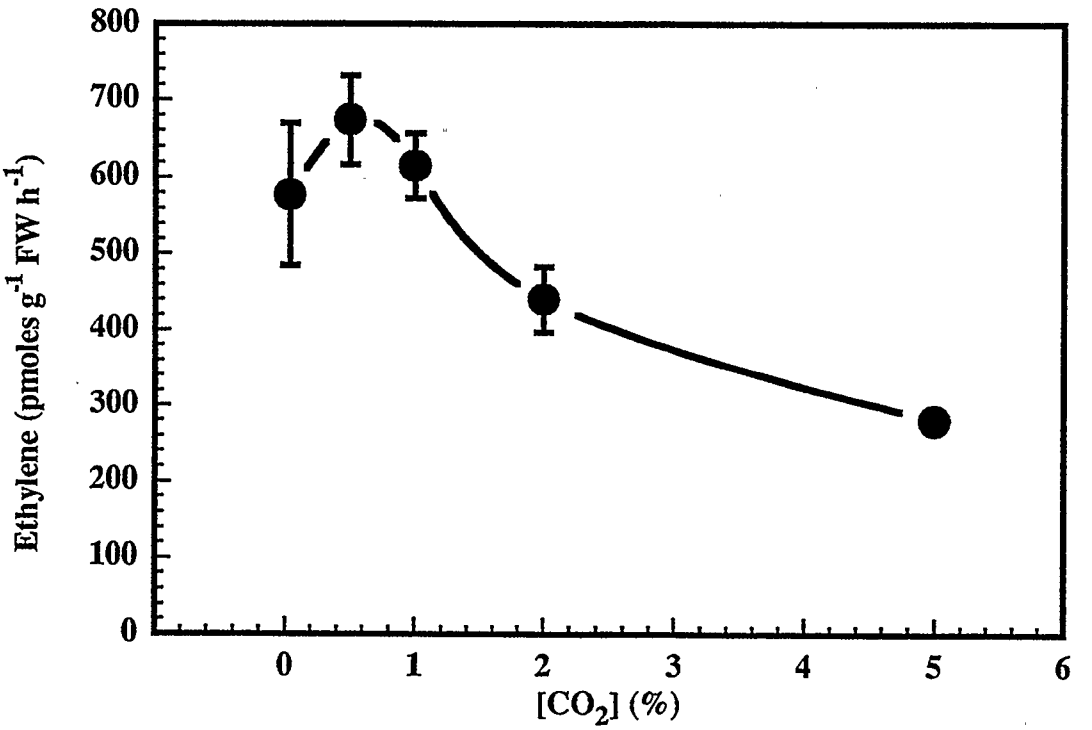

Figure 34. The effect of CO_2 on ethylene production of excised roots of 4 day old sunflower seedlings after 30 min incubation. n= 5, mean ± standard error.

Ethylene production was also monitored over a range of times with varying concentrations of CO_2 (fig. 35). Total ethylene production by roots incubated with ambient CO_2 increased with time, but at a decreasing rate. Total ethylene production by roots incubated with 2% CO_2 was inhibited compared to ambient roots, and this inhibition was magnified with increasing time. Roots incubated with 0.5% CO_2 showed an intermediate trend. Roots pre-incubated with 30 mM $NaHCO_3$ for 0.5 h were also inhibited with respect to ethylene production (fig. 35), however 5 mM $NaHCO_3$ had no effect.

Total ethylene production by roots pre-treated with 1 mM ACC for 1 h increased linearily over the 3 h time-span monitored (fig. 36). CO_2 at either 0.5 or 2% had no effect on ethylene production by roots pre-treated with ACC.

The effect of CO_2 on ACC and MACC levels of excised roots.

ACC content in untreated roots increased over 3 times during the 3 h course of the experiment, while MACC levels increased only 1.5 times (table 2.). ACC content of roots treated with 2% CO_2 was only 80% of the levels measured in untreated roots, however no difference in MACC levels was observed. Ethylene production by 2% CO_2-treated roots was 75% that of untreated roots at 3 h (table 2.).

Table 2: Ethylene production by, and ACC/MACC levels in, roots of sunflower with 2% CO_2-treatment (± standard error).

	untreated 0 h	untreated 3 h	2% CO_2-treated 3 h
Ethylene (pmoles g-1 FW)	N/A	2213.9 ± 229.0	1663.5 ± 100.5
ACC (nmoles g-1 FW)	0.42 ± .07	1.51 ± .12	1.23 ± .16
MACC (nmoles g-1 FW)	13.19 ± 2.79	18.67 ± 3.85	19.08 ± 2.57

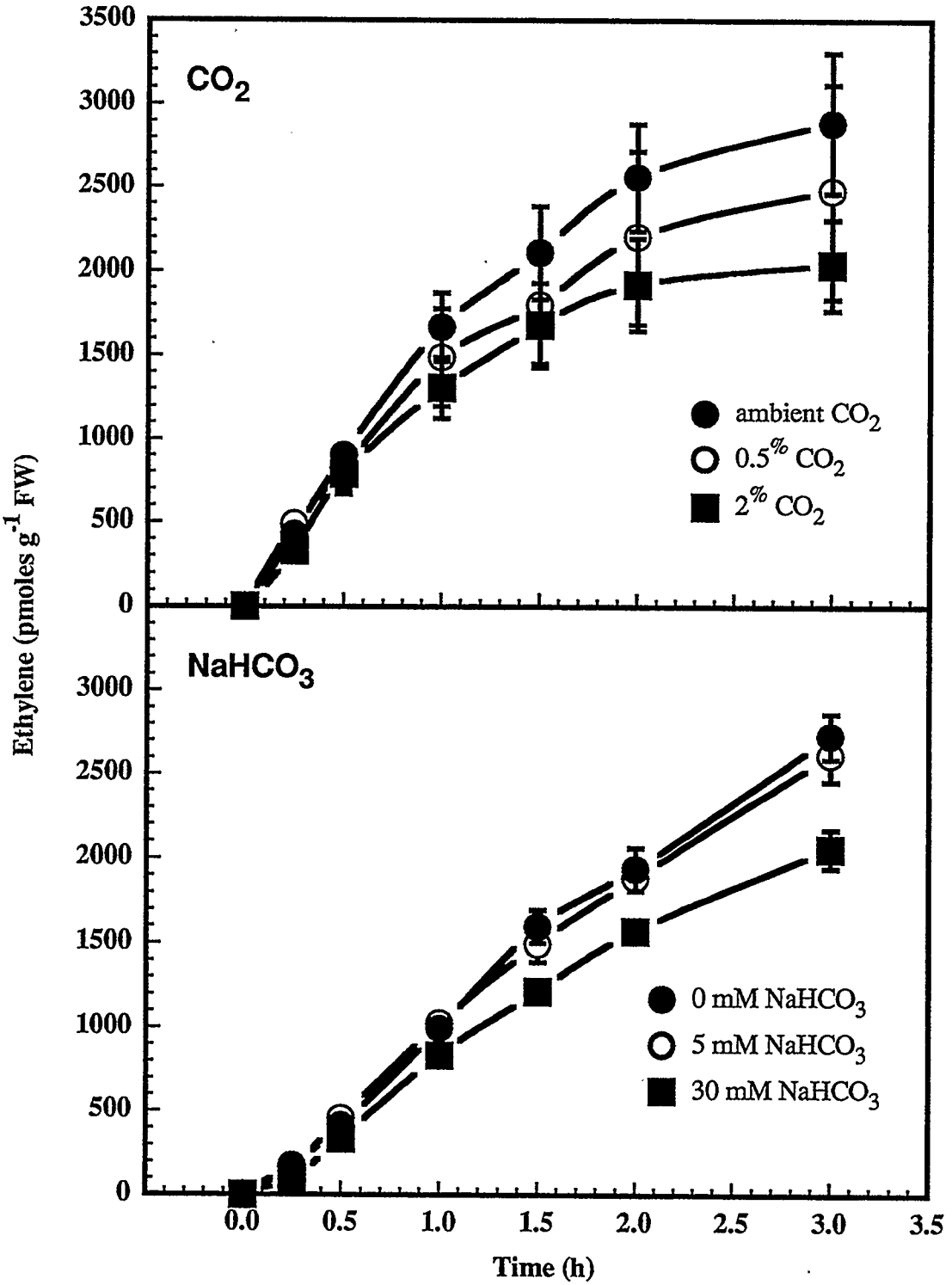

Figure 35. Ethylene evolution with time from excised roots of 4 day old sunflower, incubated with different levels of CO_2, or pre-incubated with different levels of $NaHCO_3$. n= 5, mean ± standard error.

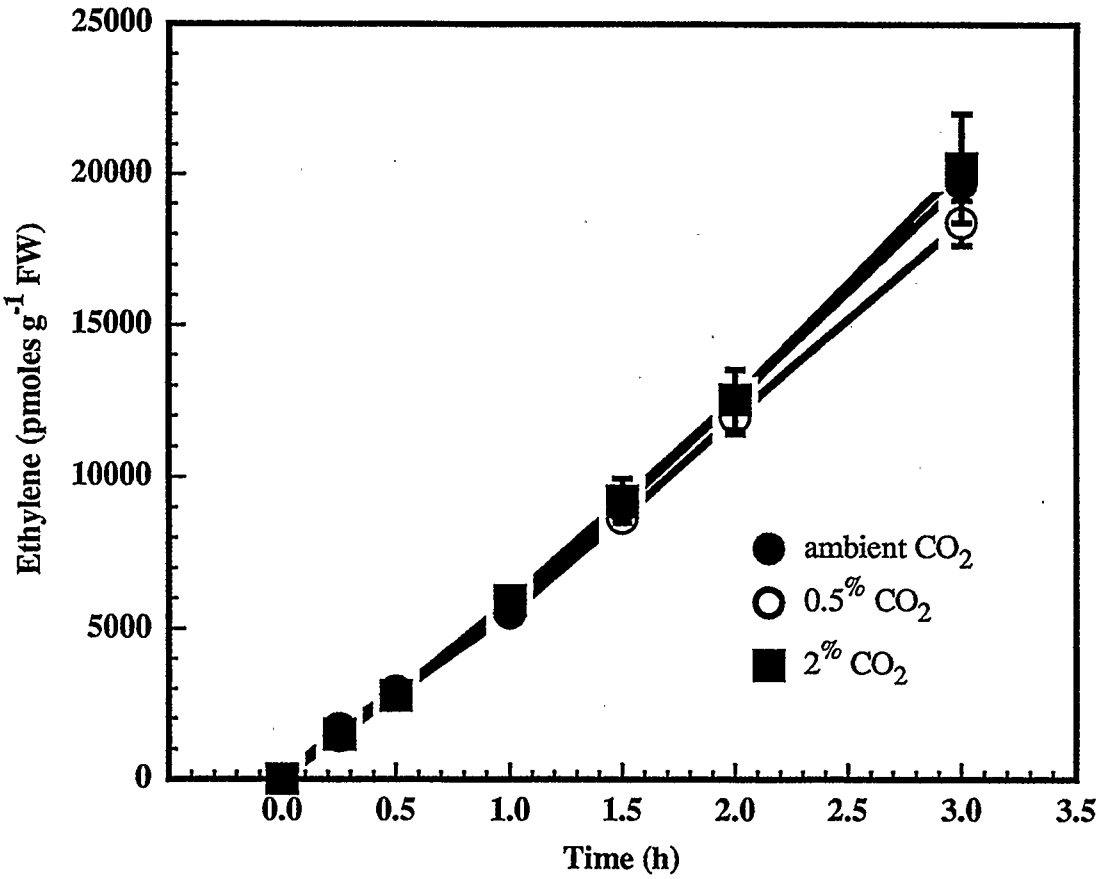

Figure 36. Ethylene evolution with time from excised roots of 4 day old sunflower seedlings pre-treated with 1mM ACC, incubated with different levels of CO_2. n= 5, mean ± standard error.

Ethylene production, elongation rates and ACC oxidase activity of roots of intact seedlings treated with elevated CO_2.

The effect of 2% CO_2 treatment on ethylene production by roots of intact seedlings is illustrated in figure 37. No difference in ethylene production in response to CO_2 is observed.

Figure 37 also represents the effect of 2% CO_2 treatment on the root elongation rate. At 1 and 0 hours prior to CO_2 treatment, roots destined for CO_2 treatment are elongating slightly slower than untreated roots, but by 1 hour after the beginning of CO_2 treatment, treated roots elongate at only 43% of the rate of observed at 0 hours, and 38% of the rate of the untreated roots. The inhibition of elongation gradually diminishes to 6 hours when treated and untreated roots are again elongating at comparable rates.

In vitro analysis of EFE activity from tips of roots treated with 5% CO_2 for 6 hours gave 5612.7±711.7 pMoles ethylene g-1 FW h-1 for untreated roots and 4232±389.4 pMoles ethylene g-1 FW h-1 for CO_2 treated roots, a 25% reduction in EFE activity. No difference in EFE activitiy was observed in roots treated with high CO_2 for either 3 or 24 h.

Discussion

Ethylene production by excised roots was inhibited by high concentrations of CO_2. A slight promotion of ethylene production was observed with 30 min of incubation at 0.25 to 0.5% CO_2, but this effect was not observed in an alternative system over a longer time period. The inhibition of ethylene production observed at high (2%) CO_2 levels was magnified with increasing time: only small differences in ethylene production could be observed up to 0.5 h. Other research has shown that very low oxygen levels in the root medium reduce ethylene evolution in intact roots (Bradford and

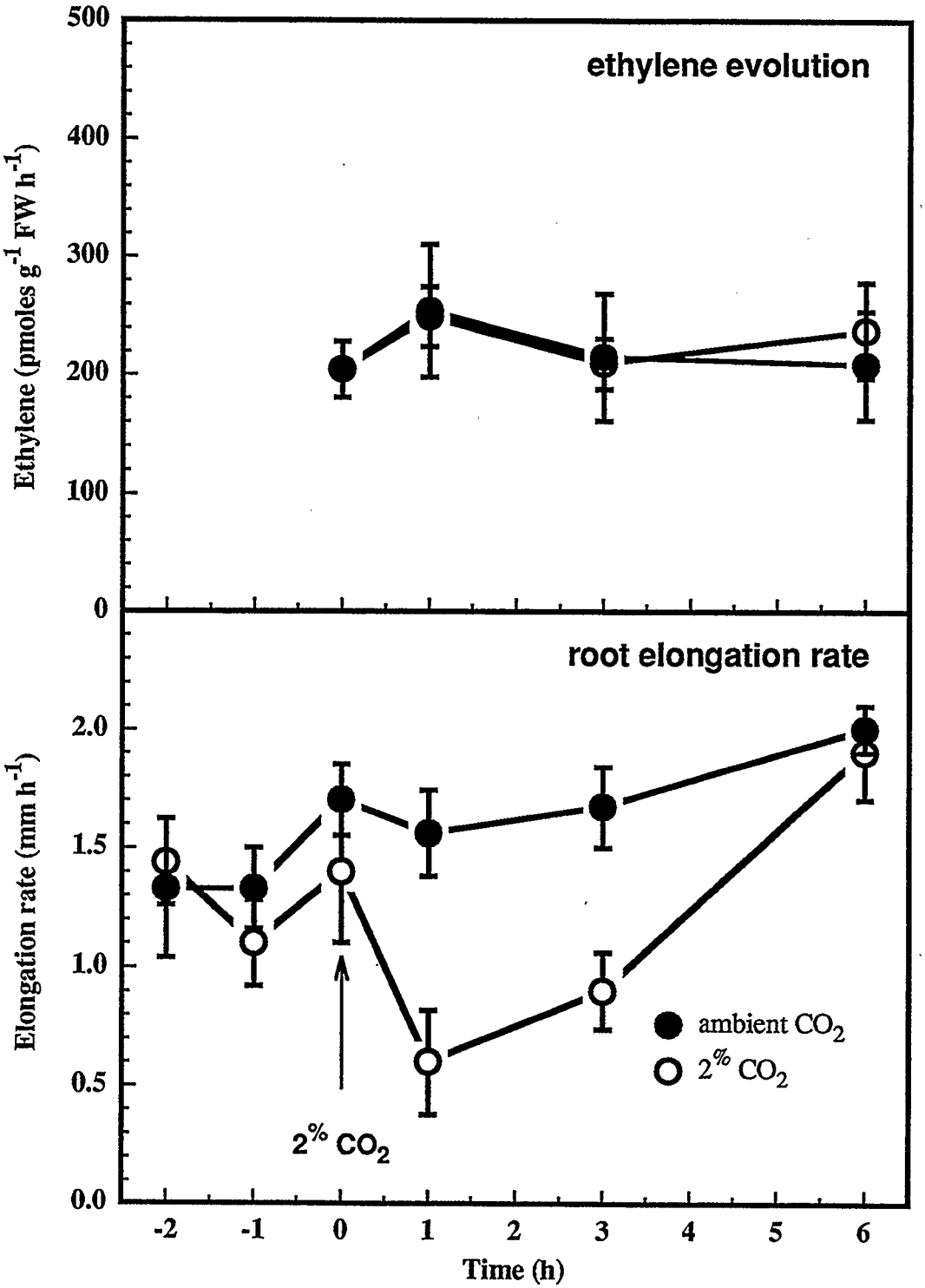

Figure 37. The effect of $2^{\%}$ CO_2 on ethylene production and root elongation rate of 4 day old intact sunflower seedlings. For ethylene n= 5, for root elongation n= 10, mean ± standard error. Arrow indicates transfer to $2^{\%}$ CO_2.

Yang, 1980b, Wang and Arteca, 1992). This inhibition has been attributed to inhibition of ACC oxidase activity, as O_2 is a co-substrate for ethylene production (McGarvey and Christoffersen, 1992; Yip *et al.*, 1988). Atwell *et al.* (1988) on the other hand show that low O_2 (as little as 3 kPa) stimulates ethylene production in maize roots. The inhibition seen here was not due to low oxygen inhibition of ACC oxidase activity, as 20% O_2 was maintained in all cases. To test whether the inhibitory effect of CO_2 on ethylene production could be attributed to an inhibition of ACC oxidase activity, roots were pre-treated with 1 mM ACC for 1 h. CO_2 had no effect on ethylene production by roots pre-treated with ACC suggesting that the observed inhibition was not due to inhibition of ACC oxidase activity, but possibly was due to a decrease in available ACC for conversion to ethylene. This hypothesis fits well with the long-time course data showing a magnification of inhibiton of ethylene production with increasing time. ACC levels measured in excised roots were in agreement with this hypothesis; a 20% reduction in ACC levels were observed in 2% CO_2-treated roots at 3 hours, compared to untreated roots. No difference in MACC levels were observed with or without CO_2 treatment, suggesting that the decrease in ACC levels are likely due to reduced ACC synthesis in the root rather than enhanced conjugation to MACC. How CO_2 exerts its effect on ACC availability is not known. It is possible that CO_2 directly affects biosynthetic enzymes, or it may reflect a more general effect on the metabolism of the root. Possibly the high external CO_2 concentrations prevent diffusion of CO_2 out of the root, slowing respiration which in turn could affect other aspects of metabolism.

Root elongation in the system used was inhibited by elevated CO_2 levels. These results are in agreement with earlier work on lettuce seedlings (Abeles and Wydoski, 1987) and on radish roots *in vitro* (Radin and Loomis, 1969), while an investigation on pea root sections showed no effect of high CO_2 on elongation (Chadwick and Burg,

1967, 1970). Stolwijk and Thimann (1957) demonstrated strong inhibition of root growth in sunflower and pea with 1 to 6.5% CO_2, but this same inhibition was not observed in oats or barley. The inhibition of elongation observed in this study was rapidly induced by elevated CO_2 and was transient, showing maximum inhibition within 1 hour after beginning treatment, and then returning to untreated rates by 6 hours. These findings suggest that the physiology of the root adjusts to the elevated CO_2 levels and mitigates the elongation response.

Ethylene levels from the roots of intact seedlings did not change with high CO_2. A tradition of literature shows that CO_2 increases ethylene production in plant tissues, Kao and Yang (1982) demonstrated that light inhibition of ethylene synthesis can be attributed to depletion of CO_2, and Dhawan *et al.* (1981), and Bassi and Spencer (1982) found that high CO_2 promoted ethylene evolution in intact sunflower shoots. CO_2 is thought to promote EFE activity in shoots *in vivo* (Kao and Yang, 1982, Philosoph-Hadas *et al.*, 1986) and has been demonstrated to increase EFE activity dramatically *in vitro*, promoting activity from 8 to 10 times (chapter 5; Dong *et al.*, 1992). The actual mechanism of CO_2 activation of EFE activity is not known, however the experiments described in chapter 5 have shown that CO_2 changes substrate and co-factor binding properties, as well as the pH optimum. Presumably CO_2 could be acting as an allosteric effector or it might form a carbamate with the enzyme. CO_2 levels within the cells of the root are not known, although levels of CO_2 extracted from roots of green ash was found to vary from 2.4 to 10% with flooding (Good and Patrick, 1987). How exogenous CO_2 levels affect intracellular CO_2 levels is also unknown at this time. If data of this type was made available, many of the mysteries surrounding CO_2 and ethylene in plant tissues would be cleared up. Given the lack of promotion of ethylene production by roots of intact seedlings with high CO_2 levels, and the lack of CO_2 promotion of ACC

oxidase activity in excised roots, it is possible that CO_2 levels within roots cells, at the site of ACC oxidase activity, may be saturating for ACC oxidase activity. The inhibition of ethylene production observed in the excised root system was not apparent with intact seedlings. Transport of ACC from the shoot to the root has been reported in other systems (Amrhein *et al.*, 1982) and it is possible that such transport is occuring here.

Elevated CO_2 levels were shown to decrease extractable ACC oxidase activity by 6 hours after treatment. Although the decrease is small, it is puzzling in view of the fact that high CO_2 does not significantly affect ethylene production by roots of intact seedlings. These results are not in agreement with those of Philosoph-Hadas *et al.* (1986) who found that ACC oxidase levels increased with prolonged exposure to exogenous CO_2 (up to 20 hours CO_2 treatment). Many factors could account for this discrepancy, including the type of tissue used being different, and the fact that the tissue in their study was not intact but very artificial (leaf discs incubated for extended periods of time). Additionally these authors did not have the benefit of an *in vitro* ACC oxidase assay at their disposal. If ACC oxidase levels are being modified by high CO_2 concentrations, then presumably some compound is acting as a signalling mechanism to regulate ACC oxidase synthesis. Previous work has shown that at 1 or 3 hours after ACC treatment (which produces high levels of ethylene) there is no change in extractable ACC oxidase activity (chapter 4). Similarily neither wounding or treatment of roots with IAA (both of which also increase root ethylene production) have been shown to change ACC oxidase activity (chapter 5). In view of these results, and especially since ethylene levels were not enhanced by 2% CO_2, it is possible that CO_2 itself is somehow acting as a signal to reduce ACC oxidase synthesis, though this is not necessarily the case.

The 2% CO_2 applied here had a dramatic effect on root elongation. It is conceivable that the high levels of CO_2 in the atmosphere could also inhibit root growth by reducing the diffusion of endogenous CO_2 out of the root, possibly leading to a diminished respiratory capacity (by stalling the citric acid cycle), however if this were the case then the recovery of the roots' elongation rates would not be expected at 6 h. CO_2 has long been reported to be an inhibitor of ethylene binding (Burg and Burg, 1967), and possibly ethylene binding is required for normal root growth. Very low levels of ethylene have been proposed to actually stimulate root elongation in some species (Konings and Jackson, 1979) and therefore it is conceivable that the inhibition of binding of endogenously produced ethylene might reduce root growth. The recovery of root elongation by 6 h could reflect a mitigation of ethylene binding inhibition, by increasing the number of binding sites, or by some other means alleviating the effect of CO_2.

In summary, in excised roots 2% CO_2 inhibits ethylene production by reducing the synthesis of ACC. In intact seedlings 2% CO_2 does not affect ethylene production by roots, but does transiently inhibit root elongation.

Chapter 7.

General Discussion.

Most of the work performed on ethylene physiology has centered on the well-documented climacteric of certain fruits. The climacteric system is an ideal one for study for several reasons; first, climacteric fruits are economically important, and research on them is more likely to attract research dollars than more esoteric investigations; second, these fruits are readily available in local supermarkets or are easily picked off of trees and thus afford easy access; and third, climacteric fruits make an abundance of ethylene at a specific, easily characterizable time and therefore measurement of the gas or its precursors is simplified. The climacteric fruit system however is a very specialized system and should not be interpreted as a model for ethylene physiology in other parts of the plant. The scarcity of general research performed on roots, and specifically research on ethylene physiology of roots can be explained by the phrase "out of sight, out of mind". The root is invisible in most instances, and is definately harder to manipulate than an apple.

The objective of this thesis was broad in its scope; to study the involvement of ethylene in root growth. In order to examine this phenomenom it was first necessary to develop a system in which the effect of ethylene on root growth could be characterized. To this end, the Germ-a-Tron, an aeroponic germination system, was developed. This device went through several evolutions with the final result, the v. 3.0, proving itself a reliable, efficient germination system. Seedlings from this system were easy to manipulate and and the straight root growth of these plants made assessing the effects of various treatments on root elongation easy to measure. It was observed that ethylene, via ACC, inhibited root elongation substantially in our system, as it has been observed

to do in others. A comparison of Germ-a-Tron germinated plants to those germinated in trays showed that Germ-a-Tron plants developed faster, and the roots of Germ-a-Tron seedlings also produced more ethylene. The reason for the increased ethylene production by these plants is not clear, however increased ethylene production by faster growing tissues has been documented in several systems (Huxter et al., 1979, Jayasekera, pers. comm.). It is possible that the rapid growth exhibited by Germ-a-Tron plants is actually checked by higher levels of ethylene. A slight, but statistically not significant, promotion of root elongation was observed in roots treated with silver thiosulphate, an inhibitor of ethylene action. While ambiguous in that the promotion of elongation observed was small, this result could suggest that endogenous ethylene might regulate root elongation somewhat, especially in rapidly growing roots. It would be worth studying the effect of silver thiosulphate or other inhibitors of ethylene action on root elongation in both fast growing (Germ-a-Tron) vs. slower growing (tray) systems.

During the course of characterizing the rate of ethylene evolution by the two types of growth systems, a preliminary study (not included in this thesis) was performed on the response of seedling roots from the two germination systems to thigmostimulation. As enhanced rates of ethylene evolution have been reported in response to thigmostimulation of roots (Kays *et al.*, 1974; Sarquis *et al.*, 1991), ethylene evolution was monitored as a marker for sensitivity to thigmostimulation. Seedlings were removed from their germination environments and then the roots were gently stimulated with wet Terra-Green. Inconclusive evidence was obtained suggesting that Germ-a-Tron seedling roots were more sensitive to thigmostimulation than tray-grown roots. Possibly, roots that are constantly in contact with a solid substrate become accustomed to this type of stimulation, certainly the morphology of the roots from the two systems are different, probably as a consequence of the difference in the

thigmostimulation encountered. While the results obtained from these experiments were intriguing, this examination was terminated as it became apparent that stimulating the roots of the two systems equally was difficult, due to the variation in their morphologies, and also because the tray-grown seedlings were heavily stimulated when they were removed from the tray. Again, further study of this phenomenom is warranted once this logistic problem has been dealt with.

In an effort to better understand the involvement of ethylene in root growth, the relationship between the root and shoot with respect to ethylene physiology was studied. To accomplish this, a study of ACC metabolism and transport of ACC in intact, unstressed plants was investigated. This study was the first comprehensive investigation of its kind, and employed the use of 2,3-^{14}C ACC. This study showed that ACC was readily transported from roots to shoots, and that metabolism of ACC to both MACC and ethylene occured rapidly in both roots and shoots. Evidence was also presented illustrating the transport of MACC in the xylem stream, which previously had appeared unlikely. A second volatile compound which was not ethylene was also produced from the radioactive ACC in both roots and shoots, and based on its solubility properties we proposed this second gas to be CO_2. MACC was not likely to be the source of this gas, as peak MACC levels occurred well after production of this gas was decreasing. It is possible that an alternate pathway exists for the metabolism of ACC to a product other than ethylene, and potentially this pathway may be important in regulating ethylene production under some conditions. In a case where there is an abundance of ACC, shunting some to the production of CO_2 may reduce the amount of ethylene produced, ameliorating some of the effects of high levels of ethylene on the tissues. Certainly, the amounts of this gas measured would be effective in reducing ethylene biosynthesis if this actually is the case. A fellow researcher in The

Netherlands, Ernst Woltering, has shown that ACC can be metabolized to CO_2 by bacteria (Overbeek and Woltering, 1990), and has suggested that this is what we were observing in our system (pers. comm.). We can not rule out the possibility of CO_2 production from carbons 2 and/or 3 of ACC by bacteria in our system, as we did not test for this. However the copious quantities of putative CO_2 we observed from both roots (on which bacteria might be expected) and from shoots (in which one might not expect a lot of bacteria) compels us to believe that this was not the case.

The results from chapter 2 gave an overall picture of ethylene metabolism in the intact plant as presented in figure 38. This diagram gives an overall description of the fate of exogenously fed ACC. Both ACC and MACC were observed to be transported from the root to the shoot, but no attempt was made to ascertain whether or not transport of these compounds occured in the reverse direction. It has previously been reported that MACC (Fuhrer and Fuhrer-Fries, 1985) and ACC (Amrhein *et al.*, 1982) can be transported from the shoot to the root, and it would be interesting to discover if similar transport of ACC, and possibly MACC, occurs in this system.

The investigation of ACC transport and metabolism in the intact plant showed some variations in the pattern of metabolism of ACC in the two organs. It was then postulated that ACC metabolism in different parts of the root would also be different, reflecting the diverse functional roles of the different root tissue and the diversity in their development. Since radiolabelled ACC was no longer available at a reasonable price, GC-SIM-MS was used, in conjunction with conventional GC techniques to localize ethylene, ACC and MACC levels from different parts of the root. Variations in endogenous levels of ACC and MACC were found to correspond to variations in ethylene production by these tissues. Treating roots with exogenous ACC increased ACC, MACC and ethylene production in all parts of the root with disproportionately

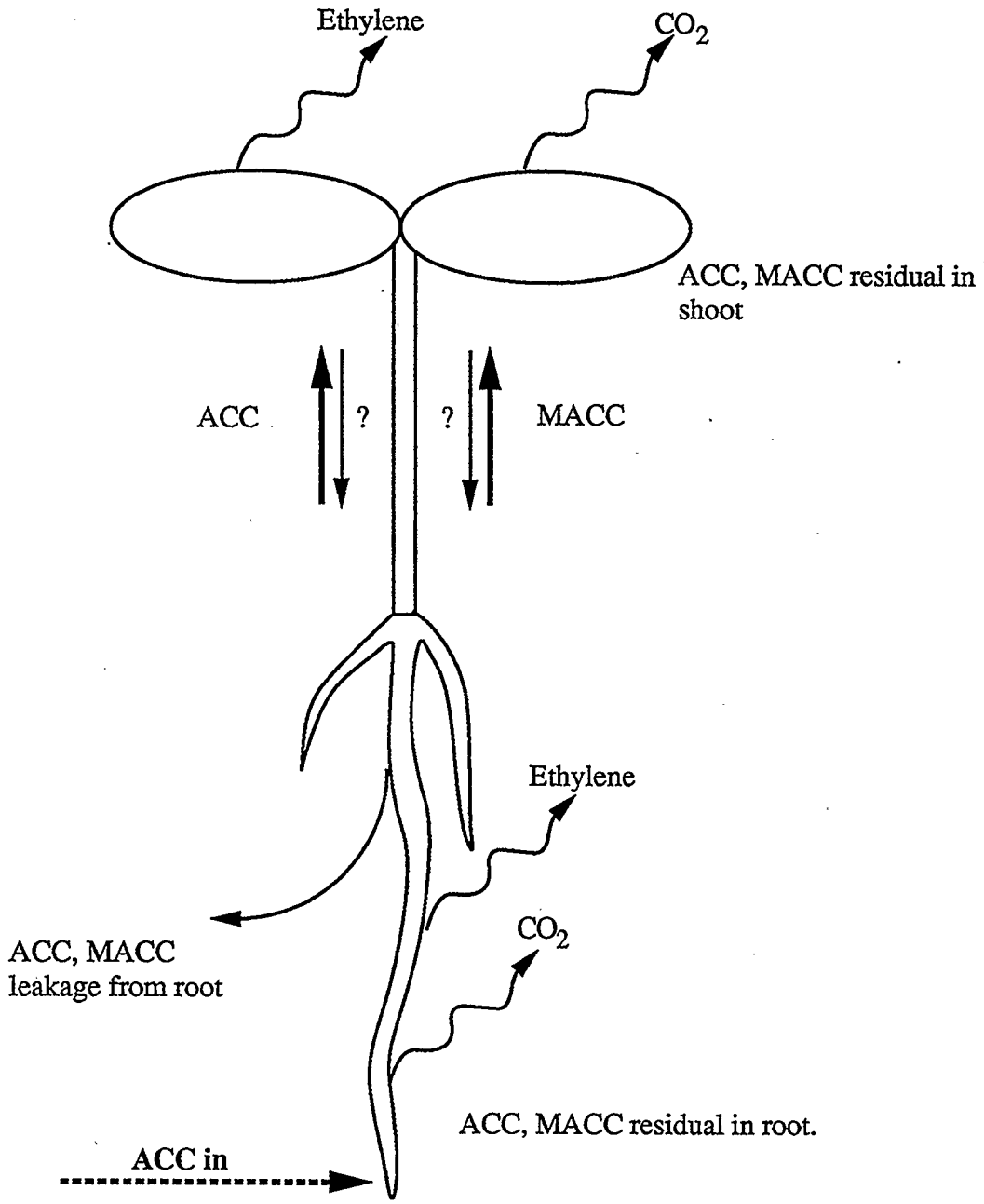

Figure 38. Fate of ACC fed to seedlings of 4 day old sunflower.

large increases in ethylene production by root middles and bases, which show the least ethylene production endogenously. Using a recently developed assay, ACC oxidase activities were determined in the different parts of the root with and without ACC treatment. No difference in ACC oxidase activity levels could be detected with or without ACC-treatment in any of the root parts, however greater endogenous activity was observed in the middles and bases compared to the tips and laterals. In an unstressed, un-treated (without exogenous ACC) situation, ACC oxidase may not limit ethylene production, probably it is the level of ACC, or availability of ACC that regulates ethylene evolution. When plants are fed exogenous ACC, ACC oxidase activity may limit ethylene production, and this may explain the disproportionately larger amount of ethylene emanation from ACC-treated middles and bases, as they posses higher ACC oxidase activities. This study reinforced an earlier theme in that the most actively growing tissues (tips and laterals) produced the most ethylene on a fresh weight basis, calling in to question the importance of ethylene in the regulation of root growth once again. Also, because these tissues contain large amounts of ACC, and because ACC oxidase activities measured show that the potential rate of ethylene production is higher than the actual observed production, we suggest that ACC and ACC oxidase are spacially separated, and that possibly restricted access of ACC to ACC oxidase actually limits ethylene production here. Figure 39 illustrates the levels of ethylene evolution, ACC and MACC within roots of unstressed 4 day old sunflower seedlings.

During the course of experimentation on ethylene, ACC and MACC levels in different parts of the root an *in vitro* ACC oxidase assay was developed, which allowed us to measure ACC oxidase activities from different parts of the root as mentioned above. The work of McGarvey and Christofferson (1992) and Dong *et al.* (1992) showed that CO_2 stimulated ACC oxidase activity *in vitro* strongly. In other systems

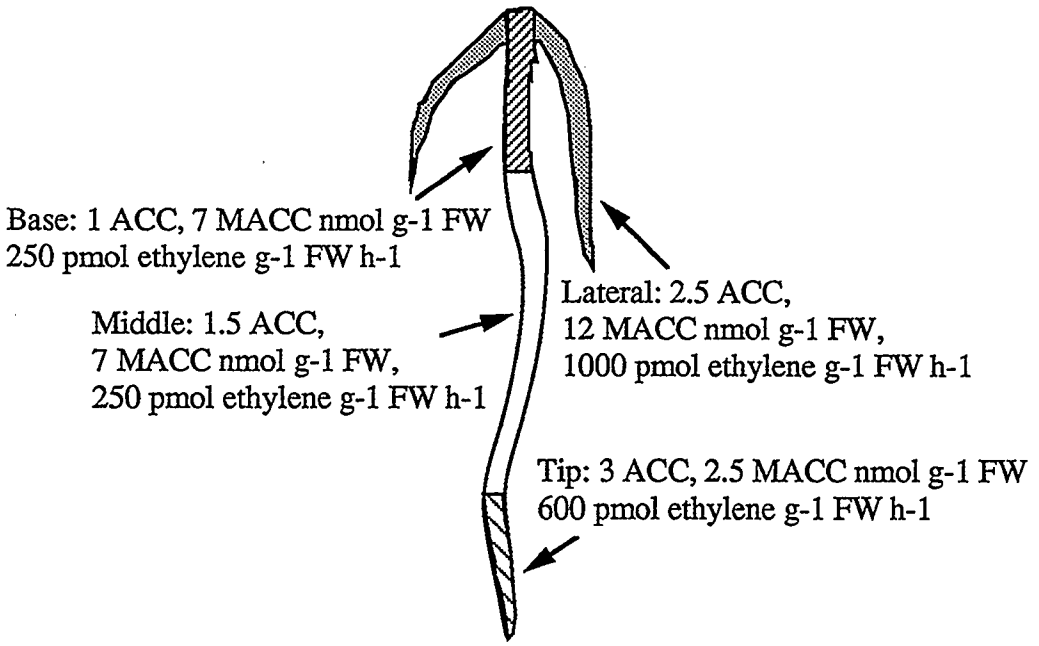

Figure 39. Endogenous levels of ACC, MACC and ethylene in different regions of the root.

such as haemoglobin (Kilmartin and Rossi-Bernardi, 1973) and RUBISCO (Lorimer and Miziorko, 1980) CO_2 modifies enzyme activity by binding as a carbamate to a terminal amino group, which may possibly be the case here, though it is also possible that CO_2 is acting as an allosteric effector. We investigated some of the parameters of the enzyme activity in response to ambient or 5% CO_2 and found that in virtually every case, changes in CO_2 altered these parameters. The effect of treating the roots with IAA or wounding on ACC oxidase activity was also studied. No induction of the enzyme was evident with either of these treatments, and when we take into account the lack of inducibility by ACC (chapter 4) we must conclude that there is always an abundance of potential activity, enough to produce copius quantities of ethylene in response to any situation. In view of the fact that CO_2 enhances ACC oxidase activity *in vitro*, the possible involvement of CO_2 in regulating ethylene production *in vivo* must be considered. Unfortunately we have no data on intracellular CO_2 levels in root tissue. Depending on the CO_2 levels within the root, the potential actvity could be near zero at (0% CO_2) or up to 10,000 pmoles g-1 FW h-1 (5%). All cells make CO_2 by respiration, and presumably this CO_2 is dissolved in the cytolplasm where ACC oxidase may also be present. It is possible that the high ethylene production by fast growing tissues reflects CO_2 production by more rapid respiration in these parts, however, elevated ACC levels are also observed in tips and laterals where ethylene production is highest.

The changes in ACC oxidase parameters with high CO_2 might play an important part in regulating ACC oxidase activity (and ethylene production) *in vivo*. To test this hypothesis, excised roots were incubated with varying concentrations of CO_2. Elevated CO_2 levels did not increase ethylene production as we expected, but were shown to actually inhibit ethylene production. This inhibiton was apparently due to a reduced availability of ACC, through a reduction in ACC synthesis rather than enhanced

conjugation. In view of the fact that ACC oxidase activity is apparently not affected by CO_2 *in vivo* (chapter 6, fig. 36) it is unlikely that the reduced availability of ACC is a compensating mechanism for CO_2 promotion of ACC oxidase activity. These results suggest that either the enhancement of ACC oxidase activity *in vitro* is an artifact, or that CO_2 levels in root cells are already saturating ACC oxidase. Elevating the CO_2 level of shoots has previously been shown to increase ethylene production by these tissues, so the possibility that the CO_2 effect *in vitro* is an artifact is unlikely, it is more likely that CO_2 levels are already saturating ACC oxidase. The roots used in these studies are respiring rapidly as evidenced by their very rapid rate of growth, and thus CO_2 production by these tissues should be high. Possibly an enhancement of ACC oxidase activity with exogenous CO_2 could been seen in slower growing roots, or in roots in which respiration was inhibited.

Treating roots of intact plants with 2% CO_2 did inhibit root elongation, however ethylene production by the root was not affected. In view of the results obtained in chapter 3 where root to shoot transport of ACC was observed, and the results of other studies in which basipetal transport of ACC has been measured (Amrhein *et al.*, 1982), it is possible that the shoot transports ACC to the root, thereby ameliorating the effect of CO_2 on availability of ACC. How does CO_2 decrease root growth? It is likely not due to increased ethylene production, but perhaps through inhibiting the diffusion of endogenous CO_2 out of the root, and thus slowing respiration. Alternatively it is possible that some endogenous ethylene is required for root elongation, and ethylene binding inhibition by CO_2 disrupts normal growth.

In conclusion, ethylene biosynthesis in roots is linked to shoots. Within roots the physiology of ethylene varies, perhaps reflecting the roles of the different root parts studied. ACC oxidase from roots was strongly promoted by CO_2 *in vitro*, but not

affected *in vivo*. Ethylene production by excised roots was decreased by CO_2 but in intact plants ethylene evolution by roots was not affected at all. CO_2 appears to inhibit root growth, but probably not via an effect on ethylene production.

This thesis has dealt with several facets of root physiology simultaneously. The effects of ethylene on root elongation in the system developed was characterized. The relationship between the root and shoot with respect to the transport and metabolism of the ethylene precursor ACC was investigated. The variation in ethylene, ACC, MACC and ACC oxidase levels in different parts of the root was documented. The effect of CO_2 on ACC oxidase activity and the effect of CO_2 on ethylene production by roots *in vivo* was studied. From the wide variety of experiments conducted I have come to the conclusion that more work is required to elucidate the role of ethylene in root growth, and the regulation of ethylene production by roots. In attempting to answer some simple questions about root growth and ethylene biosynthesis this thesis has uncovered some interesting problems for further study. High levels of ethylene induced by ACC treatment in roots inhibits root growth, however the most actively growing portions of the root also exhibit much higher rates of ethylene production than less active parts. It is difficult to see how the two phenomena are co-related. We observed that high levels of CO_2 dramatically promote ACC oxidase activity *in vitro*, yet these same levels have no effect *in vivo*. Why? Although in excised roots ethylene production was actually inhibited by high CO_2, ethylene production was not affected by CO_2 in intact roots. An important message arising from these experiments is that conclusions obtained from isolated systems do not necessarily reflect the physiology of whole plants growing in their native environment.

Chapter 8.

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