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Hormonal Controls of Adventitious Root Initiation in Hypocotyls of *Helianthus annuus* Seedlings

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Hormonal Controls of Adventitious Root Initiation in Hypocotyls of *Helianthus annuus* Seedlings" submitted by Jin-Hao Liu in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

A system for studying the initiation of adventitious root formation (ARF) in hypocotyls of derooted seedlings of sunflower (*Helianthus annuus* L.) is described. It was found that pH of the test solutions and any gravistimulation of cuttings affected ARF. Removal of original root system of the seedlings elicited changes in the levels of endogenous plant hormones and ARF in the basal portion (rooting zone, or RZ) of hypocotyls. Before the excision of original roots, endogenous indole-3-acetic acid (IAA) level in the RZ was higher than that in the non-rooting zone (NRZ) (apical portion of hypocotyls). After the excision of original roots the quantity of IAA decreased in the RZ, but increased steadily in the NRZ. A transient increase in ethylene production in the RZ occurred following root removal, peaking at 3 h. The increase in ethylene production may be due to a wound-induced stimulation in synthesis of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid. This increase in ethylene production was absent in the NRZ.

Exogenous auxins applied for short periods during the early and critical stages of ARF enhanced ARF. When the inhibitors of auxin transport were applied to the hypocotyl just below cotyledons, the number of root primordia was reduced. If the source of endogenous auxin was removed by decapitation (removal of cotyledons and apical bud), the number of root primordia decreased dramatically. Exogenous auxins completely or partially overcame the inhibitory effect of the inhibitor of auxin transport or decapitation.

The inhibitors of ethylene biosynthesis/action reduced ARF in non-decapitated cuttings. Ethylene-releasing compounds enhanced endogenous ethylene production and rooting and they counteracted the effect of ethylene inhibitors. If the source of endogenous auxin was removed by decapitation, ethylene-releasing compounds showed no promotive effect on ARF, but they enhanced auxin action. Surgical removal of a part of hypocotyl tissues

showed that ARF occurred only in the regions where auxin could accumulate although the excision of the tissues might also enhance ethylene biosynthesis in other regions.

All these findings strongly suggest that endogenous auxin, mainly from cotyledons and apical bud, is the primary stimulator of the initiation of ARF. Decrease in free IAA in RZ after the excision of original roots may be due to the 'use' of IAA in ARF. Wound ethylene caused by the excision of original roots is also important in the initiation of ARF, but its action depends on auxin and perhaps other substances.

[^{3}H]-IAA applied to one cotyledon of the seedlings was transported basipetally down the hypocotyls. After the excision of original roots the RZ of hypocotyls was a major sink for this [^{3}H]-IAA. The products of [^{3}H]-IAA metabolism were identical in the hypocotyls of derooted and intact seedlings, as well as in the RZ and the NRZ of the hypocotyls of derooted seedlings. The results suggest that IAA, but not its metabolites acts in ARF.

Ethylene had no effect on auxin transport and metabolism in the hypocotyls, and thus it cannot stimulate rooting via the alteration of auxin level and distribution along the hypocotyls. Ethylene-releasing compounds enhanced the rooting response of hypocotyls to exogenous IAA and decreased the inhibition of rooting by IAA transport inhibitor N-1-naphthylphthalamic acid (NPA). The inhibitor of ethylene action, silver thiosulphate, reduced the rooting response of hypocotyls to exogenous IAA and increased the inhibition of rooting by NPA. It is concluded that ethylene promotes ARF by enhancing the sensitivity of tissue to auxin. Exogenous auxins stimulated ethylene production in the RZ, thus this ethylene may have a promotive feedback effect on the stimulation of ARF by auxin.

Promotion of auxin transport to the RZ of hypocotyls and stimulation of ethylene production may account for the promotive effect of acidic pH on ARF. The promotive effect of piperazine on ARF may be partially due to its ability to induce ethylene synthesis. A scheme for the interaction of various hormones and other factors in ARF is proposed.

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LIST OF ABBREVIATIONS

ABA ..... abscisic acid

ACC ..... 1-aminocyclopropane-1-carboxylic acid

ARF ..... adventitious root formation

AVG ..... aminoethoxyvinylglycine

BA ..... benzyladenine

BITC ..... benzyl isothiocyanate

CK ..... cytokinins

DMGA ..... dimethylglutaric acid

ethephon ..... 2-chloro-ethylphosphonic acid

FP 1-formylpiperazine

GA ..... gibberellin(s)

GC ..... gas chromatography

GC-MS ..... GC-mass spectrometry

GC-MS-SIM ..... GC-MS-selected ion monitoring

HEP N-2-hydroxyethylpiperazine

HEPES ..... N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HPLC ..... high performance liquid chromatography

IAA ..... indole-3-acetic acid

IAAsp indole-3-acetyl-L-aspartate

IBA ..... indole-3-butyric acid

MACC ..... 1-(malonyl)-ACC

MTBSTFA ..... N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide

NAA ..... 1-naphthaleneacetic acid

NPA ..... N-1-naphthylphthalamic acid

NRZ ..... non-rooting zone

P-GG ..... piperazine-glycylglycine

PIPES ..... piperazine-N,N'-bis(2-ethanesulfonic acid)

RZ ..... rooting zone

SA ..... salicylic acid

STS ..... silver thiosulphate

TBDMS ..... tert-butyldimethylsilyl

TIBA ..... 2,3,5-triiodobenzoic acid

Chapter 1. GENERAL INTRODUCTION

Adventitious roots are the roots that occur in any parts of the plant other than the tap root (originates from the embryonic root) or lateral roots (Cutter 1971). Adventitious roots are commonly seen in nature. For example, in many species, adventitious root primordia can form near the stem bases of intact plants. These root primordia may emerge as the prop-roots under certain environmental conditions. Adventitious roots can also be seen on the lower surface of stems or branches placed horizontally and kept moist. This trait of a plant has sometimes been used by nursery people to produce new plants by the method called "layering" (Hartmann & Kester 1975). A major proportion of the root system in many water plants is made up of adventitious roots. In addition to their nutritional function, adventitious roots of these plants also hold the stems in an erect position. The vegetative parts of many species are able to develop adventitious roots. This is the basis for the common practice of vegetative propagation of many species. A central reason for using vegetative propagation is to maintain genetic purity of the plants, especially ornamentals. Vegetative propagation also offers an advantage of reduced lag time between seed germination and attainment of sexual maturity (several years in many trees). A greater understanding of the underlying physiological processes of adventitious root formation (ARF) may reveal possibilities for manipulation of ARF in many economic plants, some of which are difficult to root. Research on ARF is not only commercially, but also scientifically important. Adventitious root formation in tissue culture and lateral root formation show much similarly to ARF in cuttings. What is learned about ARF may also yield new information directly about rooting in tissue culture and lateral root formation. A study of ARF is also a good model of a developmental process. Understanding this developmental process perhaps will aid in the understanding of other developmental

processes. The studies described below were undertaken in order to gain better understanding of the regulatory systems of ARF.

Adventitious root formation has by some author been divided into initiative phase and developmental phase. Initiative phase usually refers to cellular differentiation that directly yields initial cells of root primordia. Developmental phase refers to division of the initial cells and of cells adjacent to the initial cells to form roots. The regulatory system for each specific phase of ARF could differ. The present study focused on initiative phase of ARF.

Adventitious root formation is controlled by a complex interaction between endogenous and environmental factors. Various types of endogenous factors might be involved in the control of ARF. We can distinguish them as plant hormonal and nutritional ones. Hormonal factors may include all five groups of well-accepted plant hormones, i.e. auxins, gibberellins (GA), cytokinins (CK), abscisic acid (ABA) and ethylene (more discussion see next paragraph). Although polyamines and phenolic compounds may also play a role in ARF. There is a controversy whether polyamines should be classified as hormones. Nevertheless, exogenous polyamines have been shown to promote rooting from the hypocotyl cuttings of mung bean under some circumstances (Jarvis et al. 1983, Shyr & Kao 1985), while in other cases they have no effect (Friedman et al. 1982, Biondi et al. 1990). Phenolic compounds also promoted root initiation in some cases (Kawase 1964, Fadl & Hartman 1967, Roy et al. 1972). They may fall into this catalogue, as these substances might influence ARF via an inhibition of indole-3-acetic acid (IAA) oxidase activity (Zenk & Muller 1963, Jarvis 1986). Nutritional factors are carbohydrates and mineral nutrients such as nitrogen, phosphorus et al. Considering carbohydrates, numerous studies have demonstrated that exogenous sugars, including sucrose, glucose, myo-inositol, enhanced the rooting response of cuttings (e.g. Nanda et al. 1971, Eliasson 1978, Jarvis & Booth 1981). However, after comprehensive studies, Veierskov et al. (1982ab,

Veierskov & Anderson 1982) were not able to demonstrate any obvious relationship between irradiance, carbohydrate content and rooting from pea cuttings. They suggested that carbohydrates might be of importance for root growth. There is sufficient experimental evidence to confirm the need for nitrogen, phosphorus, potassium and sodium during root growth and development, especially boron is an essential micronutrient (Hemberg 1951, Gorter 1958). However, the importance of various mineral nutrients in the initiation of ARF is not clearly understood. The environmental condition of stock plants and cuttings is also of importance for ARF. Light, water, nutrients, temperature and wounding of the cuttings all influence the ARF. In this study, I report on the investigations into some of the hormonal controls of ARF, particularly in the initiative phase, in the hypocotyls of derooted sunflower (*Helianthus annuus* L.) seedlings.

Through numerous investigations, after the early work of Went (1935), it has been well established that exogenous auxin is of crucial importance in the initiation of ARF in cuttings. Supplied auxins are effective in stimulating adventitious rooting (e.g. Katsumi et al. 1969, Haissig 1970, Smith & Thorpe 1975, Fabijan et al 1981b, Jarvis & Booth 1981). Synthetic auxins indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) are usually more effective than the natural one, IAA (e.g. Zimmerman & Wilcoxon 1935, Kawse & Matsui 1980, Geneve & Heuser 1982). It is thought that this may be related to the greater resistance to degradation of synthetic auxin in plant tissues (Fawcett & Wightman 1960). There is evidence that oxidation of IAA in plant tissues was much more rapid than that of indole acids with different length of side chain. Recently, Wiesman et al. (1988) found the rates of metabolism of applied IAA and IBA in mung bean cuttings were similar. Thus the assumption that the greater ability of IBA over IAA to promote ARF is due to its reduced degradation is questionable. There is limited evidence relating levels of endogenous IAA to rooting ability of tissues (see reviews by Gaspar & Hofinger 1988,

Jarvis 1986). Whether endogenous auxin is involved in rooting process is still uncertain (see Chapter 3. Introduction). Apical or basal application of kinetin or benzyladenine (BA) generally inhibit ARF (e.g. Humphries 1960, Eriksen 1974, Smith & Thorpe 1975, Fabijan et al. 1981b). Exogenous GA_3 has been widely reported to inhibit adventitious rooting in cuttings of a variety of species (e.g. Kato 1958, Brian et al. 1960, Krishnamoorthy 1970, Fabijan et al. 1981b). In most experimental systems exogenous ABA promotes adventitious rooting (Chin et al. 1969, Basu et al. 1970, Hartung et al. 1980, Rasmussen & Anderson 1980). But unlike auxin, the promotive effect of ABA is too small to have any commercial value. Ethylene and ethylene analogues were found to stimulate ARF as early as 1930s by scientists at the Boyce Thompson Institute for plant research (Zimmerman & Wilcoxon 1935). Although this observation has been frequently corroborated, there are also numerous reports to the contrary (see Chapter 5. Introduction). The induction of ARF often includes a necessary step of stress or injury which in turn causes an increase in ethylene production. For instance, Wample & Reid (1979) observed that flooding the roots of sunflower plants induced ARF in hypocotyls and an enhancement of ethylene production in the hypocotyls preceded ARF. Drew et al. (1979) reported that flooding the roots of maize caused endogenous produced ethylene to accumulate in the roots and stimulated the emergence of adventitious roots from the base of the shoot. Excision of original root system induces the formation of adventitious roots. Wounding due to the excision resulted in increased ethylene biosynthesis (Saltveit & Dilley 1978). Therefore, stress and wound-induced ethylene production may play an important role in rooting.

In view of the presumptive central role of auxin and the contradictory data on the role of ethylene on rooting, this investigation was focused on the control of ARF by auxin and ethylene. This study has designed to answer the following questions:

1) Is endogenous auxin involved in the initiation of ARF?

2) Does the wound ethylene caused by excision of original roots have a role in the initiation of adventitious roots?

3) What are the possible relationships between endogenous auxin and ethylene in the control of ARF?

The writer's hypothesis is that auxin is directly involved in the control of ARF as a primary trigger, while the involvement of ethylene in the initiation of ARF is indirect. One mediator of ethylene action might be auxin. To test this hypothesis, I firstly investigated the possible role of endogenous auxin and ethylene on rooting in sunflower hypocotyls. I chose the sunflower system because the laboratory already had much experience and data on this system. The developmental sequences of adventitious rooting in this species have described in detail by Fabijan et al. (1981a). The first visible event could be seen 24 h after removal of the original root system. There was a swelling of cell nuclei in the root initials. Distinct small root primordia can be seen by 48 h. In my studies the status of the endogenous hormones was compared between the rooting zone (RZ) and non-rooting zone (NRZ) of the hypocotyls at the time of and after root removal. Various exogenous substances were applied to enhance or reduce hormone levels in the RZ of the hypocotyls, or inhibit hormone action. Their effects on the number of root primordia were observed. Then, the effect of ethylene on rooting in auxin-depleted hypocotyls was examined. The effect of exogenous auxin on ethylene production, the effect of ethylene on auxin transport, metabolism and tissue responsiveness were also studied.

Chapter 2. EXPERIMENTAL SYSTEM

INTRODUCTION

The experimental system used was a modification of that used by Fabijan et al. (1981a). In that system (details given later) sunflower (*Helianthus annuus* L.) seedlings were grown until 6 days old. At that stage the roots were excised 5 mm above the root/hypocotyl transition zone and the cut end of the hypocotyls of the resultant cuttings was placed into a vial containing 20 ml of the appropriate test solutions for various periods (Plate 1, left). However, there were two potential problems with this system. Firstly, although the densely sown seedlings in the germination tray stood upright, as soon as they were placed in the rooting vials the tops of the seedlings tended to bend over. Secondly, in the original Fabijan rooting system, and the system used by many other workers, unbuffered test solutions were used to treat cuttings and I wondered if pH might influence rooting.

In many laboratory experiments the potential problem of gravistimulation of plants, by placing them in abnormal orientations, has been overlooked. When many plant organs are reoriented with respect to gravity, they are gravistimulated, and this can lead to many endogenous changes (Phillips 1972, Slocum & Roux 1983, Pharis et al. 1981, Bandurski et al. 1984, Feldman et al. 1985) including an increase in ethylene production (Abeles 1973, Clifford et al. 1983, Kaufmann et al. 1985, Wheeler et al. 1986). In the rooting system as used by Fabijan et al. (1981a), the tops of the seedlings tend naturally to bend over to various degrees (Plate 1, left). This bending will result in gravistimulation of the upper parts of the seedlings that could influence the outcome of the experiments. Since I suspected (see Chapter 1) that ethylene played a role in ARF, I decided to investigate the effect of gravistimulation of the derooted seedlings on ethylene production and ARF,

using a modified system as a control. The modified system used 5 small glass tubes placed vertically in a vial and held together with a rubber band (Plate 1, right). In this way all portions of the hypocotyl were kept upright. Another experiment was also designed to separately gravistimulate top half of the hypocotyl, the bottom half, and both halves. Their effects on ethylene production and the number of root primordia were investigated.

In many rooting experiments, a wide range of unbuffered solutions of different compounds have been used to treat plant tissues. The pH of these will vary depending not only on the nature of the compounds, but also on their concentration. It is thus important to know if there was any effect of pH on ARF in these studies. It was suggested as far back as the 1920s, that rooting of cuttings might be influenced by the pH of the rooting medium (Smith 1926, Hitchcock 1928). Since then, however , there have been relatively few reports on the effect of pH on adventitious rooting and the results have been very variable. Furthermore, much of the previous work on pH and rooting was on hard-to-root woody species and little is iknown of the response of easy-to-root herbaceous species. The latter are commonly used as experimental systems when studying adventitious rooting. For these reasons, I decided to investigate the effect of pH on ARF in sunflower hypocotyls. To see at what stage of ARF the pH may affect, a buffer was also supplied to the hypocotyls at different times after the start of the experiment and for differing periods during the root initiation process.

MATERIALS AND METHODS

Seedling preparation and rooting system

Fruits of the sunflower (*Helianthus annuus* L. var. Dahlgren 131, Dahlgren and Co., Inc. Crookston, MA, USA) were sown in moist Teragreen at 23°C day and 18°C night with a 16 h photoperiod (Sylvania F48T12/ws fluorescent tubes). Six days later cuttings were produced by excising the roots at 5 mm above the transition zone of hypocotyl and roots under water. The cut ends of the hypocotyls were then placed in brown 30 ml glass vials containing deionized water or the test solutions (20 ml). When the test solutions were removed the hypocotyls were well rinsed with deionized water. The two different rooting systems are shown in plate 1. In the "old" system (left) based on that described by Fabijan et al. (1981a) the weight of the cotyledons causes the hypocotyl to naturally bend over. In the 'modified' system (right) each hypocotyl was supported by a glass tube and had a vertical position. The vials were covered by aluminum foil. The irradiation at the cotyledons was 32 W m^{-2}. In the experiment designed to explore other variations in gravistimulating the hypocotyls, the hypocotyls of the derooted seedlings were held in four orientations: 1. top half inclined with vertical bottom half; 2. bottom half inclined with vertical top half; 3. both halves vertical; 4. both halves inclined. The inclination was 45° and for orientation 1 and 3 the lower portion of the tubes was held in a vertical position and for 2 and 4 in an inclination of 45° (Plate 2). The hypocotyls were very flexible and naturally tended to bend over and no substantial amount of force was necessary to hold the hypocotyls in these various positions. For instance, there was no difference in the force needed to hold treatments 1 and 2 in their different orientations. The straight and bent glass tubes were then placed into small test tubes containing deionized water. The derooted seedlings were carefully inserted into the glass tubes. It took 10 - 15 min for making all

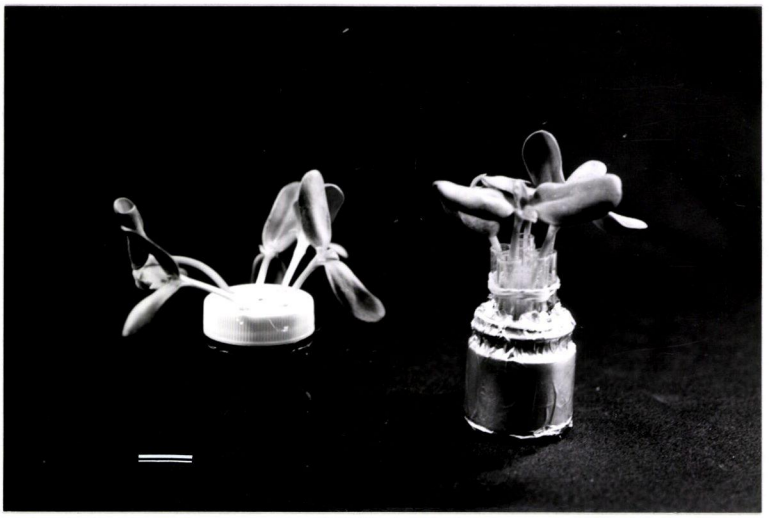


Plate 1. Left: "old" system of holding sunflower cuttings for adventitious rooting experiments, in which the cuttings naturally tend to bend over under their own weight. Right: "modified" system, in which cuttings are held vertical by means of each seedling being placed individually in an upright glass tube. Bar at lower left indicates 2 cm.

3 1 2 4

Plate 2. Devices for holding the cuttings in 4 orientations. Bar at lower left indicates 2 cm.

derooted seedlings to be placed in different orientations. The derooted seedlings were then placed in the growth chamber with conditions identical to those used for raising seedlings.

Ethylene measurements

Ethylene production from the tissues was estimated at 6 h after roots excision. Ethylene samples were taken by using a device made by connecting two 10-ml syringes with a 3-way valve. In this apparatus, hypocotyls were placed in syringe A, while syringe B was used for withdrawing sample gases. Syringe B was first flushed with ethylene-free air and then sealed. Hypocotyls were cut and immediately put into syringe A. This syringe was flushed with ethylene-free air. At time zero the apparatus was closed from contact with outside air. The plunger of syringe A was in the 10 ml position and syringe B in zero position. Precisely 10 min later, gas samples were withdrawn from syringe A into B by pulling out the plunger of syringe B and at the same time pushing in the plunger of syringe A. The sample gases from syringe B were injected into a gas chromatograph (GC) with a flame ionization detector (Varian 3700, Varian Instrument Division, Walnut Creek, CA, USA) for ethylene analysis. This procedure was used to eliminate the possibility of contamination from ethylene air pollution. In Calgary the ambient ethylene can occasionally reach levels as high as 100 nl. l^{-1} (Reid & Watson 1985). Columns (2 m X 2 mm I.D.) of Poropak N (80-100 mesh, Analabs Inc, North Haven, CT, USA) were used under the following conditions: column temp 40°C, detector temp 120°C, injector temp 30°C. Carrier gas was N_2 with a flow rate of 30 ml min^{-1}.

Buffers and their application

In one experiment (Fig. 3 A) four buffers were used: Na_2HPO_4-citric acid (phosphoric

acid, pK_1=2.15, pK_2=7.20, pK_3=12.33; citric acid, pK_1=3.13, pK_2=4.76, pK_3=6.40), citric acid-sodium citrate, Sörensen (Na_2HPO_4-KH_2PO_4) and Na-borate (boric acid, pK=9.23). All chemicals used in this experiment were purchased from Fisher Scientific Co. (Fair Lawn, NJ, USA). In another experiment (Fig. 3 B), some potentially less metabolically active buffers were used. These were: KH phthalate (phthalic acid, pK_1=2.95, pK_2=5.41); dimethylglutaric acid (DMGA) (pK_1=3.70, pK_2=6.34); maleic acid (pK_1=2.00, pK_2=6.26)-Tris (pK=8.06); Tris and piperazine (pK_1=9.81, pK_2=5.55)-glycylglycine (pK_1=3.14, pK_2=8.25) (P-GG). All chemicals used in this experiment were purchased from Sigma Chemical Co. (St. Louis, MO, USA). In both experiments all buffers were made following the methods described by Perrin and Dempsey (1979). The pH of the buffers was measured using an Accumet Model 230 A pH/Ion meter (Fisher Scientific Co.). Twenty ml of buffer (5 mM) was supplied to the base of the hypocotyls from 0 - 5 h after derooting. After 5 h in the buffer, the basail cut ends of derooted seedlings were placed in deionized water (pH 5.9) throughout the remainder of the experiment. After 72 h, hypocotyls were harvested for the estimation of number of root primordia. All experiments presented in this thesis, in which the number of root primordia was investigated, were conducted at least twice. Each treatment in an experiment consisted of 15 - 20 hypocotyls. All repetitions for a specific investigation gave similar results. The results of one of the experiments are presented.

Estimation of number of root primordia

Since I wished to study the early stage of the initiative phase of ARF rather than the later process of development of the root primordia and its elongation, I made observations of number of root primordia formed inside the hypocotyls in cleared tissue. Four cm long sections from the cut end of the hypocotyls were fixed, cleared and stained in 5%

chromium trioxide for 24 h. The sections were rinsed with distilled water and maintained in distilled water until estimates of primordia number were made using a binocular dissecting microscope (10 X). In many cases standard errors of the means were calculated. In some experiments, the square root of the number of primordia were compared in different samples by analysis of variance. If differences were found, further analysis was performed using the Neuman-Keuls multiple-range test (Zar 1984). The 95% significance level was used.

RESULTS

Gravistimulation, ethylene production and the number of adventitious roots

Table 1 shows that the 'old system' resulted in increased levels of ethylene, especially in the top half of the hypocotyl, and in much lower number of adventitious roots. The experiment was then extended to include gravistimulation of top half of the hypocotyl, the bottom half, both halves and a control (see inset in Fig. 1). In group 1, only the top half of the seedling was gravistimulated; in group 2, only the bottom half was gravistimulated; group 3 was the control (vertical with no gravistimulation); and the entire seedling in group 4 was gravistimulated. The results of this second experiment (Fig. 1) supported the findings given in Tab. 1. They also suggested that any part of the sunflower hypocotyl that is gravistimulated (in this case, 1T, 2B, 4T, and 4B) will produce increased levels of ethylene, regardless of the orientation of the shoot apex. If one compares the ethylene production by vertically oriented bottom halves (1B and 3B), it will be seen that it is greater than that of vertically oriented top halves (2T and 3T). This is opposite to the usual pattern (slightly more ethylene production from apical regions), and can probably be accounted for

Tab. 1. Ethylene production (A) [p mol $(gFW)^{-1} h^{-1} \pm$ SE, n = 3 or 4] and number of root primordia per hypocotyl (B) in hypocotyls held in two different systems shown in plate 1.

A.

time (h)	top half		basal half	
	old	modified	old	modified
6	17.5 ± 3.02	6.9 ± 0.20	14.3 ± 1.71	9.8 ± 0.65
29	12.6 ± 1.18	6.9 ± 0.37	10.2 ± 1.55	11.8 ± 0.65
37	14.3 ± 6.30	4.1 ± 1.06	10.2 ± 0.65	9.4 ± 0.37
50	14.7 ± 1.92	5.7 ± 2.30	15.5 ± 0.86	10.2 ± 0.65

B.

	number of root primordia ± SE, n = 30
old system	28.8 ± 2.68
modified system	48.8 ± 3.79

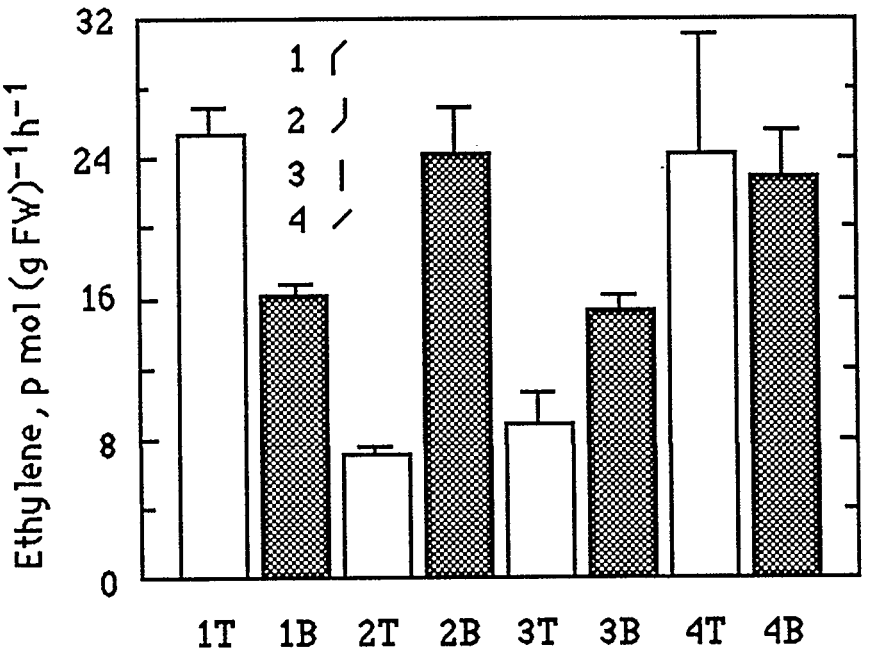


Fig. 1. Ethylene production in the top (T) and bottom (B) halves of derooted seedlings in orientations after 6 h. Means ± SE (n=3).

by the contribution of wound ethylene to the levels seen in the basal halves, since only 6 h were allowed to elapse between root excision and measurement of ethylene. In Fig. 2, the number of root primordia formed in vertical seedlings was nearly twice the number formed in any of the other three orientations, where some or all of the plant was gravistimulated.

pH and adventitious rooting

After subjecting cuttings to various buffer solutions at a range of pH values, it was observed that with each buffer, the largest number of root primordia were found at the lowest pH (Fig. 3). With all the buffers as the pH decreased, numbers of root primordia increased. At medium pH values (6.0 - 8.0), most of the buffers, eg. Na_2HPO_4-citric acid, Sörensen, DMGA and maleic acid-Tris, had no stimulatory effect on rooting compared to deionized water controls which had a pH of 5.9. Some of these buffers showed a slight promotive effect at the highest pH treatments. Treatment with P-GG buffer showed the same trends; however, it stimulated a larger number of primordia than other buffers. It was observed that P-GG at pH 4.5 resulted in severe necrosis of the hypocotyls. The difference in the number of root primordia between P-GG and other buffers and the generally stimulatory effect of low pH, suggests two effects, (a) a pH effect, the acid condition being stimulatory and (b) a separate effect of buffer components. This idea led the writer to examine the effect of the components of P-GG buffer. The solution of piperazine or glycylglycine at pH 5.5 was then applied to the base of hypocotyls separately. Figure 4 shows that piperazine was active, while glycylglycine was inactive in promoting adventitious rooting.

Treatment with the buffer at pH 4.0 for the first 5 h was the most promotive (Fig. 5). Increasing the exposure time to 10 or 24 h did not significantly promote rooting above the levels found in the 0 - 5 h treatment.

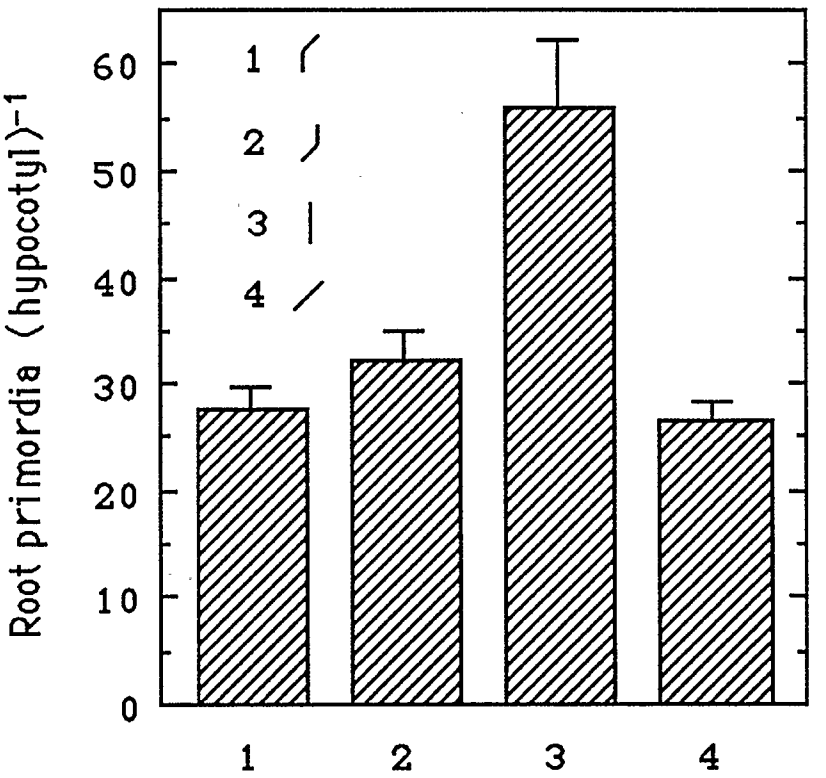


Fig. 2. Number of root primordia per hypocotyl formed in seedlings held at four orientations. Means ± SE (n=20)

Fig. 3. Effect of pH on formation of root primordia in hypocotyls. Buffers (all 5 mM) were applied from 0 - 5 h. A. deionized water (pH 5.9), ■; Na_2HPO_4-citrate, ◊ ; sodium citrate-citric acid, ○ ; Sörensen (Na_2HPO_4 -KH_2PO_4), Δ ; Na-borate, • . B. deionized water, □; KH phthalate, ○ ; dimethylglutaric acid (DMGA), ◆; maleic acid-Tris, • ; Tris, ◊ ; piperazine-glycylglycine (P-GG), ■ . Means ± SE (n = 15 - 20).

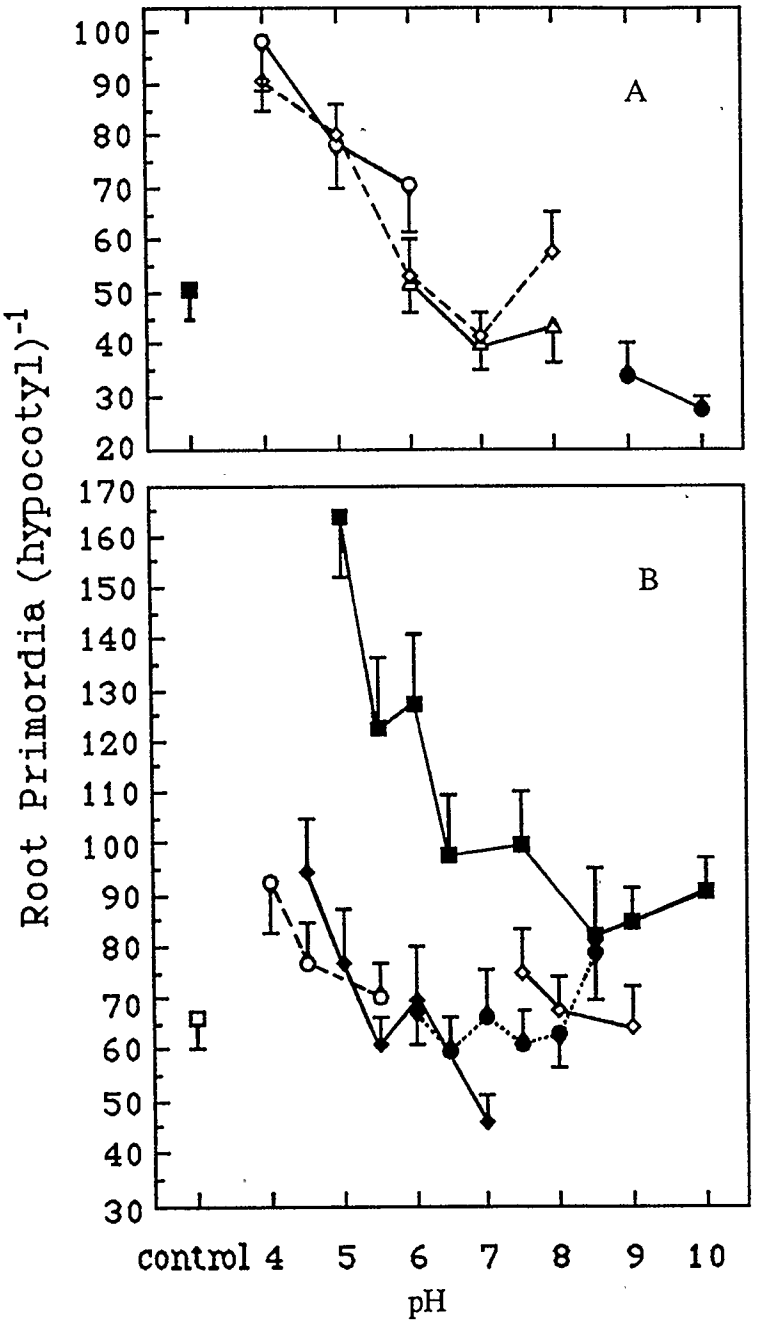

Root Primordia (hypocotyl)$^{-1}$
A
B
100
90
80
70
60
50
40
30
20
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
control 4 5 6 7 8 9 10
pH

Fig. 3.

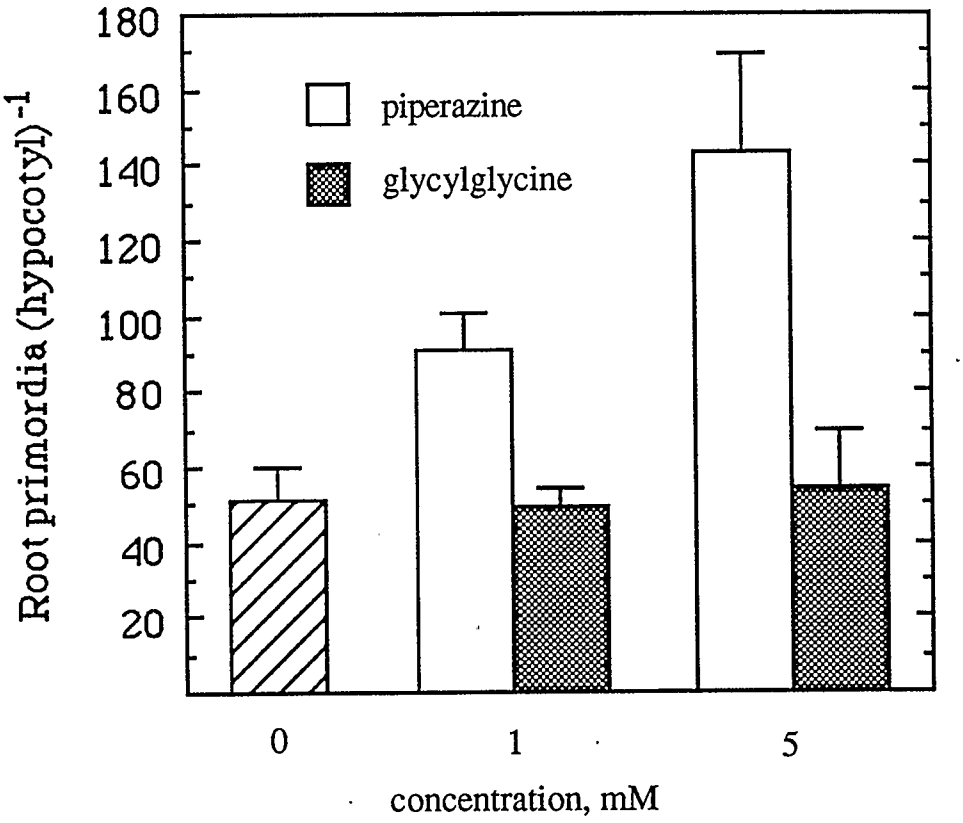


Fig. 4. Effect of piperazine and glycylglycine on the formation of adventitious root primordia on the hypocotyls. Solutions of the chemicals at pH 5.5 were applied to the base of the hypocotyls from 0 -5 h. Means ± SE (n = 20).

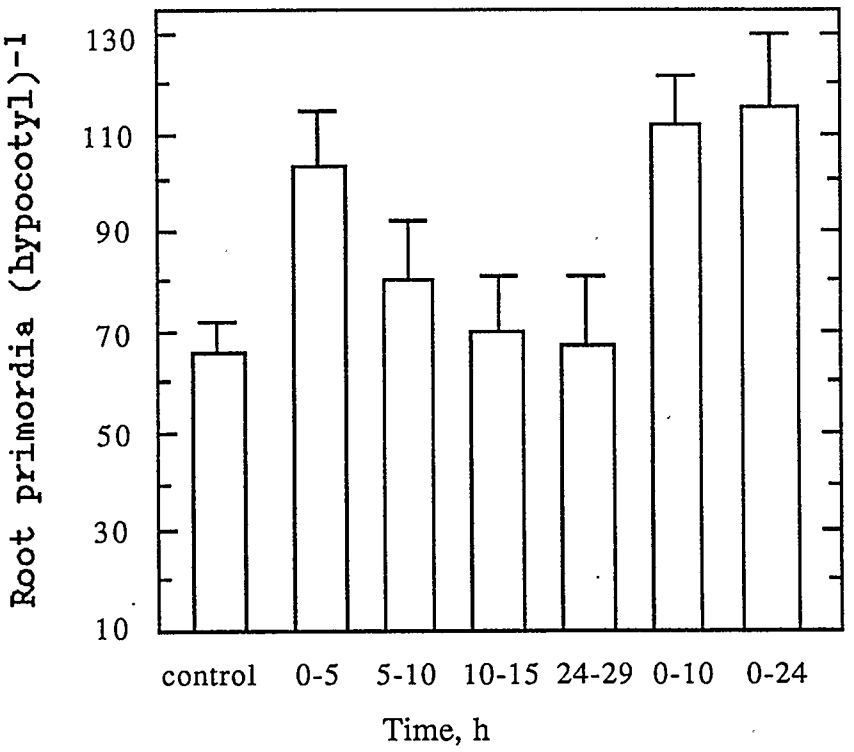


Fig. 5. Effect of Na_2HPO_4 -citrate buffer, supplied at pH 4 for various periods after original root removal, on the production of adventitious root primordia in hypocotyls. When cuttings were not in the buffer they were placed in deionized water. Means ± SE (n = 20).

DISCUSSION

The reduction of adventitious rooting by gravistimulation of derooted sunflower seedlings suggest that gravistimulation might be a problem in the study of ARF in that upright and bent cuttings produce different numbers of adventitious roots. The experiments also showed an increase in ethylene production in the hypocotyl regions that naturally bend over. Presumably bending resulted in gravistimulation, which in turn promoted ethylene production (Wheeler & Salisbury 1981, Clifford et al. 1983, Kaufmann et al. 1985). The results presented here lend support to the ideas (Firn & Digby 1980, Britz & Galston 1982, Hart & MacDonald 1984) that gravitropism in some plants is not restricted to the apical regions, as suggested by Iwami & Masuda (1974). Gravistimulation might also cause changes in endogenous auxin (Wright et al. 1978, Bandurski et al. 1984). In an experiment with [^{3}H]-IAA applied to one cotyledon, I observed that less [^{3}H]-IAA was found in the RZ of the hypocotyls held with "old system" than in the hypocotyls held with "modified system" after a specific period of transport (data not shown here). Auxin has been suggested to have a central role in the control of adventitious rooting (see Chapter 1). Whether the inhibition of rooting by gravistimulation is caused by these endogenous changes is unknown.

Gravistimulation of plant tissues may also influence the response of the tissues to exogenous plant hormones, for example, reduced effectiveness of ethylene on epinasty of tomato and African marigold plants by gravistimulation (Abeles 1973), and altered growth response to exogenous auxin and GA_3 by gravistimulation in pulvini of oat plants (Brock & Kaufman 1988). It is possible that gravistimulation in sunflower hypocotyls may also alter rooting response of the hypocotyls to applied plant hormones.

Thus, gravistimulation is a potential and unwanted component in the interpretation of results of investigation of ARF. I thought it best to eliminate the complicating effect of gravistimulation.

The effect of pH on rooting seems to have been largely overlooked, which is unfortunate, as unbuffered solutions of varying pH are sometimes used in studies on adventitious rooting. The experiment described here shows that as the external pH around the cut ends of sunflower hypocotyls is lowered, adventitious rooting in the basipetal portion of the hypocotyls is greatly promoted. These acidic conditions initiate this effect within the first 5 h after the derooted seedlings have been made. It is during these first few hours when the critical events, such as increased ethylene evolution concerning root primordia initiation, occur (Fabijan et al. 1981a, Chapter 5). Thus when studying the effects of any applied chemicals on rooting, one must take this pH effect into consideration.

Some buffers are more effective than others. It is clear that the stimulatory effects of acidic buffer solutions are not straightforward. For instance, when any of these buffers were used as acidic solutions, rooting was promoted. However, some buffers were much more effective than others. This suggests that there may be two promotive effects. Firstly, H^+ may stimulate rooting and secondly, specific chemical components in the buffer mixtures may have an effect. This was particularly evident in the case of P-GG. As yet it is difficult to explain this latter effect. It is possible that the promotive effects of buffer components were caused by the simple addition of metabolic substrate as phosphate or citrate. However, as the less metabolically active P-GG was the most effective substance, the writer is not inclined to favour that idea. Further, the observation that deionized water at pH 5.9 generally had the same effect as the potentially metabolically active buffers at that pH, indicates that these buffers are not promoting rooting at lower pH simply by acting as metabolites. Five mM piperazine and glycylglycine solutions were tested separately with

the pH being adjusted to 5.5. Piperazine alone was found to stimulate adventitious rooting, while glycylglycine had no effect on rooting.

For all experiments described in the rest of this thesis the modified system was used in which the hypocotyl of the derooted seedlings was held as near as possible in a vertical position, and the pH of the solutions was adjusted to 5.9 (same as in our deionized water) using NaOH. The gravitropism experiments did, also suggest that alterations in ethylene concentration in tissues might affect the initiation of roots. This will be discussed further in Chapter 5.

Chapter 3. THE ROLE OF ENDOGENOUS AUXIN

INTRODUCTION

Exogenously applied auxins, especially some of the synthetic ones IBA and NAA, consistently stimulate ARF in cuttings of many species (Blazich 1988). However, the role of endogenous auxin in ARF is still not well understood (Jarvis 1986, Gaspar & Hofinger 1988). There is other evidence suggesting that endogenously produced auxin is involved in adventitious rooting. For instance, the inhibitors of auxin transport, 2,3,5-triiodobenzoic acid (TIBA, Niedergang-kanueb & Skoog 1956) and N-1-naphthylphthalamic acid (NPA, Morgan & Söding 1958), decrease ARF when applied to the tissue between shoot apex and the zone of maximal rooting of cuttings (Katsumi et al. 1969, Aung 1972, Fabijan et al. 1981b). Number of roots per cutting is also reduced when endogenous source of auxin was removed by decapitation and/or debudding (Haissig 1970, Mohammed & Eriksen 1974, Fabijan et al. 1981b, Dembny et al. 1988).

There is a paucity of published data on the relationship between the levels of endogenous auxin and the ability of non-woody cuttings to form adventitious roots (see reviews of Jarvis 1986, Gaspar & Hofinger 1988). Much of this work either concerns woody tissues (Smith & Wareing 1972ab, Alvarez et al. 1989) or has used methods of auxin quantification and identification that are less rigorous than gas chromatography-mass spectrometry (GC-MS) (Hemberg 1954, Wample & Reid 1979, Weigel et al. 1984). The GC-MS method has recently been used for estimates of auxin levels in woody tissue (Alvarez et al. 1989) but I am unaware of such work with non-woody tissues. Despite this, there is evidence indicating that an accumulation of auxin in the rooting region is a prerequisite for root initiation. Unfortunately, apart from the work of Maldiney et al.

(1986) who determined IAA by immunoassay, there are few studies which make use of rigorous methods of IAA quantification to measure changes in IAA levels in the hours prior to the initiation of root primordia. These early events are likely of importance in initiating adventitious root primordia.

The specific objective of the experiments in this Chapter was to study the possible role of endogenous auxin in rooting in the hypocotyls of sunflower seedlings. To achieve this objective a number of strategies were used. (i) A key strategy was to compare the changes in endogenous auxins in the basal one third (the RZ) of the hypocotyls of derooted seedlings that normally produces most of the root primordia, to that in the apical portions (the NRZ) of the hypocotyls, which do not normally produce roots. (ii) The alterations in endogenous free and conjugated IAA, that occur early on in the rooting process, were examined using rigorous method of GC-MS-selected ion monitoring (GC-MS-SIM) using 3 characteristic ions with an internal standard of [$^{13}C_6$]-IAA. (iii) It is believed that auxin is synthesized in the young leaves and meristem and transports basipetally (Scott & Briggs 1960, Matthysse & Scott 1984). A variety of surgeries were made on the hypocotyls of intact or derooted seedlings in an attempt to intercept endogenous IAA from the cotyledons of seedlings (see Fig. 9). (iv) Transport of endogenous IAA was inhibited with known inhibitors of IAA transport and by removal of the organs thought to be IAA sources. The effects on adventitious rooting were then followed. (v) I also observed the abilities of various exogenous auxins to promote rooting and to overcome the treatments in (iv) above.

Although some of these techniques have been individually used on a wide range of species, as far as I am aware, not all of these approaches have been used on one particular species and in one tissue type under exactly the same conditions.

MATERIALS AND METHODS

Plant material, growth conditions and modified rooting system were as described in Chapter 2.

Analyses of free and conjugated IAA

Free and conjugated IAA levels were determined in three equally divided sections of the hypocotyls (apical, middle and basal; each about 2 cm in length) sampling at different times after cuttings were made. Each section of hypocotyl was analysed 2 or 3 times (entire sampling, extraction, purification and determination procedure).

Two g of freshly harvested hypocotyls were frozen in liquid nitrogen and stored in -70°C until analysis. Tissues were cut into small pieces and extracted with 3 ml of 80% aqueous methanol containing 200 mg butylated hydroxytoluene and 100 mg ascorbic acid in 1 l solution for overnight at 4°C. The tissues were reextracted with 2 ml 80% aqueous methanol for 4 h. The extracts were combined and 100 ng [$^{13}C_6$]-IAA (gift of Dr R. P. Pharis) was added as internal standard for GC-MS. Methanolic extracts were reduced to about 0.2 ml (now in aqueous phase) under reduced pressure at 35°C and 0.1 ml was withdrawn for analysis of free plus conjugated IAA. The remainder was for the analysis of free IAA. IAA conjugate hydrolysis was carried out in 7 N NaOH (freshly made) in a sealed Wheaton heating module purged with nitrogen gas. The module containing the sample was kept in a Pierce reacti-Therm™ at 100°C for 3 h. The solution was neutralized with 6 N HCl. Both non-hydrolyzed and hydrolyzed extracts were loaded onto a Sep-Pak C_{18} cartridges (Waters, Associates, Milford, MA, USA) pre-wetted with 0.1 M acetic acid. IAA was eluted with 50% aqueous MeOH in 0.1 M acetic acid. Second and third ml of eluate were collected. Eluates were reduced to dryness in a dessicator (water-driven

vacuum). Samples were redissolved in 100 µl 24.4 % aqueous methanol : 0.86% glacial acetic acid and filtered with 0.5 µM FH Millipore filter.

High performance liquid chromatography (HPLC) was performed in a reverse-phase C_{18} column (110 X 7.94 mm, Whatman Partisphere 5, Clifton, NJ, USA) with a precolumn of the same material. About 15,000 DPM of [^{14}C]-IAA (55 mCi/mmol, Amersham Co., Arlington Heights, IL, USA, to facilitate peak detection) was added to these preliminary purified samples before they were injected onto the HPLC column with an isocratic solution mixture of 24.4 % aqueous methanol : 0.86 % glacial acetic acid. The flow rate was 1 ml/min. Fractions (1 ml) were collected and aliquots of fractions were counted by a Mark III 6881 scintillation counter (Tracor Analytic Inc. Elk Grove Village, IL, USA). The fraction(s) containing significant radioactivity at the retention time (Rt) of [^{14}C]-IAA were again dried in a dessicator. Samples intended for GC-MS analyses were silylated with [N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide] (MTBSTFA) (Pierce, Rockford, IL, USA). 160 µl acetonitrile and 80 µl MTBSTFA were added to each sample, and the mixtures were heated to 70°C for 20 min. Under this condition MTBSTFA's tert-butyldimethylsilyl (TBDMS) group derivatized hydroxyl and amine groups of IAA. After evaporation, the silylated samples were dissolved in haxane and introduced by cool on-column injection into a retention gap of 0.5 m X 0.32 mm deactivated fused silica tubing coupled to a DB1-15N column (15 m, 0.25 mm i.d., 0.25 µm methyl silicone film; J & W Scientific, Folsom, CA, USA) installed in a Hewlett Packard 5790 GC. Temperature was programed from 15°C to 195°C at 15°C/min, then by 5°C/min to 275°C. Helium flow was 30 - 35 cm/sec at 275°C. The GC was directly interfaced to HP 5970 Mass Selective Detector. Data acquisition was controlled by a HP 300 Series computer. For identification of IAA the mass selective detector scanned between 50 and 500 m/z. For quantification of IAA the following ions were recorded: 403/409, 346/352,

244/250. The ratio of 403:409 was used to calculate the content of IAA and the ratio of 346:352 and 244:250 was used for confirmation. Retention time for di-TBDMS-IAA was 18.2 min. Values reported for the conjugated IAA were obtained by subtraction.

Surgical experiments

Various kinds of surgeries on the hypocotyls were conducted with a razor blade. The types of the surgeries are shown in Fig. 9. The original roots were removed in all seedlings except for type I. From type I to III the transverse incisions were made to the centre of the hypocotyl. The distances between 2 transverse incisions were 3 mm. In type III the tissue was not removed. For type IV the basal half of the hypocotyls was removed. Five days after the surgeries the visible roots were observed and the distribution of the roots was recorded. Each treatment consisted of 10 seedlings. IAA transport was studied with type II derooted seedlings. One µl aqueous solution of [^{3}H]-IAA (1.04 TBq/mM, Amersham Co., Arlington Heights, IL, USA) containing 0.052 µCi was injected into one cotyledon through abaxial surface immediately after the surgery. Nine h after [^{3}H]-IAA application, sections 1 to 4 of hypocotyl (shown in Fig. 10) were collected and analyzed for radioactivity with an OX500 oxidizer (R. J. Harvey Inst. Corp. Hillsdale, NJ, USA).

Studies with the inhibitors of auxin transport

A 1 % ethanol solution of NPA (Pfaltz & Bauer, Inc. Waterbury, CT, USA) or TIBA (Sigma Chemical Co., St. Louis, MO, USA) was applied on cotton wool wrapped around the apical portions of hypocotyls 5 mm below the cotyledons in a width of about 1 cm. NPA or TIBA solution was periodically added to the wool throughout the experiment to maintain a continuous supply of the chemicals. The control cuttings were treated with 1 % pure ethanol.

Their effect on auxin transport was tested using [^{3}H]-IAA. One and a half h after the initiation of treatments with inhibitors, an equal amount of [^{3}H]-IAA (1.04 TBq/mM) was applied to one cotyledon as described above. Six h later hypocotyls were excised and dissected into 2 portions: the portions above and below the position of NPA or TIBA treatment. Hypocotyls were analyzed for radioactivity by oxidizing as described above. The effect of NPA and TIBA on the number of root primordia was also observed.

Decapitation experiments

To produce a decapitated hypocotyl segment, the cotyledons and apical bud were removed at the time original roots were excised. The hypocotyl segments were stood with their bases in half strength Hoagland's solution throughout experiment. IAA and IBA (Sigma Chemical Co., St. Louis, MO, USA) were dissolved in minimum amount of ethanol and then made up to desired concentration with deionized water. Ethanol at these concentrations had no effect on rooting in either decapitated or non-decapitated sunflower hypocotyls. IAA or IBA in half strength Hoagland's solution was applied to the basal end of the hypocotyls from 0 -5 h. Estimation of number of root primordia was as described in Chapter 2.

RESULTS

Endogenous free and conjugated IAA in relation to the initiation of ARF

Free and conjugated IAA were determined in the RZ and NRZ of hypocotyls using GC-MS-SIM. A typical mass spectrum of putative IAA from the extract of hypocotyls containing [$^{13}C_6$]-IAA is shown in Fig. 6. Ions at m/z 403/409 are molecular ions of di-TBDMS-IAA and [$^{13}C_6$]-di-TBDMS-IAA. The peak of m/z 346 results from the loss of one tert-butyl group, while the peak of m/z 244 from the loss of two tert-butyl and three methyl groups.

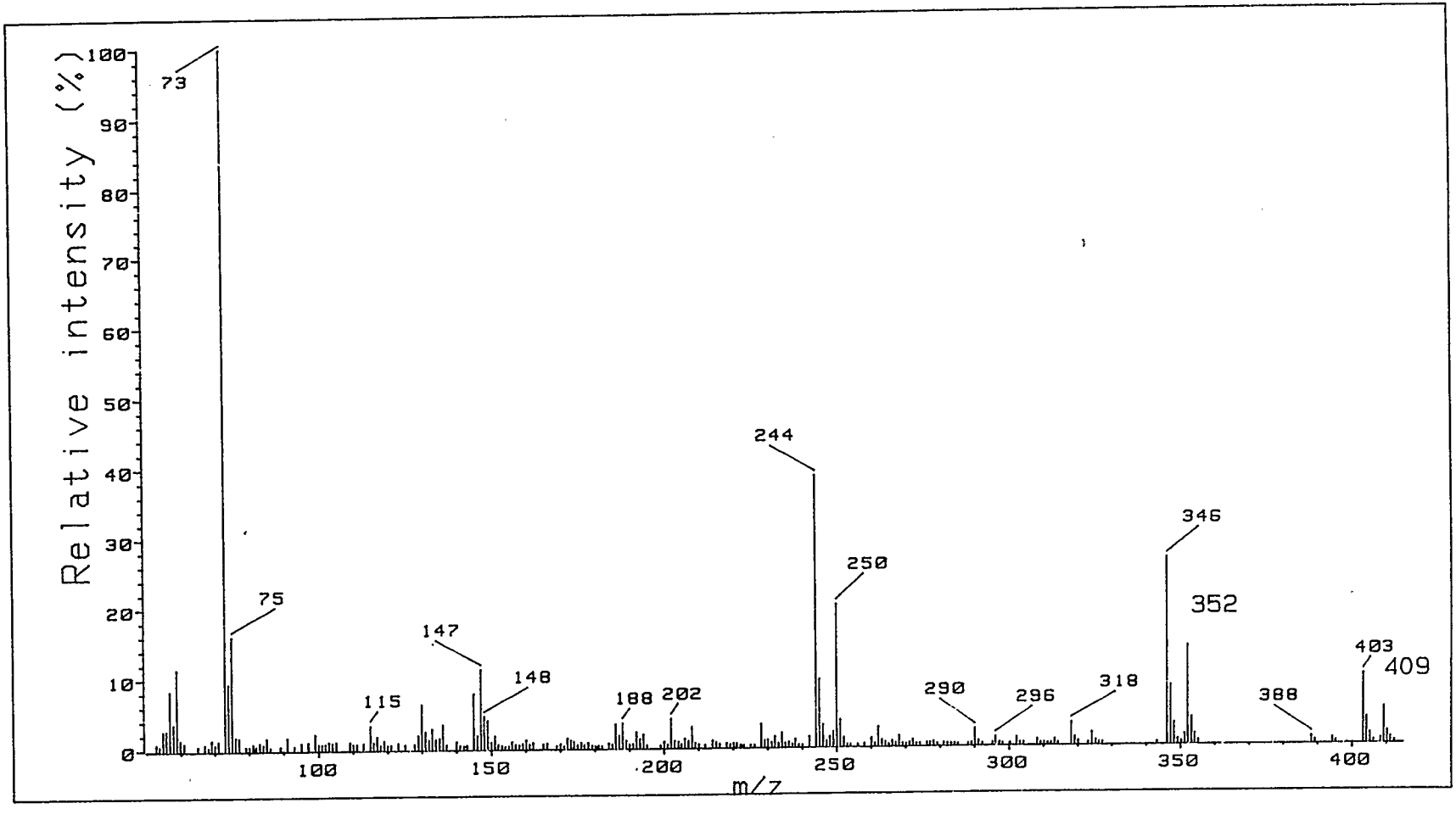


Fig. 6. Mass spectra of putative IAA peak from extract of the basal portion of hypocotyls. [$^{13}C_6$]-IAA was added as internal standard.

A selective ion chromatogram from an extract of hypocotyls is shown in Fig. 7. The ratio of ions was 0.53 at m/z 244/250, 0.51 at m/z 346/352 and 0.52 at m/z 403/409.

Fig. 8 shows the free and conjugated IAA levels in 3 equally sized hypocotyl portions (apical, middle and basal) measured at the time of root removal (0 h) and for up to 48 h after the cuttings were made. At time zero free IAA level was highest in the basal portion (RZ) of the hypocotyls and lowest in the apical portion (NRZ), while at that time the amount of conjugated IAA was lowest in the basal portion of the hypocotyls and highest in the apical portion. In the middle portion of the hypocotyls both free and conjugated IAA levels were in between those of the basal and apical portions. Thus, the ratio of free to total IAA was highest in the RZ of the hypocotyls at the time original roots were removed. Figure 8 also shows that free IAA steadily decreased in the basal portion of the hypocotyls up to 48 h. Free IAA steadily increased in the apical portion of the hypocotyls. In the middle portion of the hypocotyls free IAA increased in the first 15 h and then tended to decrease. Conjugated IAA showed a small increase in the basal portion of the hypocotyls after 15 h. In the apical portion of hypocotyls the quantity of conjugated IAA decreased dramatically to a minimum at 9 h and then increased again. For the middle portion of the hypocotyls there was a transient increase in conjugated IAA at 5 h, however the quantity then decreased to a level of near that found in the RZ.

Surgeries on hypocotyls and distribution of adventitious roots

Figure 9 shows the various types of surgeries in intact and derooted seedlings and the distribution of adventitious roots. In type I, adventitious roots formed in the base of the hypocotyl half disconnected to the original roots. In types II and III, adventitious roots initiated in the hypocotyl regions above the surgery, but not from the regions below. In

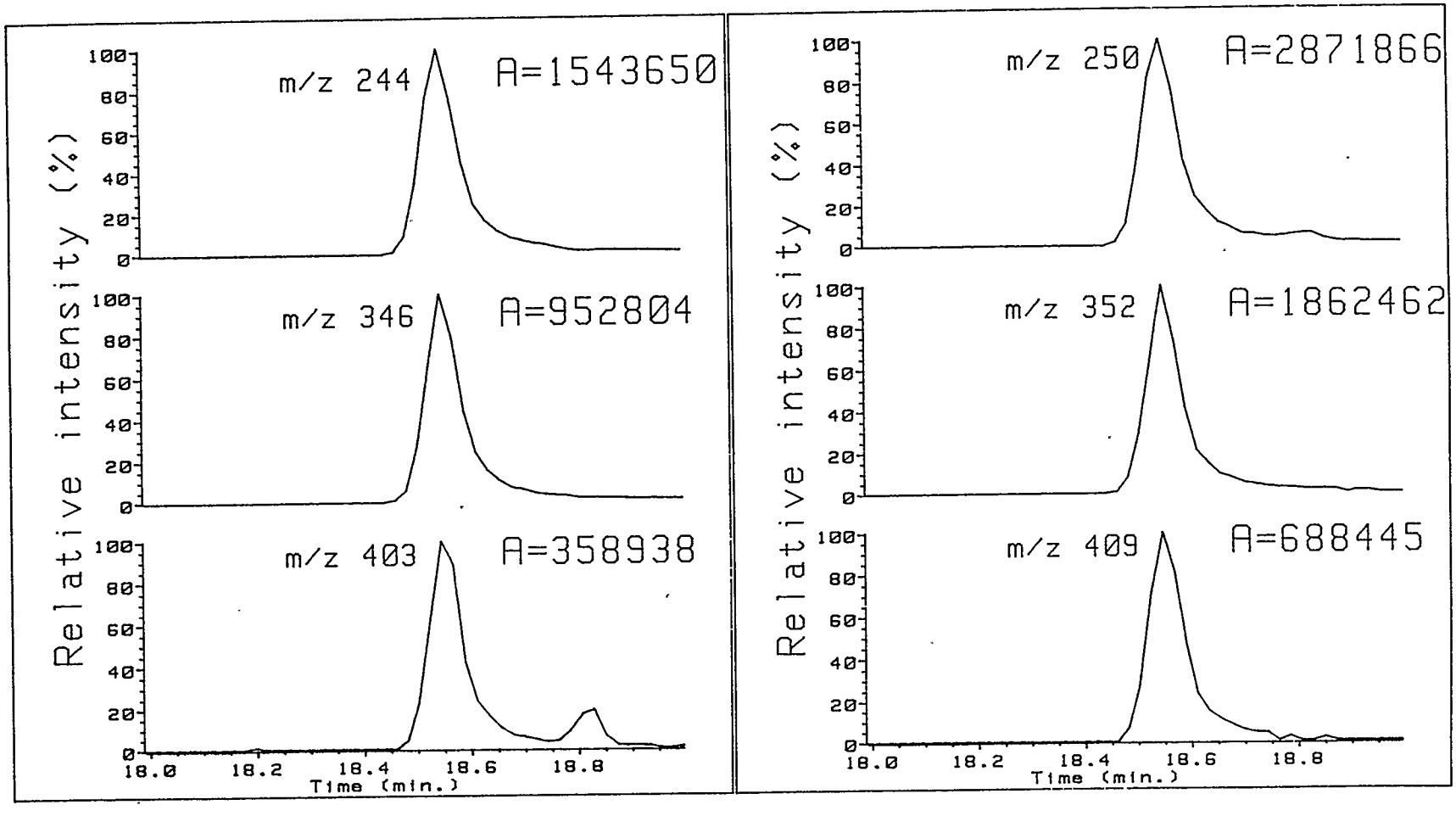


Fig. 7. Selected ion chromatograms of extract from the basal portion of hypocotyls. [$^{13}C_6$]-IAA was added as internal standard.

Fig. 8. The levels of endogenous free and conjugated IAA in apical (◊) middle (∘) and basal portions (•) of the hypocotyls at (0 h) and up to 48 h after the excision of original roots. Free IAA was measured in samples that were not hydrolyzed. Conjugated IAA was calculated by subtracting corresponding values for free IAA from results obtained by hydrolysis of samples in 7 N NaOH for 1 h at 100°C. Samples of conjugated IAA for basal portion of hypocotyls at 5 h and for middle portion at 48 h were lost during the assay. Means ± SE (n = 2 or 3).

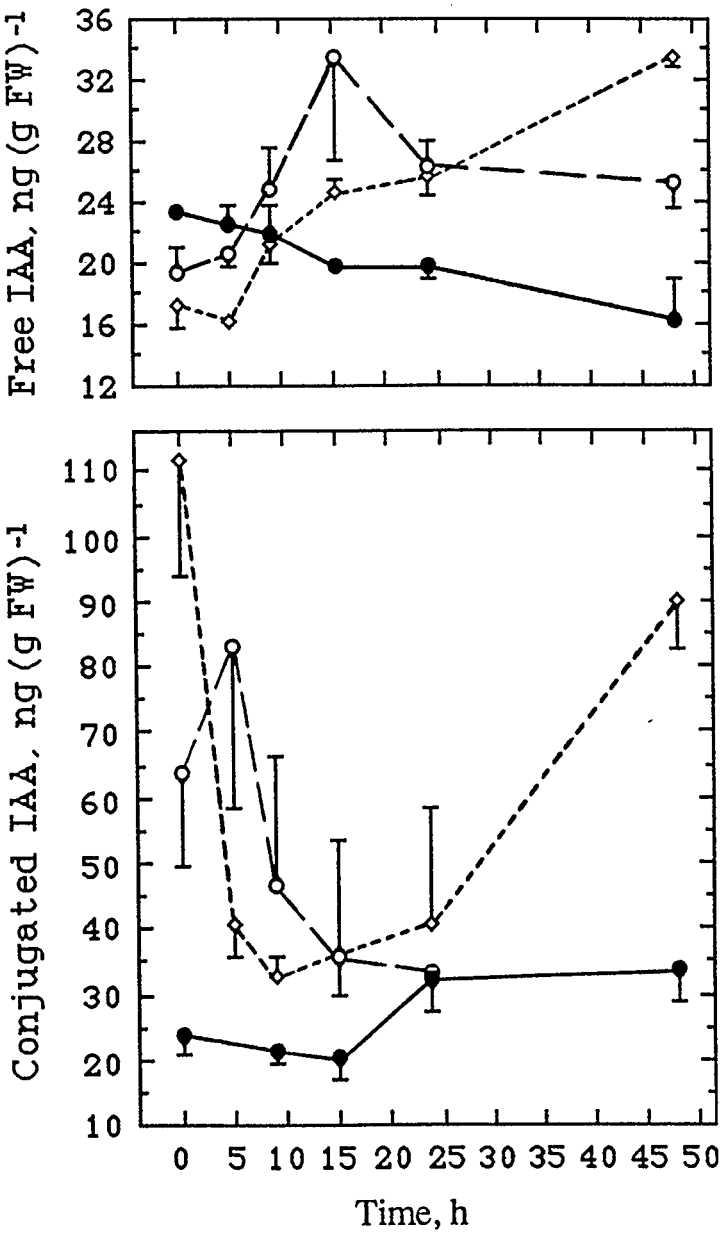

Free IAA, ng (g FW)-1
36
32
28
24
20
16
12
Conjugated IAA, ng (g FW)-1
110
100
90
80
70
60
50
40
30
20
10
0 5 10 15 20 25 30 35 40 45 50
Time, h

Fig. 8

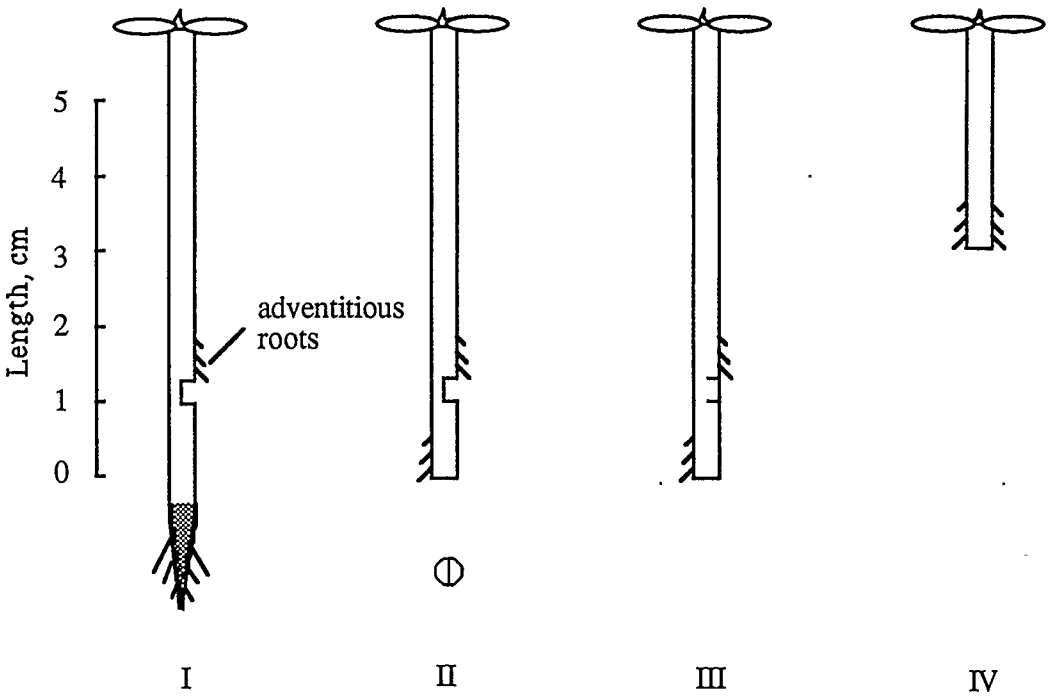


Fig. 9. Effect of various types of surgeries on hypocotyls on the distribution of adventitious roots in 6 day old seedlings. I, original roots still attached; II to IV, original roots removed.

type V, in which basal half of hypocotyl was removed, adventitious roots were developed in the middle region of hypocotyls which do not normally produce roots. The results suggest that adventitious roots form in the hypocotyl regions where auxin may accumulate. This conclusion was supported by the result shown in Fig. 10. In this experiment, The transport of [^{3}H]-IAA was investigated in type II surgery. It was found that more radioactivity was found in sections 2 and 3 where most adventitious roots were formed.

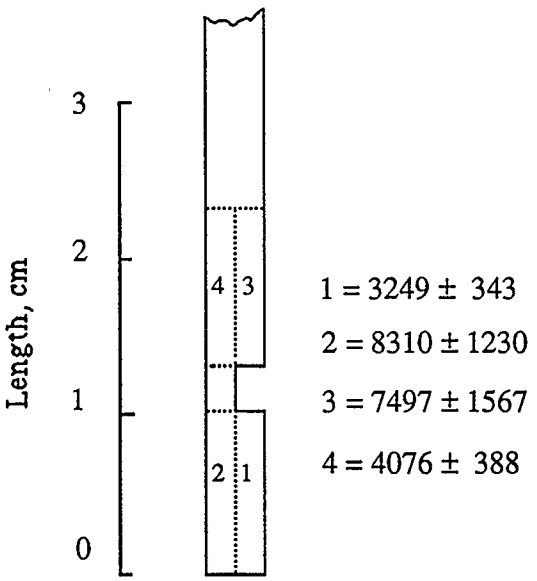


Fig. 10. Distribution of radioactivity (DPM, means ± SE, n = 6) in 4 sections of the hypocotyl with type II surgery 6 h (see Fig. 9) after [^{3}H]-IAA was applied to one cotyledon.

Endogenous source of auxin and ARF

Treatment with NPA or TIBA reduced [^{3}H]-IAA transport (Tab. 2). In comparison to control, there was less radioactivity in the hypocotyl regions below the zone of the treatments and more radioactivity above the treatments. Thus these two inhibitors of IAA transport appeared to reduce the movement of [^{3}H]-IAA to the RZ. Accumulation

Tab. 2. The effect of inhibitors of auxin transport on the distribution of radioactivity in different portions of hypocotyls 6 h after [^{3}H]-IAA was applied to one cotyledon. NPA or TIBA was applied to the hypocotyls 5 mm below the cotyledons.

		radioactivity, DPM X 10^3 (segment)$^{-1}$		
		hypocotyl segment		
		above the treatment	below the treatment	total
control		10.8 ± 0.53	13.6 ± 1.11	24.4
NPA, μM	1	11.5 ± 0.82	12.7 ± 0.68	24.1
	10	17.6 ± 1.07	8.44 ± 0.76	26.0
	50	19.8 ± 1.15	6.12 ± 0.60	25.9
	100	16.3 ± 1.62	5.04 ± 0.56	21.3
TIBA, μM	10	12.0 ± 0.85	12.0 ± 0.94	24.0
	100	14.6 ± 0.93	10.8 ± 0.88	25.4

of IAA above the treatments of NPA and TIBA agree with known characteristic of these inhibitors, i.e. they work by blocking the efflux of IAA from the basal ends of cells but not uptake (Hertel & Leopold 1963, Rubery 1988). NPA was more effective than TIBA in inhibiting [^{3}H]-IAA movement. Total quantity of radioactivity transported from cotyledon down the hypocotyls showed little difference regardless of the treatments. Both NPA and TIBA significantly reduced the number of root primordia (Figs 11 & 12). Within the concentrations tested the optimal concentration of inhibition of NPA was 50 μM. At the concentration of 100 μM no further inhibitory effect on rooting was seen. The inhibitory effect of TIBA on root formation can be completely eliminated by application of IBA at the basal end of the hypocotyls (Fig. 12).

The removal of cotyledons and apical bud, which are thought to be the source of auxin that aids in the induction of ARF, the number of root primordia was reduced by about 80% (Fig. 13). IAA (50 μM) or IBA (10 μM) partially overcame the inhibitory effects of decapitation.

Exogenous auxins and ARF

IBA applied to the base of the hypocotyls from 0 h (immediately after original root was removed) to 3 h after cuttings were made greatly stimulated adventitious rooting (Fig. 14). The naturally occurring auxin, IAA, applied for the equal length of time showed little effect on rooting (data not shown). However, when the exposure time of IAA was extended to 5 h, there was pronounced stimulation of ARF (Fig. 14).

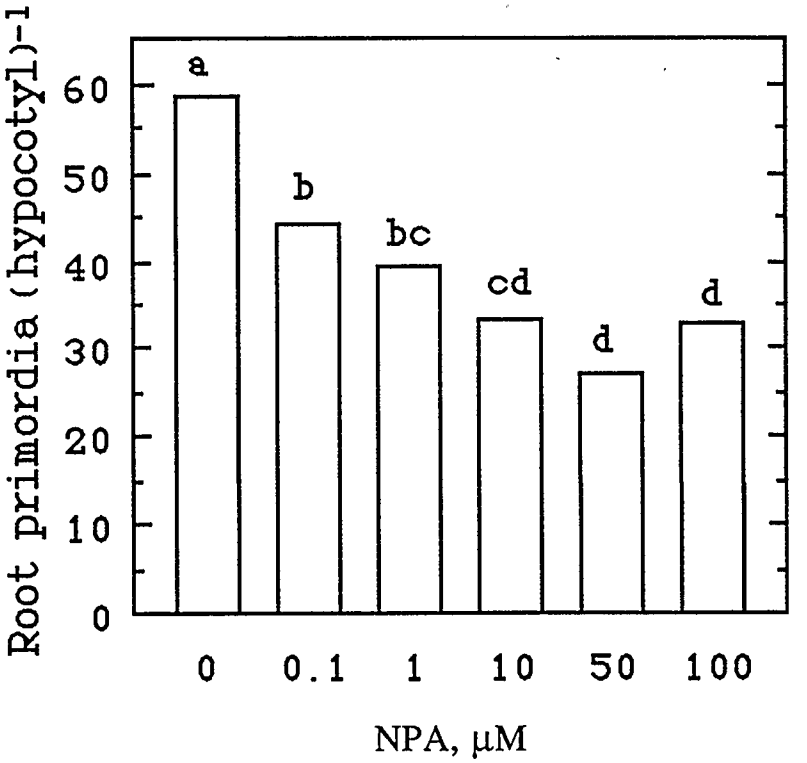


Fig. 11. Effect of NPA supplied to the apical portion of the hypocotyls below the cotyledons on the number of root primordia. Bars with different letters are significantly different (P = 0.05).

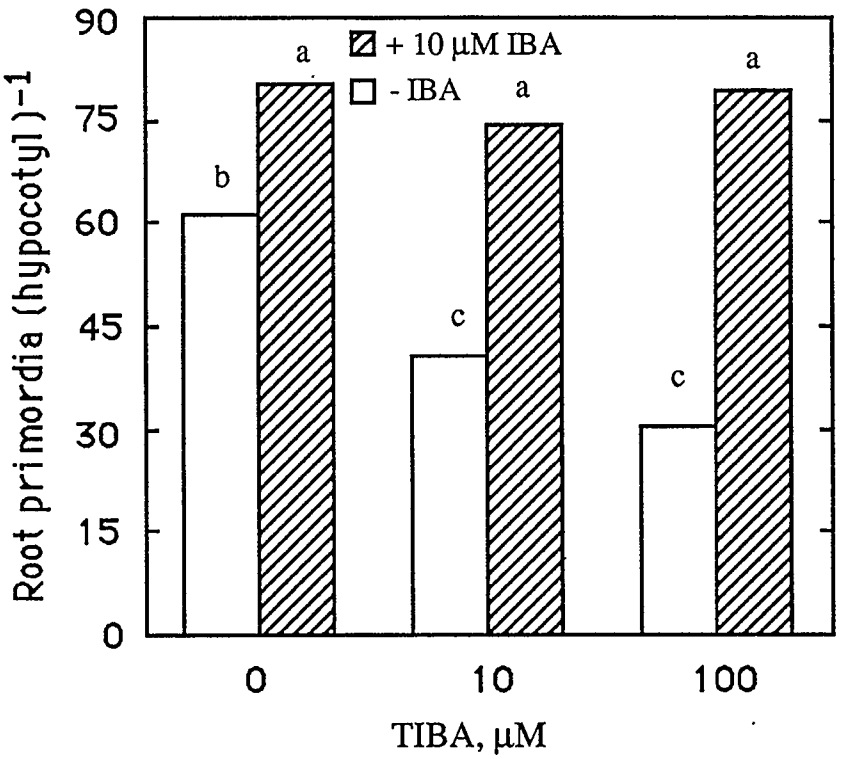


Fig. 12. Effect of TIBA alone, and together with IBA, on the number of root primordia in the hypocotyls. TIBA was applied to the apical portion of the hypocotyls below the cotyledons. The control with 0 TIBA was treated with 1 % EtOH. IBA was applied to the base of the hypocotyls from 0-3 h. Bars with different letters are significantly different (P = 0.05).

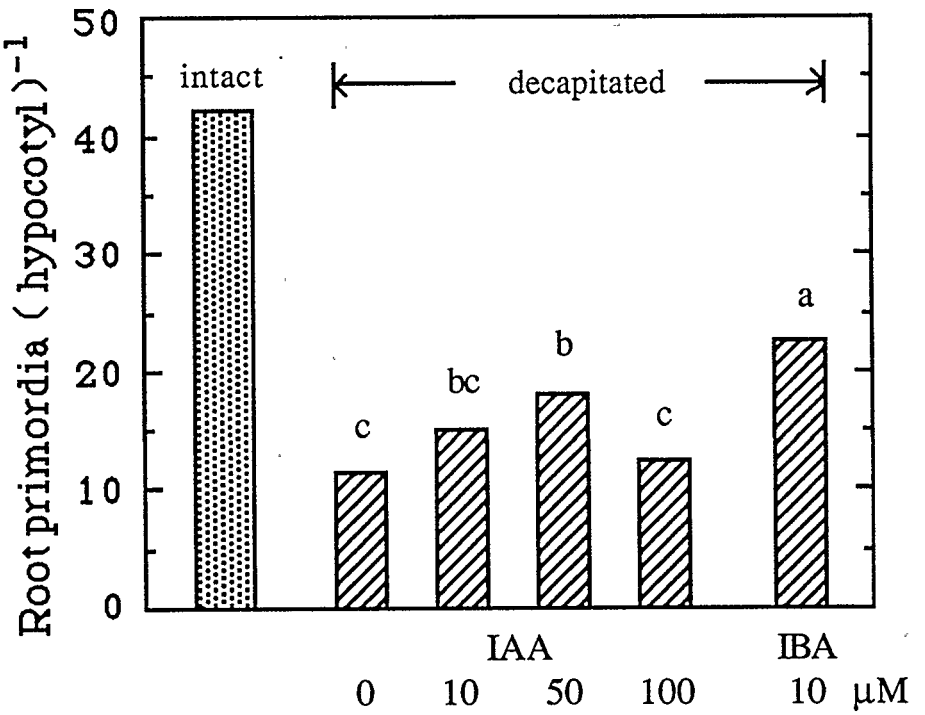


Fig. 13. Effect of auxins on rooting in decapitated (cotyledons and apical bud removed) hypocotyls. IAA or IBA solutions were supplied from 0 - 5 h. Half-strength Hoagland's solution was applied throughout the experiment. Bars with different letters are significantly different (P=0.05).

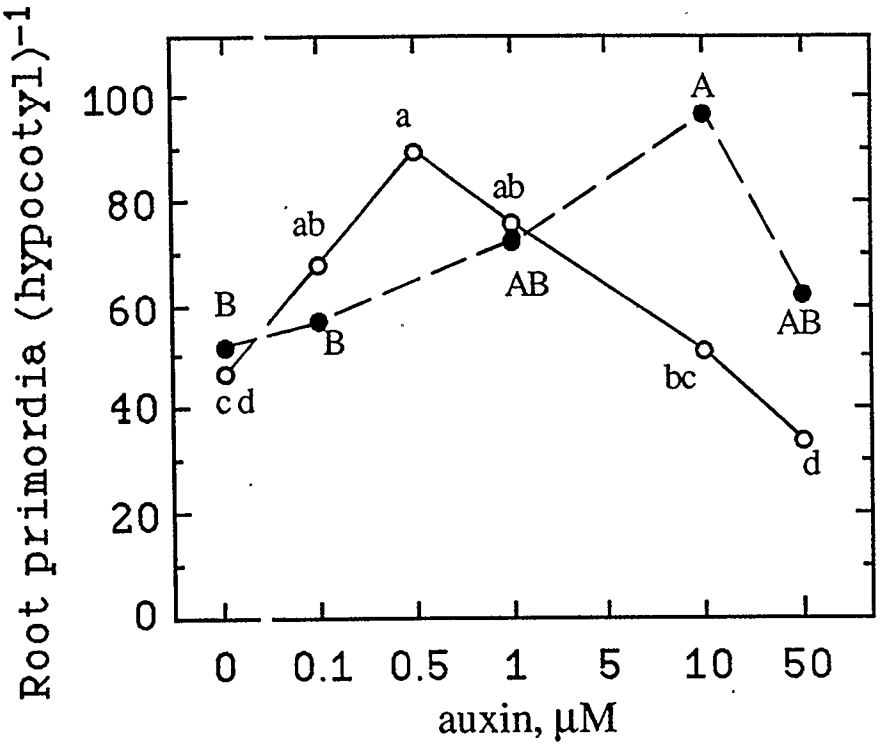


Fig. 14. Effects of IBA, supplied from 0 - 3 h (•), and IAA from 0 - 5 h (∘) on the number of root primordia. Figure shows 2 separate experiments. Points with different letters are significantly different (P = 0.05).

DISCUSSION

The data here indicate that in sunflower seedlings IAA moving from the cotyledons to the basal portion of the hypocotyls is one of the essential factors needed in the process of root initiation in the RZ. The writer comes to this conclusion for the following reasons: (i) At the time the original roots were removed, free IAA level in the RZ of hypocotyls was higher than that in the NRZ. Conversely, conjugated IAA level in the RZ of the hypocotyls was lower than that in the NRZ. (ii) Surgical experiments showed that adventitious roots formed in the hypocotyl regions where endogenous auxin accumulates. (iii) By using a number of techniques to reduce or increase endogenous auxin in the basal portion of the hypocotyls, it was found that endogenous auxin level in the hypocotyl tissues was positively correlated with the number of root primordia in derooted seedlings. These techniques were: (a) Reduction in the basipetal flow of IAA to the RZ by using the inhibitors of IAA transport reduced rooting. The number of root primordia was inversely correlated to the concentrations of inhibitors. (b) The number of root primordia was reduced if the major source of endogenous auxin was removed by decapitation. (c) Exogenous IAA or IBA promoted ARF. (d) Inhibition of rooting by auxin transport inhibitor TIBA or by decapitation could be completely or partially overcome with exogenous auxins. Exogenous auxins could substitute for endogenous one in the promotion of rooting.

Knowing the distribution of root primordia along the hypocotyl will help us better understand all the results presented here. I always found that when the number of roots was reduced by any particular treatment, the root primordia were only located at the very base of hypocotyls. When the number of roots was increased by other treatments, any additional roots were at a more acropetal position (data not shown). Differences in the time

of appearance of root primordia along the hypocotyls was also observed. The first few root primordia were found at the lowest part of the hypocotyls and more root primordia then developed acropetally with increased time. If the source of auxin was removed by decapitation, the root primordia formed at the base of the hypocotyls that contained high level of IAA at the time the original roots were removed (data not shown). The target cells of auxin at a more acropetal position are believed to totally depend on the subsequent auxin transported from cotyledons and apical bud after original roots were removed rather than auxin that was found in the tissues at the time of root removal.

As far as I know there is no published information on the levels of free and conjugated IAA in sunflower hypocotyl. The level of free IAA reported here is comparable to levels in whole plants of duckweed (14 ng [gFW]-1) determined by the GC-MS method (Cohen et al. 1987). Free IAA level in the RZ of the hypocotyls steadily decreased after the excision of original roots. However, other workers (Weigel et al. 1984, Maldiney et al. 1986) show an increase in IAA level after the cuttings were made. Comparison of the results presented here to earlier ones in the literature is difficult, as different techniques for quantification of IAA were applied for a number of species. It is also possible that in the base of sunflower hypocotyl there is sufficient IAA for rooting to occur,

Free IAA in the basal portion (RZ) of hypocotyls decreased steadily after root excision. This decrease was unlikely due to IAA conjugation, since conjugated IAA showed a small increase only after 15 h. I argue that decrease in free IAA in the RZ was more likely due to its utilization during ARF. Alternatively, it is also possible that IAA-receptor complex in the cells is unextractable by the solvent used in these experiments. Nevertheless, the different quantities of endogenous free and conjugated IAA between the rooting (basal portion) and non-rooting zone (apical portion) of the hypocotyls after the excision of original roots suggests the involvement of IAA in the initiation of ARF. The dramatic decrease in

conjugated IAA in the apical portion of hypocotyls in the early few hour after derooting is of interest, but it is not clear what caused this decrease.

The auxin transport inhibitor NPA was more effective in reducing IAA transport than TIBA, but TIBA and NPA were equally effective in reducing rooting (Figs 11 & 12). This is possible due to the inhibitory effect of TIBA itself on rooting. TIBA is able to transport basipetally, but unlike TIBA, NPA is not itself polarly transported (Rubery 1988). TIBA at the concentration above 10 μM inhibited ARF when applied to the base of the hypocotyls for first 3 h after cuttings were made. There was evidence that in leafy pea cuttings TIBA, in addition to its effect on inhibition of auxin transport, may move from the site of application (apical section of stem) to the base of the stem where roots formed and directly inhibited rooting (Potter 1990).

Exogenous IAA and IBA partially overcame the inhibitory effect of decapitation on rooting. The result agrees with the data obtained from other species (Haissig 1970, Mohammed & Eriksen 1974). Fabijan et al. (1981b) showed that IAA was not able to overcome the effect of cotyledon removal. It was reasoned that other necessary factors that are exported from cotyledons were still missing. Unlike Fabijan et al's (1981b) work, in the experiment showed here, a half-strength Hoaglands' solution was supplied throughout the experiment. The interactive effect of auxin and Hoaglands' solution might be due to the presence of boron or other ions in Hoaglands' solution. Boron has been reported to be essential for root growth from early stages of development ("pre-primordial") after rooting was initiated by IBA (Middleton et al. 1978).

However, high levels of IAA do not necessarily mean that a specific tissue will form roots. For example, the basal portion of hypocotyls contains a high level of IAA at the time the original roots were removed. Adventitious rooting occur only after original roots are removed. This suggests that auxin is not the sole factor in the control of ARF. It is possible

that some inhibitors, such as CK which is known to be synthesized in roots (Van Staden & Davey 1979), may be present in a large amount in the hypocotyls of intact seedlings. Exogenously applied CK, BA and zeatin, were shown to inhibit ARF in sunflower hypocotyls (Tab. 13, Fabijan et al. 1981b). Interestingly, BA applied to the base of the hypocotyl stimulated the basipetal transport of [^{3}H]-IAA from cotyledons to the base of hypocotyls (data not shown). This indicates that a high ratio of auxin to CK may be critical for the initiation of ARF.

In conclusion, these results suggest that endogenous IAA plays an important role in initiating adventitious root primordia in the hypocotyls of sunflower seedlings. However, it is possibly not the unique factor in the control of ARF.

Chapter 4. [^{3}H]-IAA TRANSPORT AND METABOLISM

INTRODUCTION

The data in the previous Chapter indicated that endogenous auxin plays an important role in ARF. Decapitation, or application of auxin transport inhibitors to the hypocotyls at a position between cotyledons and the RZ of the hypocotyls, greatly inhibited rooting. It is generally beleived that when the original roots are removed, basipetally transported IAA accumulates in the base of hypocotyls where it stimulates ARF. However, there is little actual data concerning the basipetal auxin transport and its metabolism in relation to ARF.

The objective of experiments reported here was twofold. Firstly I wished to examine how the removal of original roots affected IAA transport out of cotyledons and secondly I wanted to determine if there are differences in the type of auxin metabolism between rooting and non-rooting tissues. I thus followed the transport of [^{3}H]-IAA that was applied to one cotyledon in derooted and intact seedlings. I also examined [^{3}H]-IAA metabolism in the RZ and NRZ of hypocotyls of intact seedlings, in the RZ of derooted seedlings and basal portion of hypocotyls of intact seedlings.

MATERIALS AND METHODS

Plant material, growth conditions, and modified rooting system were similar to those in Chapter 2.

[^{3}H]-IAA transport

[^{3}H]-IAA was applied to one cotyledon at equal amounts of radioactivity as described in

Chapter 3. One group of seedlings were derooted immediately after the injection and maintained in deionized water. Another group of seedlings remained in original culture medium. Seedlings were harvested at 6, 9 and 24 h after application of [^{3}H]-IAA. Each sample contained 5 seedlings. Cotyledons, apical bud, hypocotyl and roots were collected. Hypocotyls were further dissected into 3 equal sections (apical, middle and basal). Radioactivity was extracted for HPLC by the extraction procedures described earlier for endogenous free and conjugated IAA extraction (Chapter 3). It was found that 85% of total radioactivity was extracted by this method. Small amounts of [^{14}C]-IAA was added to the samples before extraction for the determination of recovery and stability of [^{3}H]-IAA during the extraction and HPLC procedures. Extracts were filtered and reduced to a small volume under reduced pressure and dessicated to dryness, and then subjected to HPLC without preliminary purification. The eluating program was: Isocratic 24.4 % aqueous methanol : 0.86 % glacial acetic acid from 0 - 23 min; a linear gradient for 16-100 % methanol from 23 to 26 min and then pure methanol from 26 to 41 min. Preliminary experiments showed that no radioactivity was detected after 32 min . Thus, 32 fractions were collected, and 0.1 ml of each was added to 5 ml ScintiVerse for counting by the liquid scintillation counter described in Chapter 3. Sample quenching was corrected with a barium-133 external standard. The peak cochromatographing with internal standard [^{14}C]-IAA (usually 2 fractions) was identified as the "IAA peak" and the total radioactivity of all other peaks were summed and called "IAA metabolites".

[^{3}H]-IAA metabolism

The [^{3}H]-IAA metabolism of derooted and intact seedlings was examined. Sampling and experimental procedure for this study was identical to that for [^{3}H]-IAA transport. There

was no difference in the type of IAA metabolism among all samples when using eluating program described in above (program 1). Another eluating program (program 2) was then used. The solvents were 1 % glacial acetic acid (A) and acetonitrile (B). These were supplied according to the following program: pure A from 0 - 10 min, a linear gradient from 0 - 20 % B from 10 to 40 min, isocratic 80 % A : 20 % B from 40 to 45 min, a linear gradient from 20 to 100 % B from 45 to 50 min, and pure B from 50 to 60 min. Flow rate for both was 0.8 ml/min. No radioactivity was detected after 52 min. Thus, 52 fractions were collected.

From the HPLC column eluates a major peak which did not cochromotograph with authentic [^{3}H]-IAA was found. This was tentatively identified as indole-3-acetyl-L-aspartate (IAAsp) using non-radioactive standard IAAsp (Sigma Chemical Co., St. Louis, MO, USA). Eluates corresponding to this radioactive peak were pooled and analysed again by HPLC with non-radioactive IAAsp being added to them. Non-radioactive IAAsp from HPLC chromatogram was detected using a model HM Gilson UV detector (Gilson Medical Electronics, Inc. Middleton, WI, USA). Excitation wavelength was 280 nm. Radioactivity of the various fractions was detected and measured using a scintillation counter.

RESULTS

Transport of [^{3}H]-IAA in hypocotyls of derooted and intact seedlings

To meaningfully interpret the changes of IAA level in hypocotyls, its movement and metabolism were studied. [^{3}H]-IAA was applied to one cotyledon and transport was compared in derooted and intact seedlings. Derooted seedlings produce many adventitious roots but in intact seedlings few adventitious roots develop. Radioactivity was not detected in either the apical bud or the cotyledon that was not treated with [^{3}H]-IAA, suggesting that

[^{3}H]-IAA applied to the cotyledon is largely transported basipetally to the hypocotyl and roots. Table 3 shows the quantities of [^{3}H]-IAA, [^{3}H]-IAA metabolites and total radioactivity found in the labelled cotyledon and the hypocotyl (or the hypocotyl plus roots) at different times after [^{3}H]-IAA application. Radioactivity that cochromatographed with authentic [^{3}H]-IAA was identified as the IAA peak, and the radioactivity of all other significant peaks was called "IAA metabolites". Most of the [^{3}H]-IAA applied to cotyledon was metabolized in less than 6 h. There was little change in the amount of IAA metabolites in the cotyledon from 6 to 24 h. This suggests that these IAA metabolites were immobile and they were unlikely converted to free IAA within this time. Total radioactivity showed little difference between the hypocotyls of derooted seedlings and the hypocotyls together with roots of intact seedlings. This indicated that the total amount of [^{3}H]-IAA exported out of the cotyledon was similar in both kinds of seedlings. However, the distribution of radioactivity in different portions of seedlings differed. Figure 15 shows the distribution of IAA and IAA metabolites along the hypocotyl and roots at different times after [^{3}H]-IAA treatment. From this figure it is seen that: i) At 6 h after [^{3}H]-IAA treatment the quantity of both IAA and IAA metabolites in the basal portion of hypocotyls in derooted seedlings were lower than those in comparable tissue of intact seedlings. ii) IAA metabolites in the basal portion of the hypocotyl and roots increased by 24 h. iii) IAA in apical and middle portion of hypocotyls decreased from 6 to 24 h. iv) A large amount of IAA metabolites in the basal portion of hypocotyls in derooted seedlings as can be seen at 24 h.

Metabolism of [3H]-IAA in derooted and intact seedlings

The metabolism of [^{3}H]-IAA in different portions of the hypocotyls was compared in derooted and intact seedlings. Samples were collected from the 3 portions of hypocotyls 6, 9 and 24 h after application of [^{3}H]-IAA to a cotyledon. The metabolites of [^{3}H]-IAA from

Tab. 3. The quantities of [^{3}H]-IAA and [^{3}H]-IAA metabolites and total radioactivity shown as DPM X10^3 per hypocotyl in different parts of derooted and intact seedlings. Samples were taken 6, 9 and 24 h respectively after [^{3}H]-IAA was applied to one cotyledon.

		derooted seedlings		intact seedlings	
		labelled cotyledon	hypocotyl	labelled cotyledon	hypocotyl plus roots
6 h	[^{3}H]-IAA	0.86 ± 1.01	14.63 ± 0.65	1.32 ± 0.71	15.53 ± 0.19
	[^{3}H]-IAA metabolites	56.14 ± 1.20	18.26 ± 0.86	53.95 ± 3.96	22.96 ± 2.10
	total	57.00 ± 0.92	32.84 ± 2.94	56.42 ± 1.04	38.46 ± 2.50
9 h	[^{3}H]-IAA	2.13 ± 1.24	12.08 ± 2.70	3.08 ± 2.02	9.99 ± 0.53
	[^{3}H]-IAA metabolites	56.32 ± 3.19	20.83 ± 0.10	52.69 ± 3.01	29.45 ± 0.66
	total	58.46 ± 1.94	32.82 ± 2.60	57.81 ± 2.85	38.87 ± 3.14
24 h	[^{3}H]-IAA	1.72 ± 0.94	1.70 ± 0.74	0.87 ± 0.01	0.92 ± 0.12
	[^{3}H]-IAA metabolites	55.63 ± 1.09	33.36 ± 2.43	55.61 ± 2.07	36.47 ± 1.63
	total	57.36 ± 0.16	34.65 ± 2.11	56.50 ± 3.06	37.38 ± 1.76

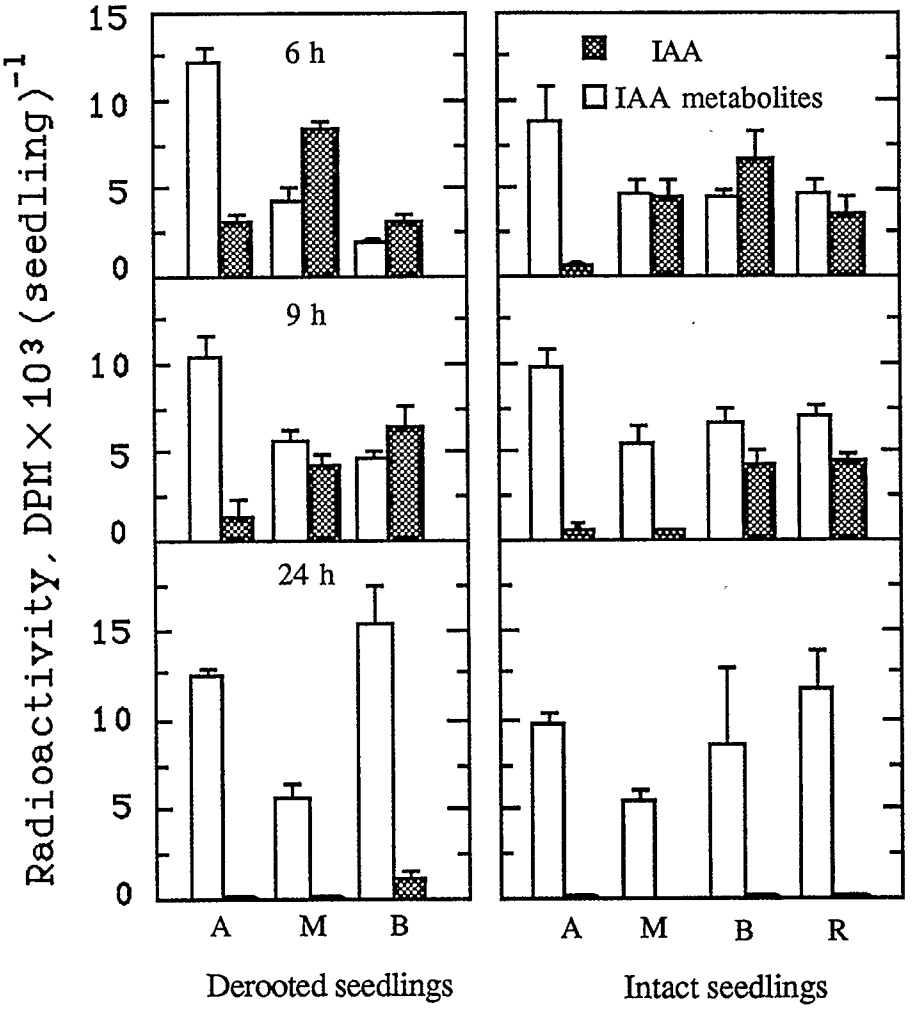


Fig. 15. Quantities of unmetabolized IAA and all other IAA metabolites in different portions of derooted and intact seedlings at 6, 9, and 24 h. [^{3}H]-IAA was supplied, at 0 h, to one cotyledon. A, apical, M, middle, and B, basal portion of the hypocotyls; R, root system.

a crude extract of the tissue were separated by HPLC with two differed solvents programs described in Materials and Methods. With either solvent system I was unable to see any significant difference in type of IAA metabolism between apical, middle and basal protions of the hypocotyls or between intact and derooted seedlings.

Figure 16 shows the pattern of metabolism of radiolabelled IAA in the RZ (basal) and NRZ (apical) of the hypocotyls of derooted seedlings and basal portion of hypocotyls of intact seedlings at 9 h. In all three tissues the largest peak of radioactivity cochromotographed with authentic IAA. The next largest peak of radioactivity was located at fractions 34-36. This cochromotographed with authentic IAAsp (detected using a UV detector). These results are consistent with a report by Norcini & Heuser (1988) that in IAA-treated mung bean cuttings the major metabolite was IAAsp. There were other smaller zones of radioactivity from fraction 24 - 28 and fraction 49 - 51.

DISCUSSION

The results presented here indicate that: i) [^{3}H]-IAA of cotyledon readily moves to the hypocotyls. At 6 h after [^{3}H]-IAA treatment the quantity of both IAA and IAA metabolites in the basal portion of hypocotyls in derooted seedlings were lower than those in comparable tissue of intact seedlings. This suggests that basipetal transport of IAA initially slowed down as the result of removal of primary root system, a known major sink for IAA. It is also demonstrated that [^{3}H]-IAA in cotyledons could arrive at the RZ of the hypocotyls in less than 6 h after application, which is the time period the critical events leading to the initiation of ARF are believed to happen. ii) The increase of IAA metabolites in the basal portion of the hypocotyl and roots by 24 h may be caused by transport of IAA

Fig. 16. Metabolism of [^{3}H]-IAA in the non-rooting zone (A, apical portion), rooting zone (B, basal portion) of the hypocotyl of derooted seedlings and the basal portion of hypocotyls of intact seedlings (C). [^{3}H]-IAA was applied to one cotyledon at 0 h. The crude extracts of the hypocotyls harvested at 9 h after [^{3}H]-IAA application were injected into HPLC. Elution was performed by solvent program 2 as described in Materials and Methods. Insert: Chromatogram of [^{14}C]-IAA which was added to the extracts to determine the metabolism and recovery efficiency of [^{3}H]-IAA during experimental procedures.

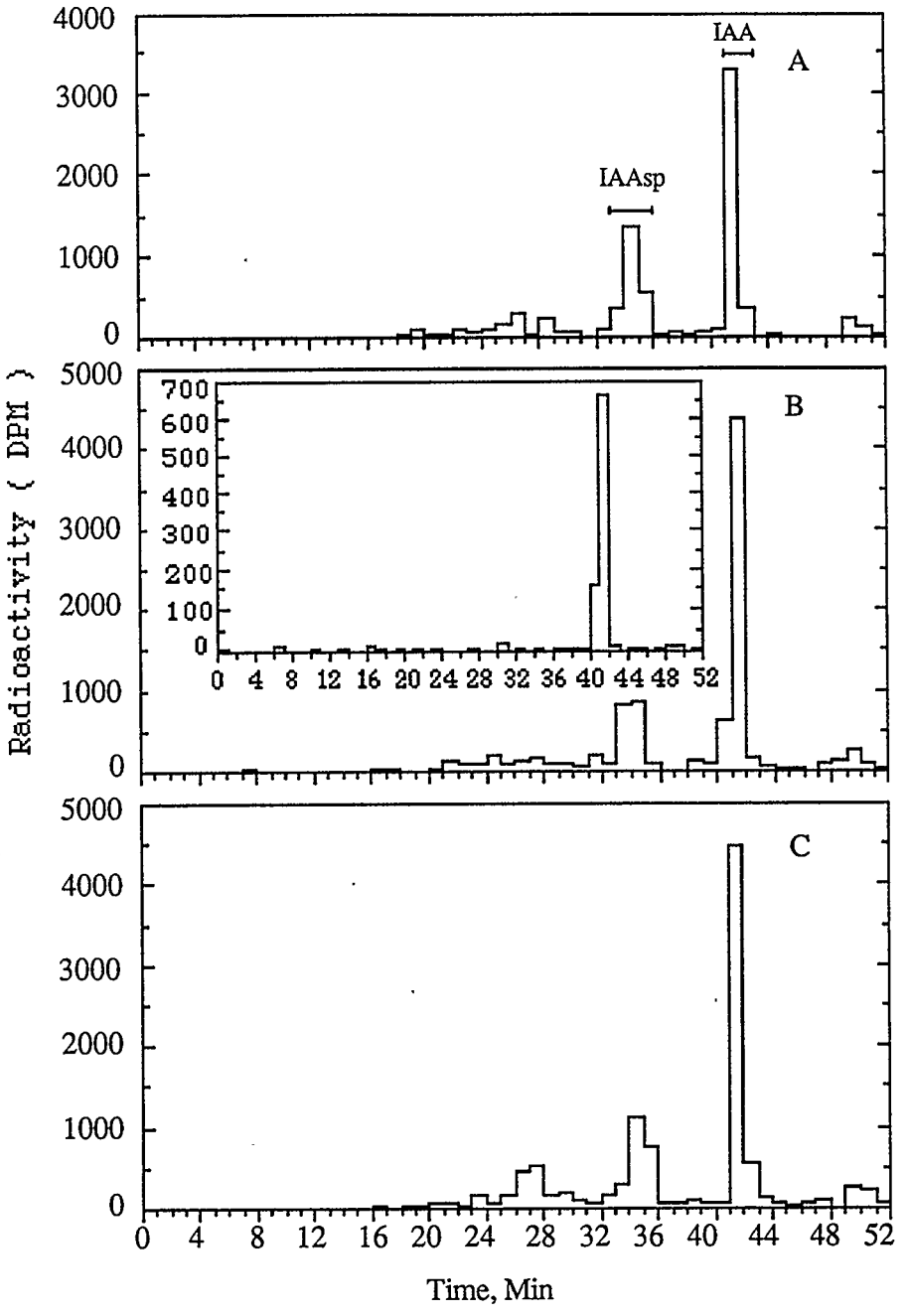


Fig. 16

from the cotyledon which is then metabolized in this basal tissues. iii) The decrease of IAA in apical and middle portion of hypocotyls from 6 to 24 h could be due to IAA being transported to lower region of the hypocotyl or roots, as well as to IAA being metabolized locally. iv) It seems likely that some IAA was metabolized during its polar transport down the hypocotyl. This finding agrees well with the data reported in etiolated lupin (Ortuño et al. 1990). v) A large amount of IAA metabolites in the basal portion of hypocotyls in derooted seedlings as can be seen at 24 h. This portion of hypocotyls thus appears to be the major sink for basipetally transported IAA in derooted seedlings.The findings support the hypothesis that IAA accumulates in the base of hypocotyls after original roots, a known major sink for IAA, are removed.

To understand the molecular mode of auxin action in controlling adventitious rooting, such as an attempt to isolate binding protein, one must know if it is IAA itself or its metabolites which are involved. There was no evidence of differences in the types of [^{3}H]-IAA metabolism between the RZ and the NRZ of derooted seedlings or between seedlings that produce roots and those do not form roots. Thus metabolites of IAA are unlikely to be involved in the control of adventitious rooting. The results support the earlier conclusion by Greenwood et al. (1974) that free auxin, and not the products of metabolism, is essential to root primordia formation. There is evidence in the literature showing that IAAsp, a major metabolite of exogenous IAA (Fig. 16 and Norcini & Heuser, 1988), did not induce adventitious roots when applied exogenously (Plücs et al. 1989).

Chapter 5. THE ROLE OF ENDOGENOUS ETHYLENE

INTRODUCTION

The data in Tab. 1, Figs 1 & 2 suggests that ethylene might have a role in ARF. However, although there are reports that ethylene may somehow be involved in adventitious rooting, the literature on the effects of ethylene on root initiation is very confusing. To quote a recent review by Mudge (1988), "The effect of ethylene on adventitious root formation is highly variable.....". Ethylene and ethylene-releasing compounds have been reported to promote (Krishnamoorthy 1970, Roy et al. 1972, Robbins et al. 1983,1985), inhibit (Mullins 1972, Coleman et al. 1980, Geneve & Heuser 1983, Nordström et al. 1984) or have no effect on adventitious rooting (Batten & Mullins 1978, Mudge & Swanson 1978). Furthermore, inhibitors of ethylene action can, when added to the hypocotyls at certain times, promote rooting yet, when supplied some hours later, appear to inhibit rooting (Fabijan et al. 1981b).

The variable response to ethylene may have many causes. A number of species at different developmental stages have been tested under a wide range of experimental conditions. Test solutions have been applied at different concentrations, for differing lengths of time and have been supplied at various times after derooted seedlings were made. It is difficult to compare and interpret such results, particularly in view of the fact that in some systems the stimulatory effects of ethylene may only be expressed over a narrow range of concentrations (Konings & Jackson 1979). A further complicating factor is that this phytohormone can promote some processes at low concentrations, yet at only slightly higher concentrations can strongly inhibit the same processes (Konings & Jackson

1979, Reid et al. 1985). There has also been careless use of inhibitors of ethylene action, and inhibitors or promoters of ethylene synthesis, in that investigators often fail to test whether these compounds are operating in the manner in which they are supposed to work. Finally, in rooting experiments there have been quite a few studies in which ethylene or ethylene-releasing agents have been applied. However, there is a paucity of data on the changes in endogenous levels of ethylene, or of its precursors, during the course of root initiation.

The objective of my experiments was to study the possible role of ethylene in ARF in sunflower hypocotyls, especially during the first few hours of primordia formation. To achieve this I used a number of chemicals which increase or decrease endogenous ethylene production, or block ethylene action. The chemicals were usually applied immediately after seedlings were derooted and the effects of these treatments on ARF and on ethylene production were observed. The changes in the endogenous levels of, ethylene, the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and the levels of one of its metabolites, 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC) were also followed. I compared the levels of these 3 substances in the lower portions of the hypocotyls that produce most of the adventitious roots (RZ), to those in the upper portions of the hypocotyls that do not produce roots (NRZ).

MATERIALS AND METHODS

Plant material, growth condition, modified rooting system, application of test solution and estimation of number of root primordia are as described in Chapter 2. ACC was purchased from Calbiochem (Behring Diagnostica, Division of American Hoechst Corp, USA); AVG from Fluka AG (NK, USA) and ethephon from Rhone-Poulenc Canada Inc.

Preparation of ethephon and silver thiosulphate (STS)

The solutions of ethephon were always freshly made just before the application. The pH of the solutions were adjusted to 5.9 using NaOH. Silver thiosulphate was prepared from stock solutions of 0.01 M $AgNO_3$ (Fisher Scientific Co., Fair Lawn, NJ, USA) and 0.04 M $Na_2S_2O_3$ (Chemonics Scientific). $AgNO_3$ was stored in dark. Equal volumes of each solution were mixed to produce STS and fresh STS was used in each experiment.

Measurement of ethylene

Most of the adventitious roots are found in the basal 2 cm (RZ) of the hypocotyls (Fabijan et al. 1981a). I compared the ethylene production in the RZ to production in the NRZ of the hypocotyls. The NRZ was 0.5 cm below the cotyledonary node and it was also 2 cm in length. Ethylene samples were taken and measured by the method described in Chapter 2.

Measurements of ACC and MACC

ACC and MACC were measured following the methods described by Jiao et al. (1986).

RESULTS

Figure 17 shows the endogenous ethylene evolution following removal of the root system. In the RZ the maximum rate of production occurred at 3 h after the excision of original roots (70% increase over 0 h). At 7 h ethylene production returned to initial values. This transient peak of ethylene production in the RZ was probably due to wound ethylene caused by excision of the original roots (Burg & Thimann 1959, Saltveit & Dilley 1978). The higher ethylene production at 3 h vs 12 h can also be seen in the first 2 histogram bars of Fig. 8. Ethylene production in the NRZ of the hypocotyls was always lower than that in the RZ.

I then measured the endogenous concentrations of the ethylene precursor ACC and its conjugate MACC in the RZ, and compared these to the levels found in the NRZ. The concentration of ACC in the RZ decreased rapidly (Fig. 18A) and failed to return to its initial level until 72 h. In the NRZ, ACC concentration also decreased but then rapidly returned to its initial level. The levels of MACC are shown in Fig. 18B. In the NRZ the concentration of MACC showed only small changes throughout the 72 h. In the RZ there was little change in the MACC concentration during the first 24 h. However, after this time MACC concentration increased (Fig. 18B), indicating that after 24 h more ACC was being converted into MACC in the RZ compared to the NRZ.

To investigate if the wound-induced ethylene is an important factor in adventitious root initiation, a number of presumptive inhibitors of ethylene synthesis, inhibitors of ethylene action and 2 ethylene-releasing compounds were applied immediately after the excision of original roots and before the early rise in ethylene levels. These chemicals were supplied to the lower end of the hypocotyls at 0 h and removed 3 h later. Their effects on ethylene production and on the initiation of root primordia were examined. The effects of these

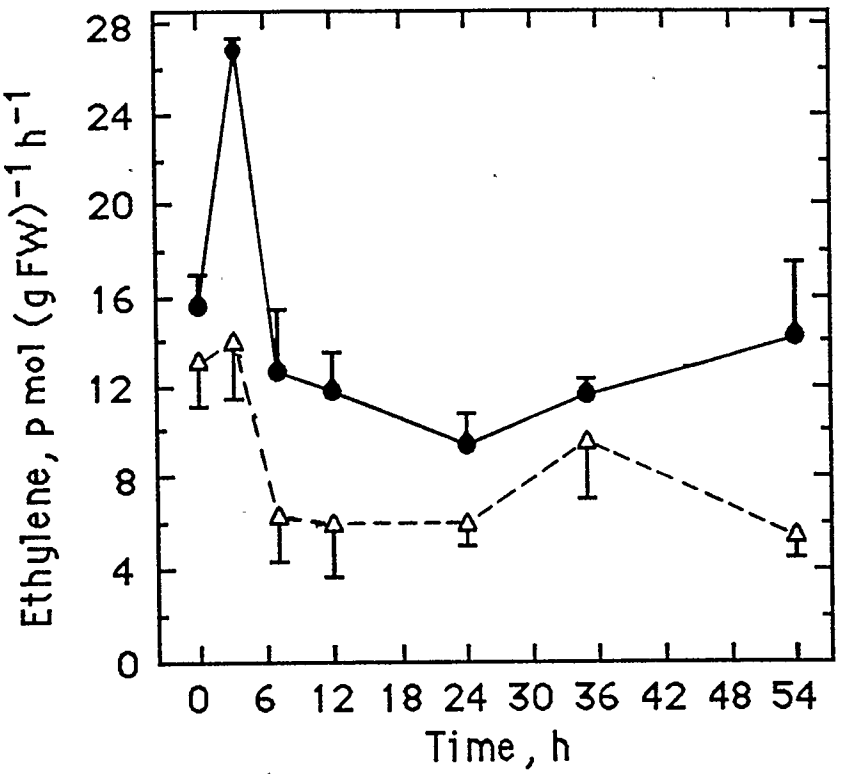


Fig. 17. Ethylene production in the rooting (•) and the non-rooting (Δ) zones of hypocotyls over the 54 h period following the excision of the original roots at 0 h. Means ± SE (n=3 or 4).

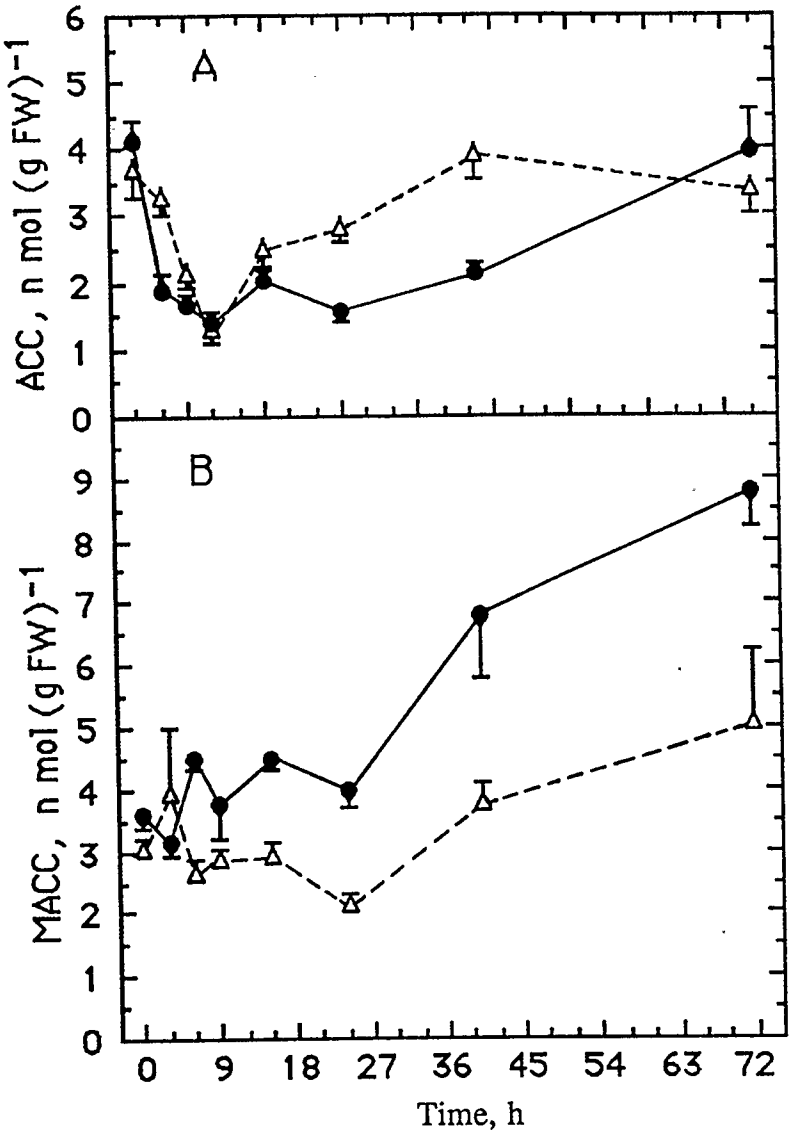


Fig. 18. The levels of endogenous ACC (A) and MACC (B) in the rooting (•) and the non-rooting zones (Δ) of sunflower hypocotyls over a 72 h period following the excision of the original roots at 0 h. Means ± SE (n=3 - 5).

compounds on ethylene production are described below.

AVG, supplied from 0 h (immediately after the original roots were excised) to 3 h, reduced the ethylene production at 3 and 12 h (Fig. 19). Since AVG inhibits ACC synthesis, this data suggested that supplies of ACC were limiting for ethylene production. This seems contrary to the data in Fig. 18 which shows ACC to be in abundant supply. The writer can only suggest that much of the ACC seen in Fig. 18, is not available for ethylene synthesis; perhaps because it is in a different cellular compartment than the ethylene-forming enzyme.

Other presumptive inhibitors, such as benzyl isothiocyanate (BITC, Patil & Tang 1974), galactose (Philosoph-Hadas & Aharoni 1987) and salicylic acid (SA, Leslie & Romani 1988) had no inhibitory effect on ethylene evolution 3 h after production of the cuttings. Rather, BITC and SA increased ethylene production (Tab. 4). Because they were ineffective in lowering ethylene production I did not further examine their effects on rooting.

Tab. 4. Effect of SA and BITC on ethylene production in hypocotyls of the rooting zone at 12 h. Both chemicals were supplied from 0 to 3 h.

treatment	concentration (µM)	ethylene, p mol $(gFW)^{-1} h^{-1} \pm SE$
control		14.7 ± 0.65
SA	10	48.1 ± 0.94
	100	288.9 ± 5.64
BITC	0.92	20.4 ± 5.47
	9.2	29.8 ± 2.37

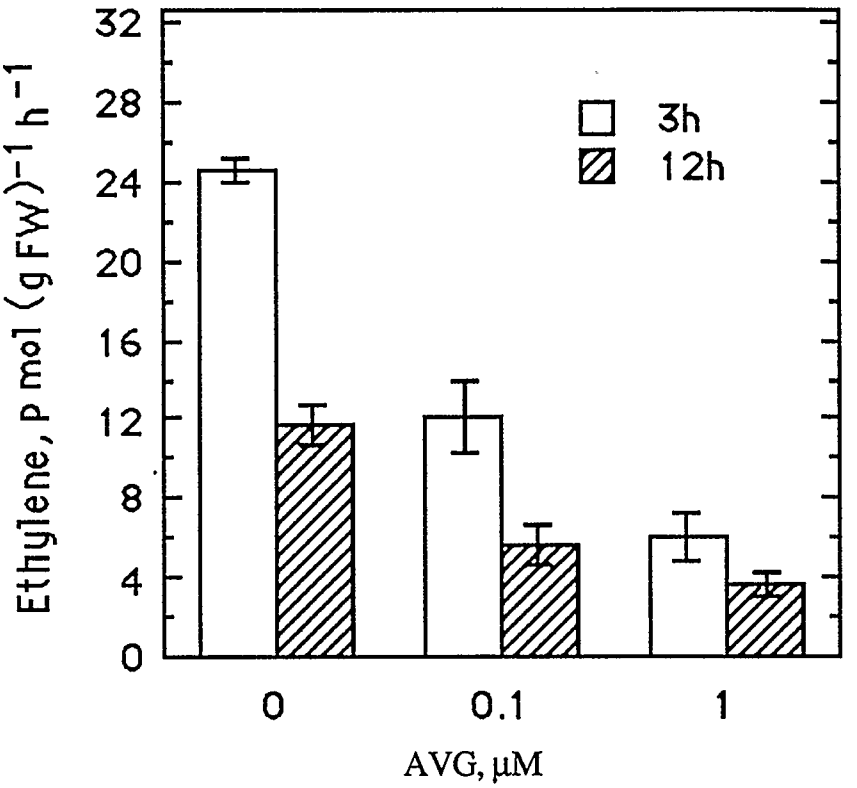


Fig. 19. The effects of AVG on ethylene production in the RZ of hypocotyls. AVG was applied from 0 - 3 h. Ethylene production was measured at 3 and 12 h after the excision of original roots. Means ± SE (n = 3 or 4).

Figure 20 shows the effects of ACC and ethephon on the 3 h peak of ethylene production found in the RZ. As expected, both ACC and ethephon increased endogenous ethylene production.

I examined the possible influence of the early peak of ethylene production (Fig. 17) that was found in the RZ on adventitious rooting. This was done by supplying AVG, $AgNO_3$ and STS immediately after the excision of original roots and removing them 3 h later.

AVG at 0.1 and 1 μM supplied from 0 to 3 h, inhibited the initiation of root primordia. The inhibitory effect of both concentrations of AVG was reversed when ACC was applied along with AVG (Fig. 21).

$AgNO_3$, which has often been used as an inhibitor of ethylene action, was supplied from 0 to 3 h. The effect of $AgNO_3$ (Fig. 22) on the initiation of root primordia was consistent with previous results (Fabijan et al. 1981b) showing that $AgNO_3$ stimulated rooting at the concentration at which the hypocotyls showed necrotic symptoms. In contrast, with STS the hypocotyls looked healthy even at a concentration of 100 μM. Also the effect of STS on root formation was opposite to that of $AgNO_3$, as STS inhibited rooting (Fig. 23). When the hypocotyls were treated with $Na_2S_2O_3$ alone, at concentrations equivalent to that of STS applied, this substance had no effect on the number of roots formed (Fig. 23), indicating that the effect of STS on rooting was caused by silver ions rather than by sodium or sulphur.

The contrary effects of the 2 silver compounds may be explained by the finding that in some tissues $AgNO_3$ can promote ethylene production (Huxter et al. 1979). Thus an explanation for the contrary effects of these silver compounds is that STS indeed acts as an ethylene blocking agent, but in sunflower hypocotyls $AgNO_3$ is somehow unable to act as an ethylene antagonist. Differences in the effectiveness of the 2 salts have been reported in cut carnations (Veen & van de Geijn 1978). Furthermore, since $AgNO_3$ caused tissue

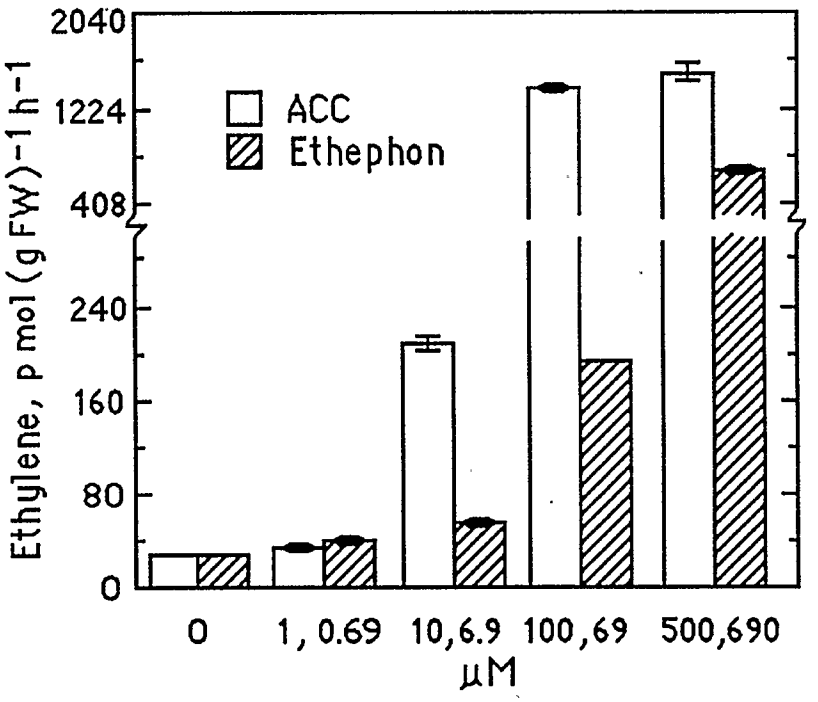


Fig. 20. The effects of ACC and ethephon on ethylene production in the RZ of hypocotyls. Ethylene production was measured at 3 h after root excision. The ethylene-releasing agents were supplied from 0 to 3 h. Means ± SE (n=3 to 4).

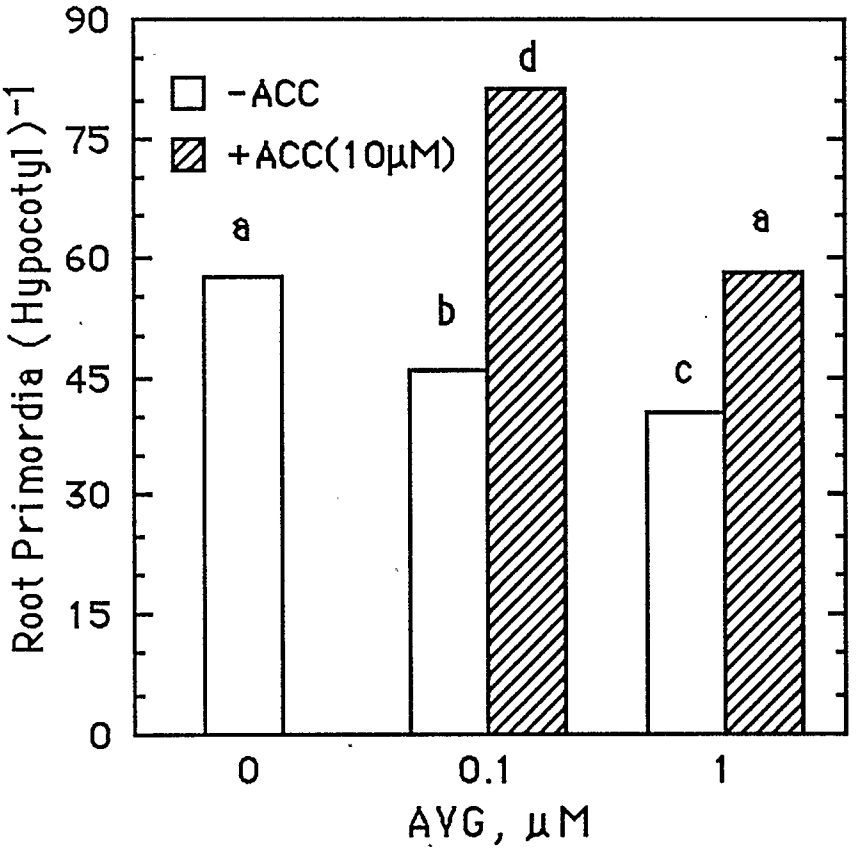


Fig. 21. The effect of AVG on the number of root primordia. AVG was supplied from 0 - 3 h. Bars with different letters are significantly different (P = 0.05).

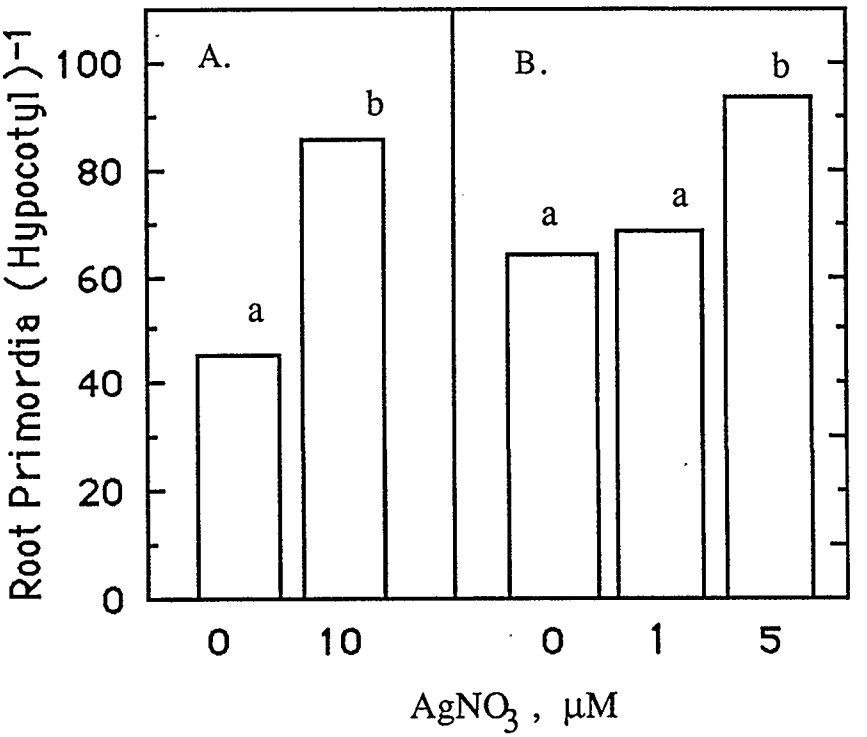


Fig. 22. The effect of silver nitrate on the number of root primordia per hypocotyl. The results of 2 separate experiments are shown. $AgNO_3$ was supplied from 0 to 3 h after root excision. Bars with different letters are significantly different (P = 0.05).

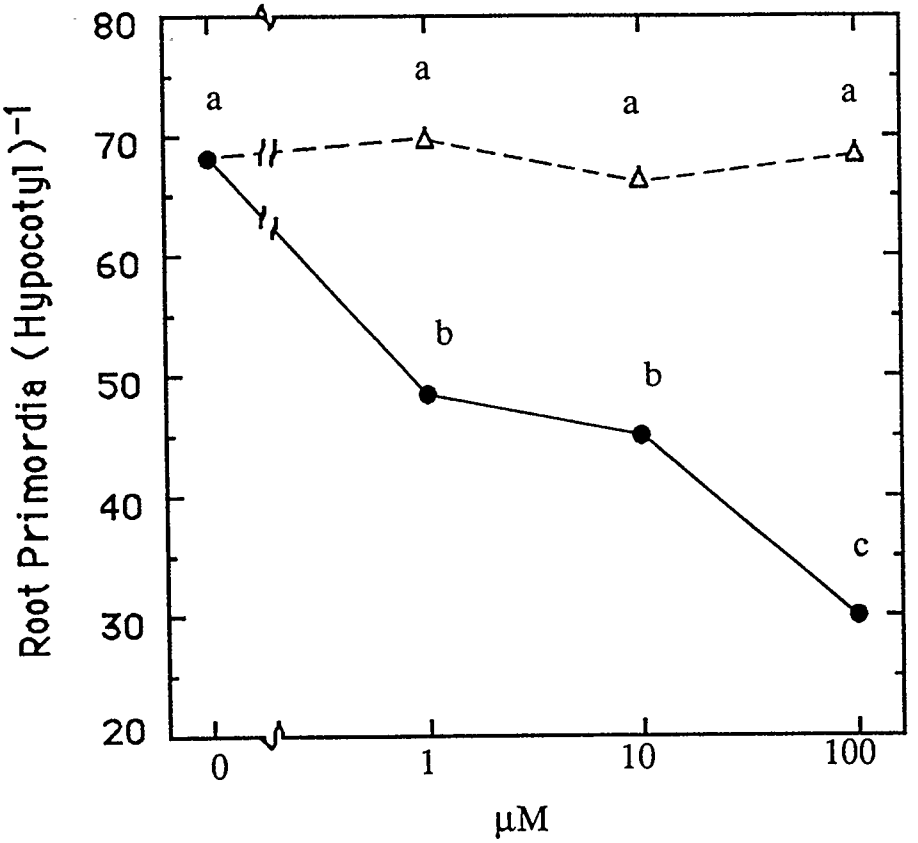


Fig. 23. The effect of STS (•) and $Na_2S_2O_3$ (Δ) on the number of root primordia. STS was supplied from 0 to 3 h after root excision. Points with different letters are significantly different (P = 0.05).

necrosis, I felt that this salt might be toxic to the tissues and, like some other toxic chemicals, promotes ethylene production which might then stimulate rooting.

The writer checked the effects of the 2 silver salts on ethylene production (Tab. 5) and it was found that $AgNO_3$ had a much larger promotive effect on ethylene production than did STS. Next the writer tested the possibility that the stimulatory effects of $AgNO_3$ on rooting (Fig. 22 and Fabijan et al. 1981b) might be due to the promotion of endogenous ethylene production, rather than its inhibitory effect on ethylene action. The data in Fig. 24 shows that STS inhibits and $AgNO_3$ promotes rooting. In the combination treatment, STS overcame the promotive effect of $AgNO_3$ perhaps by blocking the effects of $AgNO_3$-stimulated ethylene production. This result is consistent with the hypothesis that in sunflower hypocotyls, $AgNO_3$ promotes rooting because it increases ethylene production and STS reduces the rate of root formation by blocking ethylene action.

Tab. 5. Effect of $AgNO_3$ and STS on ethylene production in hypocotyls at the rooting zone at 3 h [p mol $(gFW)^{-1}$ h^{-1} ± SE]. Both chemicals were supplied from 0 to 3 h.

concentration (µM)	0	1	5	10
STS	26.9 ± 0.49	65.3 ± 12.8	66.9 ± 11.9	57.9 ± 14.5
$AgNO_3$	26.9 ± 0.49	57.1 ± 6.61	149.3 ± 26.4	219.1 ± 71.8

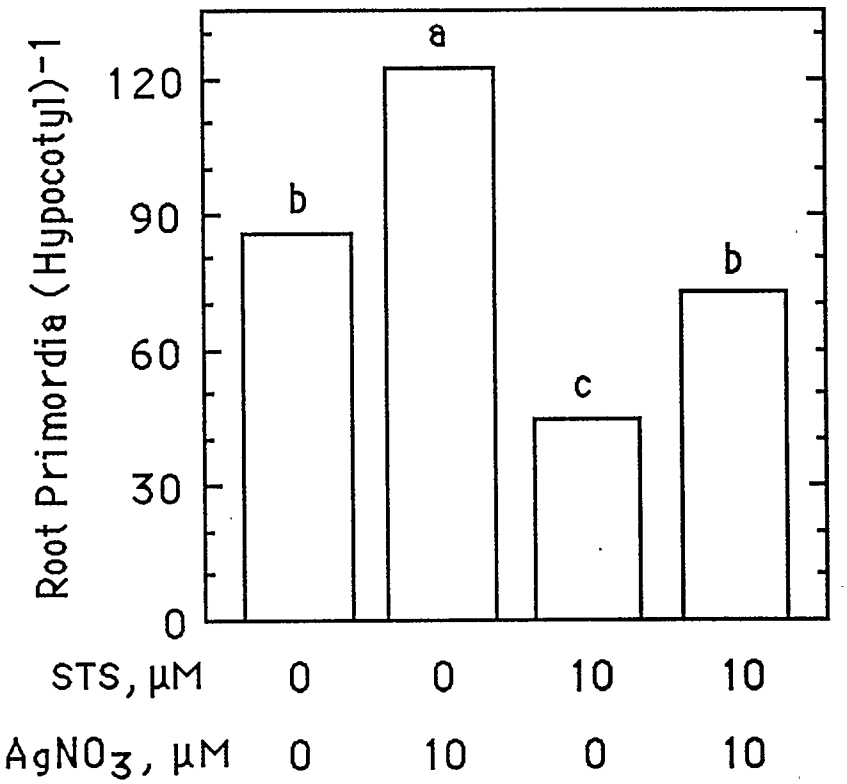


Fig. 24. The effect of STS and $AgNO_3$ alone and in combination on the number of root primordia in hypocotyls. Bars with different letters are significantly different (P = 0.05).

If the early peak of endogenous ethylene production is a promoter of rooting, then ACC and ethephon should also promote rooting. Indeed it is found that low concentrations of ACC (10 μM) supplied from 0 to 3 h stimulated root initiation (Fig. 25). Higher doses of ACC inhibited the process. The stimulatory effect of ethylene over only a narrow range of concentrations was not unexpected and has been seen in other systems (see Introduction).

In this experiment with ACC (Fig. 25) the writer also used STS. STS effectively reduced rooting in the controls (no ACC), presumably by inhibiting the action of endogenous ethylene. This inhibitor also blocked the promotion of rooting caused by 10 μM ACC. At the highest concentrations of ACC, which inhibit rooting, STS again reversed the influence of ACC. I assume that in this latter case the influence of much of the endogenous ethylene was blocked by STS, and that the amount of effective ethylene was now similar to that found due to the application of 10 μM ACC.

The effects of ethephon (Fig. 26) supplied for 0 to 3 h were similar to those of ACC and were in agreement with the observations of Robbins et al. (1983), even though the species and the duration of application were different. Low concentrations of the ethylene-releasing compound increased rooting, while higher concentrations were inhibitory. Again, as in the Fig. 25, STS tended to reverse these effects.

Finally, Chapter 2 showed that gravistimulation of any portions of the hypocotyl inhibited ARF. Gravistimulation also caused an increase in ethylene production. Silver thiosulphate was applied to the hypocotyls the bottom half of which was gravistimulated (treatment 2 in Fig. 2) and the control hypocotyls (treatment 3). Again, as in Fig. 23, STS greatly inhibited rooting in control hypocotyl. It can be seen that STS also inhibited rooting in gravistimulated hypocotyl although the effect was small (Fig. 27).

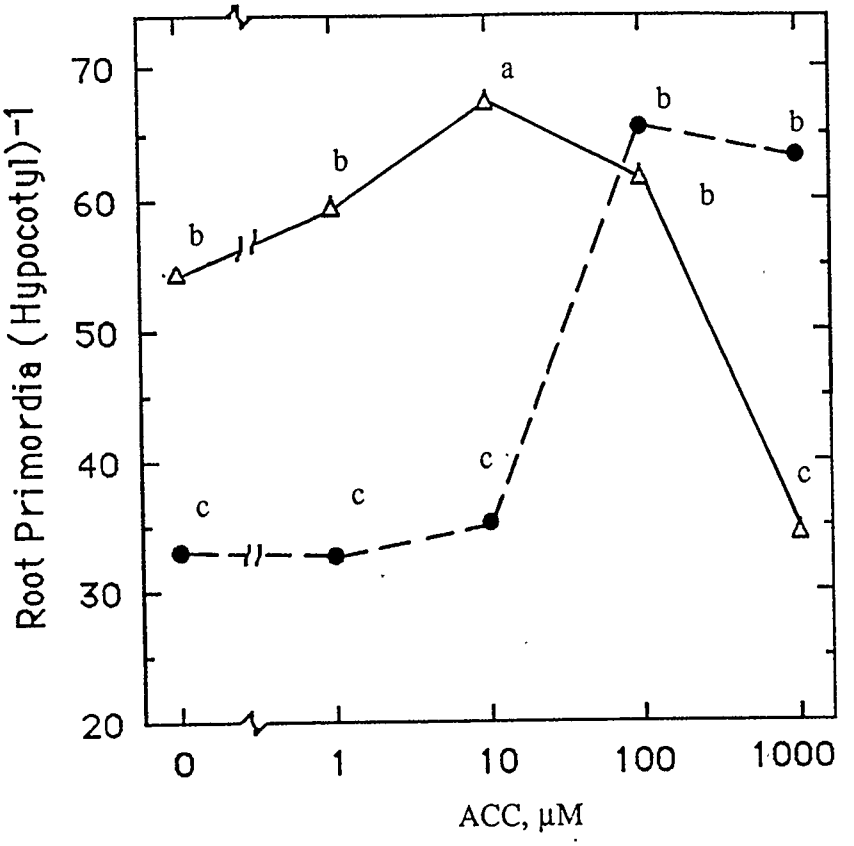


Fig. 25. The effect of 4 concentrations of ACC alone (Δ) and with 5 µM STS (•) on the number of root primordia. ACC and STS were supplied from 0 to 3 h. Points with different letters are significantly different (P = 0.05).

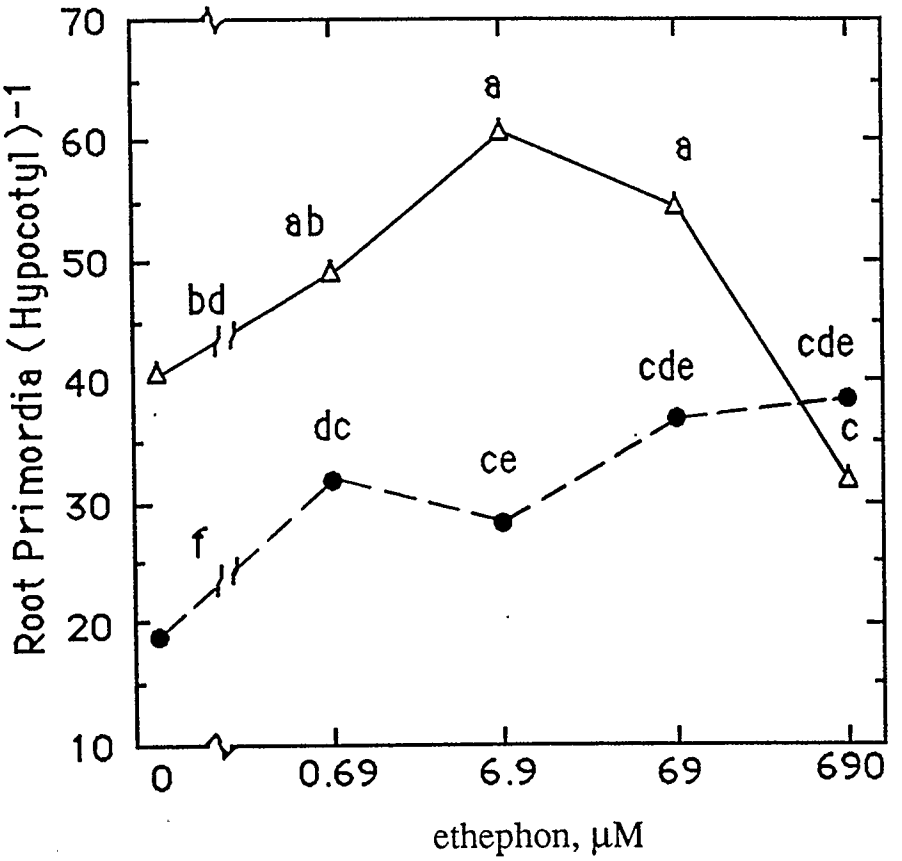


Fig. 26. The effect of 4 concentrations of ethephon alone (Δ) and with 100 µM STS (•) on the number of root primordia. Ethephon and STS were were supplied from 0 to 3 h. Points with different letters are significantly different (P = 0.05).

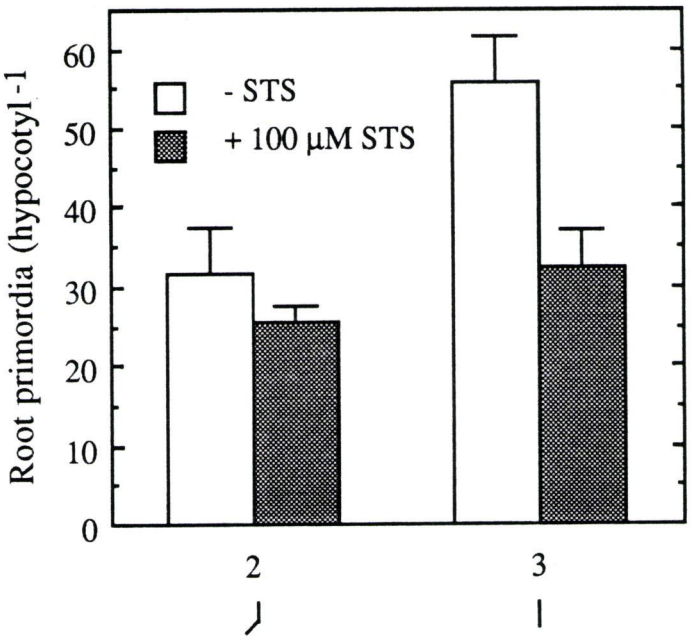


Fig. 27. Effect of inhibitor of ethylene action STS on the number of root primordia in the hypocotyls held in two orientations. Orientation 2 had the basal half of the hypocotyl bent at 45° and top hold upright. In orientation 3 all parts of the hypocotyl were upright. Means ± SE (n = 15).

DISCUSSION

The experiments described above demonstrate that: (i) the lower portion of hypocotyls in which most of the roots are formed showed an early transient increase in endogenous ethylene production. This peak occurred shortly after the removal of the original root system. The increase in ethylene was much less evident in the upper NRZ of the hypocotyl. (ii) This pulse of ethylene production was preceded by a rapid decline in endogenous ACC. (iii) AVG supplied from 0 to 3 h lowered ethylene levels and reduced numbers of primordia. These effects were reversed by exogenous ACC. (iv) Ethephon and ACC supplied from 0 to 3 h were rapidly converted to ethylene and at low concentrations promoted rooting. Higher dose rates inhibited rooting. (v) STS supplied from 0 to 3 h inhibited rooting and overcame the promotive or inhibitory effects of ACC and ethephon. It is thus concluded that the early pulse of endogenous ethylene in the basal portion of hypocotyls is the result of wounding caused by root removal. This ethylene may be the signal - that the original roots have been removed and is thus the key promotive signal for ARF.

I was tempted to suggest that the early peak of ethylene in the RZ is caused by the conversion of ACC into ethylene, rather than by a stimulation of ACC synthesis, and that the subsequent decrease in ethylene production may be due to a limiting supply of ACC caused by the conversion of ACC into MACC. However, in view of the observation (Fig. 19) that aminoethoxyvinylglycine (AVG, an inhibitor of ACC synthesis) lowers ethylene production in the first 3 h, these results do not preclude an effect on ACC synthesis. There appears to be a more than adequate supply of ACC for the amount of ethylene that is actually produced, and the amount of ACC lost from the tissue from 0 to 24 h is much greater than the quantity of ethylene that is produced. The data in Fig. 18 suggests that

between 24 h and 72 h much of this ACC may be converted into MACC, but I do not know the reason for the substantial decline in the amount of ACC between 0 h and 24 h that is neither converted into ethylene nor seems to be malonyated. Perhaps much of the ACC extracted from the hypocotyl is in some compartment where it is unavailable for ethylene synthesis. In view of the ability of ACC to move acropetally (Bradford & Yang 1980), one possibility is that between 0 and 24 h much of the ACC could be transported to the rapidly growing apical regions. It is found that in identical sunflower seedlings, [^{3}C]-ACC is very mobile and can rapidly move up the hypocotyls to the apical region (Finlayson et al. 1991).

Whatever might be the interpretation of the precise relationships between these changes in ACC, MACC and ethylene, it is clear that there are marked differences in the metabolism of ethylene and related compounds between those portions of the hypocotyl that produce roots and those that do not.

The contradictory effects of $AgNO_3$ and STS can be explained as follows. Silver can inhibit the action of ethylene (Beyer 1976). Previously Fabijan et al. (1981b) found a transient promotion of rooting by $AgNO_3$, they concluded that endogenous ethylene, at the early stages of rooting, was inhibiting the rooting process. I now argue that that conclusion was wrong. In this study $AgNO_3$ (at 5 μM) was also found to promote rooting, but at the same time it produced necrotic lesions and greatly promoted ethylene production. The effects of silver in the form of STS were however very different from those of $AgNO_3$. STS produced no visible toxic effects, it inhibited rooting and only slightly promoted ethylene production. It is believed that the reason for the different effects of the 2 forms of silver are that the nitrate form may more quickly become immobile by binding to some component inside the cells. This may have 2 consequences: (a) the immobile silver could have some toxic effect which stimulates ethylene production, and (b) not enough soluble silver remains to reach deeper into the tissues to the sites of root formation. Thus there is

insufficient silver to block the action of ethylene and the high levels of ethylene are able to activate root development. In support of this the writer point to the work of Veen and van de Geijn (1978) who found that silver, applied as $AgNO_3$, moved upward in the stem of cut carnations much more slowly than did silver in the thiosulphate form. I also found on histological examination of free hand fresh sections of $AgNO_3$ treated tissues that the necrotic cells were restricted to the outer tissues of the epidermis and the cortical collenchyma cells. Further, heavy metals are known to have a stress-like effect in that they can stimulate endogenous ethylene production (Abeles 1973). The STS on the other hand appears to be more mobile, has fewer toxic side effects, and is able to reach the site of action of ethylene and block root development.

Some of the arguments used above on the effect of $AgNO_3$ can be used to interpret the effects of BITC. Patil & Tang (1974) found that BITC inhibited ethylene synthesis in papaya and Fabijian et al. (1981b) showed that BITC, like $AgNO_3$, stimulated rooting in sunflower. They then concluded that endogenous ethylene was an inhibitor of the rooting process. However in this present study the writer showed that BITC slightly increased the rate of ethylene evolution from sunflower hypocotyls and now conclude that its stimulatory effect on rooting could be due to an increase in endogenous ethylene. This agrees with the present findings that similar small increases in ethylene are sufficient to promote ARF.

The data presented here show that only low levels of ethephon and ACC which are supplied for short periods will promote rooting, while higher levels inhibit the process. A similar response was also observed when ACC and ethephon were applied to mung bean hypocotyls under identical experimental conditions (data not shown). This kind of concentration response can explain some of the contradictory results in the literature. Geneve & Heuser (1983) reported that adventitious root initiation was reduced in mung bean cuttings treated for 3 h with 100μM and higher concentrations of ethephon. They

failed to observe any effect of ethephon at lower concentrations, possibly due to their failure to count microscopic root initials. They also observed that ethephon reduced the length of roots. The inhibitory effect of ACC and ethephon in pea cuttings reported by Nordström & Eliasson (1984) may also be due to the high concentration of the compounds. They treated the cuttings with 10 μM ethephon and 10-100 μM ACC for 4 days. In other reports in which ethephon was shown to promote ARF, the concentration of ethephon was under 100 μM, e.g. 0.34 μM (Roy et al. 1972), 69 μM (Krishnamoorthy 1970), 4 to 40 μM (Fabijan et al. 1981b), and 0.69 to 69 μM (Robbins et al. 1983). Thus the precise concentration of exogenous ethylene, or of compounds that promote ethylene production, is critical in the interpretation of the results, and this may have lead to some of the confusion in the literature regarding the effectiveness of ethylene on rooting.

The data in Chapter 2 showed that gravistimulation of hypocotyls reduced ARF (Fig. 2) and promoted ethylene production (Fig. 1). One might suggest that gravistimulated ethylene inhibits rooting. However, the inhibitor of ethylene action STS reduced ARF in gravistimulated hypocotyls. It is clear that gravistimulated ethylene, like ethylene induced by wounding or other chemicals reported in this Chapter, is a rooting stimulator. Thus inhibition of ARF by gravistimulation is not due to gravistimulated ethylene. It is possible that auxin is involved in the inhibition of ARF by gravistimulation (see p. 21).

In summary, experiments described is this Chapter support the idea that the early small increase in ethylene concentration in the basal portion of the hypocotyl probably is, along with IAA, one of the key factors that initiates root formation.

Chapter 6. RELATIONSHIP BETWEEN ENDOGENOUS AUXIN AND ETHYLENE

INTRODUCTION

The data in Chapters 3 and 5 showed that both endogenous auxin and ethylene production caused by the excision of original roots have a promotive role in the control of ARF in the hypocotyls of sunflower seedlings. It is unknown if the actions of the two plant hormones are independent of each other, or if one depends on the other.

Exogenous auxin stimulates ethylene biosynthesis in plant tissues (Zimmerman & Wilcoxin 1935, also see review by Abeles 1973). Auxin-induced ethylene synthesis may account for the ability of auxin to induce a number of physiological responses in plants, such as epinasty and inhibition of growth (Abeles 1973). However, the effect of exogenous auxin on rooting appears not to be mediated via auxin-induced ethylene biosynthesis. A number of investigators have found that in mung bean cuttings there was no correlation between auxin-induced ethylene production and the number of roots formed (Geneve & Heuer 1982, Batten & Mullins 1978, Mudge & Swanson 1978).

The experiments here were designed to explore the possible relationship of endogenous auxin and ethylene with respect to the control of ARF in sunflower seedlings. The specific objectives were to investigate (a) if there was any correlation between exogenous auxin-induced ethylene production and ARF; (b) to see if there was any relationship between ethylene production in auxin-depleted hypocotyls and the number of root primordia and (c) if the effect of ethylene on ARF might somehow be mediated by an auxin. To achieved these objectives four approaches were used. (i) Various concentration of IAA and IBA were applied to non-decapitated cuttings and their effect on ethylene production was compared with the number of root primordia. (ii) Ethylene production and ARF were

measured in the basal portion of decapitated and non-decapitated hypocotyls, those treated with NPA, the inhibitor of IAA transport. (iii) The experiments described in Chapter 3 showed that removing the source of endogenous auxin by decapitation greatly reduced ARF. If the effect of exogenous auxin on rooting is mediated via ethylene biosynthesis in the RZ, ethylene-releasing compounds could substitute for endogenous auxin. Thus the effects of ethylene-releasing compounds on rooting in decapitated hypocotyls was observed. (iv) IAA was also applied to decapitated hypocotyls in combination with ethylene-releasing compounds. The data in Chapter 3 showed that exogenous auxins could promote rooting and thus can substitute for endogenous auxins. The interaction of ethylene-releasing compounds and auxins was observed.

MATERIALS AND METHODS

Plant material, growth conditions, modified rooting system, ethylene production and estimation of number of root primordia are as described in Chapter 2. The methods for the production and rooting conditions of decapitated hypocotyl segments are described in Chapter 3. In the experiment with decapitated hypocotyls, ACC or ethephon, alone or together with 50 μM IAA, was applied to the base of hypocotyls from 0 - 5 h. Inhibitor of IAA transport NPA was applied to hypocotyls below cotyledons from 0 - 8 h as described as earlier (Chapter 3). Samples for the measurement of ethylene from the RZ of hypocotyls were taken at 3 h after treatment.

RESULTS

Auxin, ethylene production and ARF

Figure 28 shows the effects of exogenous auxins on ethylene production and ARF in sunflower hypocotyls. At concentration below 1 μM IAA was more effective than IBA in inducing ethylene production. When the concentration was above 10 μM, there was little difference in their ability to induce ethylene production. IAA produced a maximal effect on rooting at only 0.5 μM, while the concentration of IBA had to be raised to 10 μM to produce a similar promotive effect. For both IAA and IBA progressively higher concentration were less effective for ARF.

Ethylene production and ARF in auxin-depleted hypocotyls

The relationship between ethylene production and rooting was studied in auxin-depleted hypocotyls. Figure 29 shows that NPA had little effect on ethylene production, but it significantly reduced ARF. In the next experiment (Tab. 6) when the major source of endogenous auxin was removed by decapitation, ARF was reduced dramatically. Again auxin-depletion had no influence on ethylene production in RZ.

Rooting response of decapitated hypocotyls to exogenous ACC, ethephon and IAA

Figure 30 shows the response of decapitated (auxin-deprived) hypocotyls to ethylene-releasing compounds. It was found that ethephon alone from 6.9 - 690 μM and ACC from 1 - 100 μM) had little effect on root formation in decapitated hypocotyls (data not completely shown). However, ACC at 10 μM and ethephon at 69 μM enhanced ARF in those hypocotyls that were also supplied with IAA (50 μM).

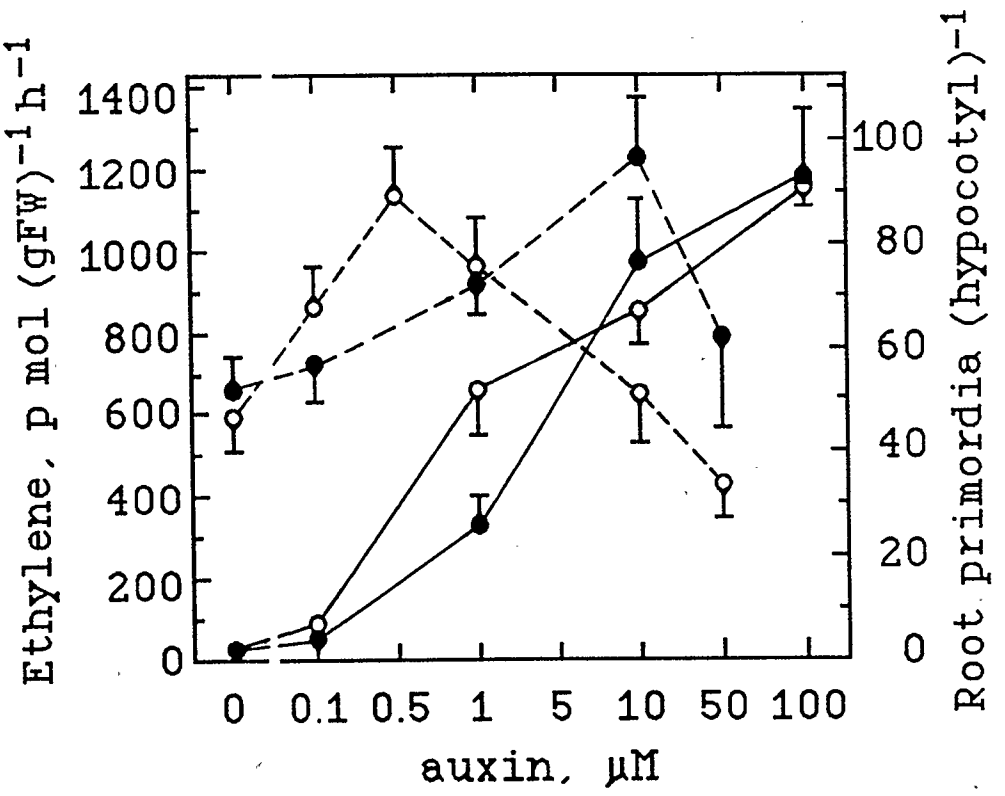


Fig. 28. Effect of exogenous auxins on the number of root primordia (broken lines) and ethylene production in the RZ of hypocotyls (solid lines). Ethylene production rates were determined at 3 h after the initiation of the treatments. Auxins were applied from 0 - 5 h. IAA, ∘; IBA, •. The error bars are the standard error of the means.

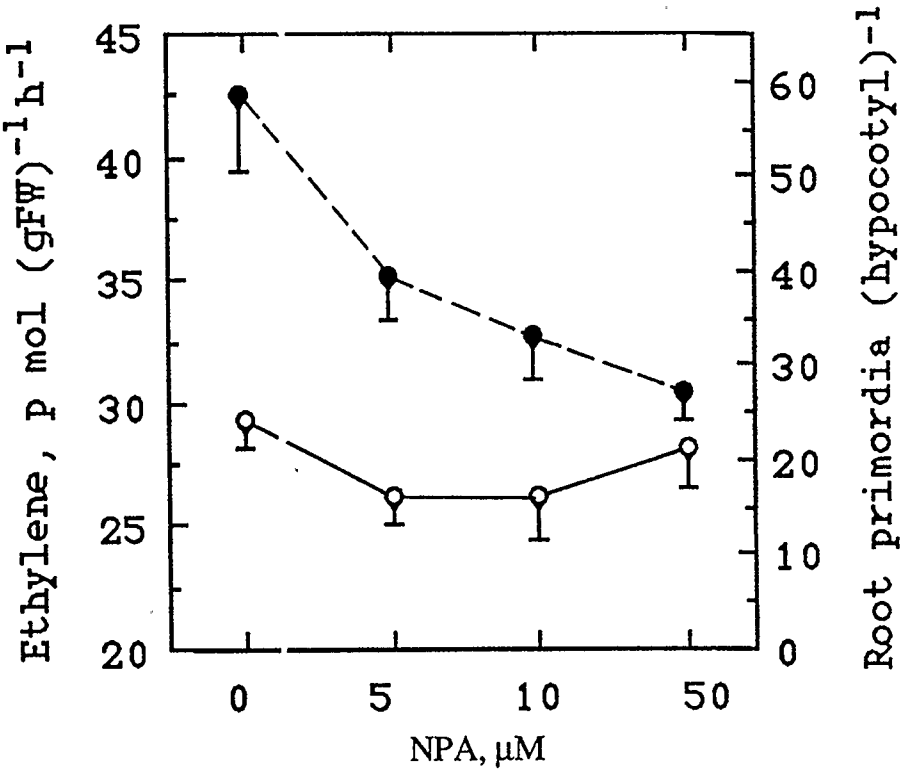


Fig. 29. Effect of NPA, the inhibitor of auxin transport, on the number of root primordia (broken line) and ethylene production in the rooting zone of hypocotyls (solid line). NPA was applied to the hypocotyl just below cotyledons. Ethylene production rates were determined 3 h after the initiation of NPA treatment. The error bars are the standard error of the means.

Tab. 6. Ethylene production in the RZ of hypocotyls, measured at 3 and 12 h after cuttings were made, and number of root primordia in decapitated and non-decapitated seedlings.

type of cuttings	ethylene, p mol(gFW)$^{-1}$ h^{-1} ± SE		number of root primordia
	3 h	12 h	
non-decapitated	23.1 ± 3.6	9.5 ± 3.4	42.0 ± 3.97
decapitated	21.7 ± 2.5	10.6 ± 1.4	10.5 ± 0.76

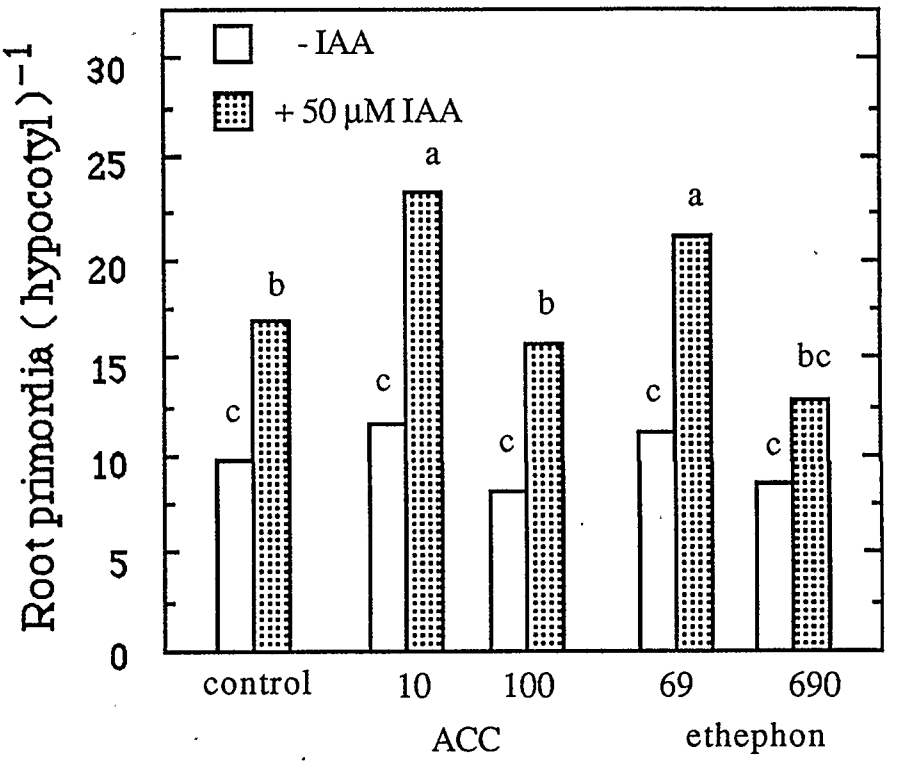


Fig. 30. The effect of ethylene-releasing compounds, alone and together with IAA, on rooting in decapitated hypocotyls. Chemicals were applied from 0-5 h. Half-strength Hoagland's solution was supplied throughout the experiment. Bars with different letters are significantly different (P=0.05).

DISCUSSION

The results reported here agree with those on mung bean seedlings (Geneve & Heuer 1982, Batten & Mullins 1978, Mudge & Swanson 1978) showing that the relationship of auxin-induced adventitious root and auxin-induced ethylene production is not straightforward. Although both IAA and IBA enhanced ethylene production and stimulated root formation, there was no clear correlation between the amount of induced ethylene and the number of root primordia. For example, different concentrations of IBA and IAA produced optimal rooting yet there was only relatively little difference in the concentration of IBA and IAA needed to promote ethylene production (Fig. 28). Auxin-depletion in the RZ of hypocotyls caused by NPA treatment inhibited rooting, but no inhibition of ethylene production was shown (Fig. 29). Auxin-depletion in the RZ of hypocotyls caused by removing the major source of auxin (decapitation) also reduced ARF to about 20 % of those found in non-decapitated cuttings. Ethylene production rate in the RZ was again unaffected (Tab. 6). Thus it can be concluded that the stimulation of auxin on rooting in sunflower hypocotyls is not simply due to the induction of ethylene production.

For the following reasons it could be argued that auxin is directly involved in the control of adventitious rooting, while the effect of ethylene is mediated by auxin. (i) Both decapitation and NPA treatment inhibited the ARF even though ethylene production rate was unaffected, suggesting that auxin, but not ethylene was the limiting factor in adventitious rooting. This idea is supported by a similar result reported in flooded *Acer negundo* in which the blocking of basipetal auxin transport with NPA reduced ARF despite the presence of large amount of ethylene in the basal portion of stem (Yamamoto & Kozlowski 1987). (ii) Exogenous auxins could substitute for the endogenous one in stimulating adventitious rooting. (iii) Ethylene-releasing compounds used alone showed no

promotive effect on root formation in auxin-deprived hypocotyls. But they at low concentration enhanced auxin-stimulated ARF in these hypocotyls. These results are consistent with the findings of Batten and Mullins (1978), that ethylene alone had no promotive effect on rooting in the decapitated mung bean cuttings. Similar conclusion was also made in a study of flood-induced ARF by Wample and Reid (1979). They concluded that ARF in the hypocotyls of flooding sunflower plants was primarily controlled by auxin, with ethylene acting only as a cofactor.

The indirect effect of ethylene and primary effect of auxin may also be seen in surgical experiment. Cuttings of hypocotyls caused ethylene production (Fig. 17). It would be expected that ethylene production would increase above and below the cut region. However, basipetally transported auxin could accumulate only above the cuts and roots were only formed above the cuts.

It was shown that with gravistimulated of hypocotyls, the enhanced ethylene production was unable to promote rooting (Chapter 2). This suggests that other rooting factor(s) which may also be influenced by gravistimulation may be more important than ethylene. The result also suggests that the effect of ethylene in the control of ARF may be indirect.

Chapter 7.

ALTERING THE SENSITIVITY OF HYPOCOTYLS TO AUXIN BY ETHYLENE

INTRODUCTION

It was shown in Chapter 6 that the action of ethylene on promoting ARF depended on the existence of cotyledons and apical bud, or presence of exogenous auxin. In other words, the effect of ethylene on rooting may be mediated by auxin. This study attempted to investigate how ethylene may influence the action of auxin.

There seem to be two likely ways in which ethylene could influence auxin action. Firstly, ethylene may change the concentration of auxins in hypocotyls. This could be achieved by affecting basipetal transport of auxin and/or its metabolism. Ethylene can inhibit basipetal auxin transport (Morgan & Gausman 1966, Burg & Burg 1967). It has been proposed that the promotive effect of ethylene on rooting may be due to its ability to inhibit auxin transport, and hence subsequent accumulation of auxin in tissue occurred above the transport block (Apelbaum & Burg 1972, Robbins et al. 1983, 1985). Accumulation of auxin caused additional ARF in this zone. As far as the writer knows, there are no reports that this hypothesis has been tested in cuttings. Ethylene may reduce the rate of auxin metabolism, including conjugation and oxidation, and hence increase the level of effective auxin in the RZ. Secondly, ethylene may alter the sensitivity of the sunflower hypocotyls to auxin, a phenomenon which was observed during root growth (Bertell et al. 1990) and gravitropism of soybean hypocotyls (Salisbury et al. 1988, Rorabaugh & Salisury 1989).

These possibilities were investigated in sunflower hypocotyls. The effect of ethylene on [^{3}H]-IAA transport was examined in isolated hypocotyl segments and the hypocotyls with

attached cotyledons and shoot apex. The influence of ethylene on auxin metabolism was investigated by comparing the rates and types of [^{3}H]-IAA metabolism between AVG, STS, or ethephon treated hypocotyls. Endogenous IAA levels in the RZ of the hypocotyls treated with AVG, STS, ACC or ethephon were measured using GC-MS-SIM. Silver thiosulphate, ACC and ethephon were also applied to NPA or IAA treated hypocotyls of the seedlings. In these experiments the number of root primordia in these hypocotyls was also recorded.

MATERIALS AND METHODS

Seedling preparation and rooting system

Plant material, growth conditions, modified rooting system, application of test solution, measurement of ethylene and estimation of number of root primordia were as described in Chapter 2. Solution of STS was prepared as described in Chapter 5. Solutions of IAA, IBA, NPA were prepared and applied to the hypocotyls of the derooted seedlings as described in Chapter 3.

Ethylene-releasing compounds, ethylene inhibitors and [^{3}H]-IAA transport

(a) Isolated hypocotyl segments

Initially, isolated hypocotyl segments were used to test the effect of ethylene on [^{3}H]-IAA transport. Hypocotyl segments (20 mm in length) were excised from the basal portion of hypocotyls of 6 day old seedlings. The seedlings were derooted and the basal portion of hypocotyls were immersed in solutions of ethylene-releasing compounds (ACC or ethephon) or ethylene inhibitors (AVG or STS) at various concentrations. After 3 h, the

basal 5 mm of the hypocotyl was removed before a 20 mm length of the hypocotyl segment was cut. This manipulation was undertaken to get a fresh cut surface. [^{3}H]-IAA (0.068 μCi) was supplied with 1.5 % agar blocks from the apical end of segments. On the basal end of the segments same concentration of investigated compounds were supplied in an agar block. Segments were kept in vertical position with their acropetal ends in their normal orientation in darkness at room temperature for 6 h. Ethylene production of segments was measured at 2.5 h after segments were made. Preliminary experiment showed no difference in radioactivity in receiver block among the treatments. I then looked at the distribution of radioactivity within the tissue. The hypocotyl segments were excised into 3 sections: the apical 5 mm, middle 10 mm and basal 5 mm. Radioactivity from apical and basal sections were extracted with 80% methanol as described in Chapter 3. The extracts were then passed through a Sep-Pek ^{18}C column to remove most of the pigments before radioactivity was counted according to the methods in Chapter 3.

(b) Hypocotyls with attached cotyledons and shoot apex

Ethephon, ACC or AVG was supplied to the apical portion of hypocotyls of derooted seedlings at 1 cm below the cotyledonary node in cotton wool as described elsewhere in Chapter 3. Ethylene production rate was measured at 2 h after treatment in three sections: (I) the portion above the cotton wool; (II) the portion in contact with the cotton wool; and (III) the portion below the cotton wool. Each section was 1 cm in length. One μl aqueous solution of [^{3}H]-IAA (1.04 TBq/mM) containing 0.052 μCi was applied to one of the cotyledons as described previously (Chapter 3). After 6 h hypocotyls below the site of treatments (cotton wool) were sampled. Each treatment contained 8 derooted seedlings. The hypocotyls were oxidized with a R. J. Harvey OX500 oxidizer (Chapter 3).

Ethylene and [^{3}H]-IAA metabolism

[^{3}H]-IAA (1.04 TBq/mM) was applied to one cotyledon of each seedling just before derooting. Ethephon, AVG or STS was supplied to the cut base of hypocotyls from 0 - 3 h after derooting. At 9 h the basal 2 cm of each hypocotyl was taken. Tissues were extracted and extracts then subjected to HPLC without preliminary purification using eluating program one as described in Chapter 3. The eluants were 24.4 % aqueous methanol : 0.86 % glacial acetic acid. Thirty-two fractions were collected, and 0.1 ml of each was added to 5 ml ScintiVerse for counting by liquid scintillation counting (Chapter 3).

Measurement of endogenous IAA

The possible effect of ethylene on endogenous IAA levels was investigated. AVG (1 μM), STS (10 μM), ACC (10 μM) or ethephon (6.9 μM) was applied to the base of hypocotyls from 0 - 3 h. Nine h after the initiation of the treatments hypocotyl segments (2 cm in length) were harvested from the basal portion of the hypocotyls for endogenous IAA quantification following the methods described in Chapter 3.

Application of ACC and STS and the rooting response of hypocotyls to exogenous IAA

The test solutions (20 ml) of ACC or STS was applied to the base of hypocotyls from 0-3 h after cuttings were made, IAA from 0 - 5 h. Hence at first 3 h test solution contained IAA plus ACC or STS at indication level. After 3 h, the cuttings were rinsed with deionized water and kept in IAA solution till 5 h. Then the cuttings were transfered to deionized water for the rest of the experiment.

Application of ACC, ethephon and STS to auxin-deprived hypocotyls

NPA was applied to the hypocotyls below the cotyledonary node from 0 - 8 h as described in Chapter 3. The test solutions (20 ml) of ACC, ethephon or STS was applied to the base of hypocotyls from 0-3 h after original roots were removed.

RESULTS

Ethylene-releasing compounds, ethylene inhibitors and [^{3}H]-IAA transport

The effect of ethylene on basipetal transport of auxin was tested in isolated hypocotyl segments and the hypocotyls with attached cotyledons and shoot apex.

From Tab. 7 it can be seen that AVG inhibited ethylene production measured at 2.5 h after segments were made, wheres both ACC and ethephon increased ethylene production. There was no significant difference in the amount of radioactivity between hypocotyls treated with these substances and control, in both the apical and basal portions of hypocotyl segments, indicating that ethylene has no effect on IAA uptake and transport.

The disadvantage of using isolated hypocotyl segments in this experiment is that unavoidable wound ethylene occurs in the segments upon the excision. A control with normally occurring level of ethylene could not be obtained. Thus the possible effect of ethylene on auxin transport was further tested in the hypocotyl region distant from the cut-end. Compounds were applied to the apical portion of hypocotyls of derooted seedlings (with cotyledons and shoot apex). Table 8 shows ethylene production and radioactivity in different portions of hypocotyls. Ethylene production rate in the hypocotyl at the site of

Tab. 7. The effect of ethylene-releasing compounds and inhibitors of ethylene synthesis and action, on the distribution of radioactivity in hypocotyl segments after [^{3}H]-IAA was applied from the apical cut-end of the segments in agar blocks. The hypocotyls were pretreated with solutions of indicated compounds for 3 h prior to excision of the segments. The compounds at the same concentrations were also applied in agar blocks placed in the basal cut-end of the segments. Ethylene production was measured at 2.5 h after segments were excised. All values are means ± SE.

		DPM$\times 10^3$ (segment)$^{-1}$		ethylene,
		apical (5 mm)	basal (5 mm)	p mol (gFW)$^{-1}$ h^{-1}
Exp.1	control	23.4 ± 1.78	5.2 ± 0.58	
	STS, 100 µM	23.0 ± 0.50	5.3 ± 0.36	
Exp.2	control	20.6 ± 0.70	4.7 ± 0.12	49.0 ± 4.90
	AVG,10µM	19.8 ± 0.51	5.2 ± 0.32	12.8 ± 0.93
	ACC,100µM	21.1 ± 0.11	4.6 ± 0.23	374.5 ± 28.15
Exp.3	control	20.2 ± 0.56	5.9 ± 0.53	44.1 ± 3.67
	ethephon, 69µM	21.0 ± 1.90	6.2 ± 1.07	205.2 ± 9.63

Tab. 8. The effects of AVG, ethephon and ACC on ethylene production and [^{3}H]-IAA transport in hypocotyls. Radioactivity was measured in the portion of the hypocotyl that was basipetal to the region to which the various chemicals were applied. The chemicals were supplied periodially to the hypocotyl 1 cm below the cotyledons (region II) of derooted seedlings throughout the experiment. Hypocotyl region I was above the treatment and III was below the treatment. Each region was 1 cm in length. The hypocotyls were pretreated with regulators for 2 h prior to the application of [^{3}H]-IAA to one cotylendon. Six h after [^{3}H]-IAA application, hypocotyl region III was harvested for determination of radioactivity.

compound		ethylene, p mol (gFW)$^{-1}$ h^{-1} I	II	III	DPM X 10^3 (hypocotyl)$^{-1}$
control		35.9 ± 3.3	36.7 ± 6.2	28.2 ± 1.3	13.7 ± 0.59
AVG, µM	5	31.4 ± 1.6	16.9 ± 1.3	15.0 ± 0.4	11.9 ± 0.91
	10	17.5± 1.6	10.8 ± 1.3	12.8 ± 0.3	12.5 ± 0.85
ethephon, µM	34	74.9 ± 3.9	48.9 ± 16.4	22.0 ± 4.9	15.1 ± 0.91
	69	105.3 ± 7.3	45.3 ± 2.4	22.4 ± 2.1	14.2 ± 1.13
	345	226.9 ± 9.7	103.6 ± 8.0	28.0 ± 5.0	12.9 ± 1.03
	690	408.8 ± 80.8	268.2 ± 16.1	42.4 ± 1.8	12.9 ± 0.63
ACC, µM	10	90.2 ± 5.8	111.4 ± 4.8	27.4 ± 2.4	15.1 ± 1.15
	50	148.6 ± 10.3	364.9 ± 25.8	27.9 ± 2.1	13.7 ± 1.41
	100	579.8 ± 24.8	1167 ± 120.0	43.5 ± 1.9	12.2 ± 0.40
	200	603.6 ± 45.4	1122 ± 74.7	61.9 ± 1.2	14.3 ± 1.70

treatment (section II) and above (section I) was greatly enhanced by ACC and ethephon. In section III (below the site of treatment) relatively small increases in ethylene production were found and only at very high concentration of ACC or ethephon. The results indicate that applied ACC or ethephon was absorbed well by hypocotyls and moved acropetally. Radioactivity from IAA found at basal portion of hypocotyls below the treatment were not significantly different over a wide range of concentration of ACC or ethephon. AVG reduced ethylene production rate to the level below its natural one, but it did not influence the movement of radioactivity. It thus seems reasonable to conclude that ethylene did not affect IAA transport in this system.

Ethylene and [^{3}H]-IAA metabolism

Ethephon, AVG and STS were applied to test the effect of ethylene on [^{3}H]-IAA metabolism. Analysis by HPLC of a crude extract of hypocotyls which had been treated with these compounds indicated that these compounds showed little significant effect on the pattern of metabolism of [^{3}H]-IAA in the hypocotyls (Fig. 31). Experiments described in Chapter 5 showed that these compounds, used at these concentrations, significantly promoted or inhibited rooting. To compare the rate of [^{3}H]-IAA metabolism among the treatments, the peak cochromatographing with an internal [^{14}C]-IAA standard was identified as the "IAA peak", and the total radioactivity of all other peaks were summed and called "IAA metabolites". Table 9 shows the amount of unmetabolized and metabolized IAA in the base of hypocotyls. There was little difference in total radioactivity, unmetabolized plus metabolized [^{3}H]-IAA, found at the basal portion of hypocotyls among the treatments. Further, the ratio of [^{3}H]-IAA to total radioactivity showed little variation between controls and any of the treatment. My interpretation of these results is that ethylene had little effect on the rate of [^{3}H]-IAA metabolism.

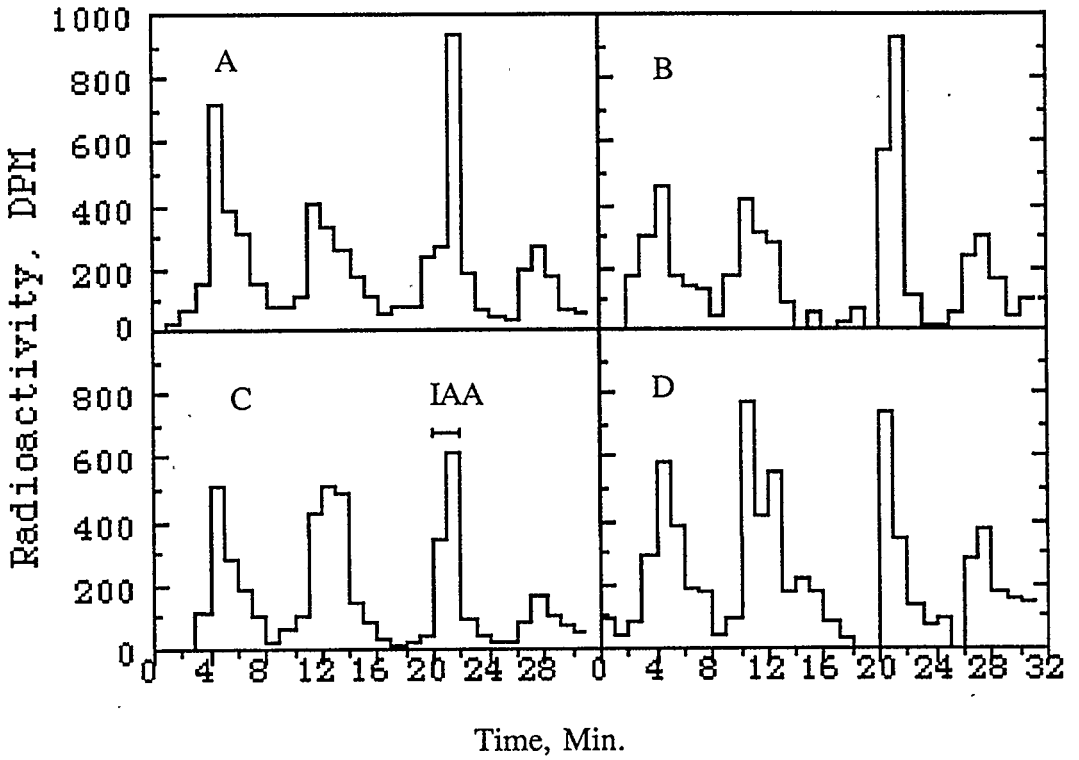


Fig. 31. Metabolism of [^{3}H]-IAA in the basal portion of control hypocotyls (A) and the hypocotyls treated with AVG (0.5 μM, B), STS (10 μM, C) or ethephon (6.9 μM, D). [^{3}H]-IAA was applied to one cotyledon at 0 h. The crude extracts of the hypocotyls harvested at 9 h after [^{3}H]-IAA application were injected into HPLC.

Tab. 9. The quantities of [^{3}H]-IAA and [^{3}H]-IAA metabolites shown as DPM per hypocotyl in the RZ of hypocotyls treated with AVG, STS and ethephon. Samples were taken 9 h after [^{3}H]-IAA was applied to one cotyledon. Figures in parentheses are the IAA and IAA metabolites as a percentage of total.

	control	AVG, 0.5 µM	STS, 10 µM	ethephon, 6.9 µM
IAA	2992±145(31.2)	2767±410(30.6)	3012±186(26.8)	3020±193(30.1)
IAA metabolites	6584±292(68.7)	6268±115(69.4)	8209±320(73.2)	7006±158(69.9)
total	9576±776(100)	9035±204(100)	11221±490(100)	10026±1506(100)
$\frac{\text{IAA}}{\text{total}} \times 100$	27.8	30.6	26.8	30.1

Ethylene and endogenous IAA level

Table 10 shows the endogenous IAA levels in the hypocotyls treated with AVG, STS, ACC or ethephon. As expected, neither the inhibitors of ethylene synthesis and action nor the ethylene-releasing compounds affected endogenous IAA concentration, since earlier experiments indicated that ethylene had no effect on auxin transport and metabolism.

Tab. 10. Endogenous IAA levels in the RZ of the hypocotyls treated with ethylene inhibitors or ethylene-releasing compounds. The chemicals were applied from 0 - 3 h. IAA was measured at 9 h after the initiation of the treatments. (means ± SE, n = 1 or 2).

	IAA, ng (g FW)$^{-1}$
control	23.8±0.88
AVG, 1 μM	23.2
STS, 10 μM	23.7±0.23
ACC, 10 μM	21.9±0.61
ethephon, 6.9 μM	24.1±2.03

ACC, STS and rooting response of hypocotyls to exogenous auxin

Table 11 shows the original data on the effect of exogenous IAA with or without the addition of STS or ACC on ARF. In order to compare the number of root primordia of STS- or ACC-treated and untreated hypocotyls from experiments carried out on different occasions, the number of root primordia was converted into the percentage response for distilled water only control. IAA concentrations were then plotted against the percentages of root primordia (Fig. 32). Response curves shifted as the result of the application of ACC or STS. For non-STS or -ACC treated hypocotyls the number of root primordia peaked at 0.5 μM of IAA. While ethylene inhibitor STS was applied, the maximum rate of root production was at 1 μM of IAA. When ACC was supplied, the number of root primordia peaked at lower level of auxin than that of untreated hypocotyls, i.e. 0.1 μM.

Tab. 11. Effect of ACC or STS, in combination with IAA, on the number of root primordia per hypocotyl. Table shows 3 separate treatments each containing a deionized water only control.

		deionized water	STS, 10 μM	ACC, 50 μM
IAA, μM	0	46.5 ± 6.25	57.3 ± 4.10	72.8 ± 9.82
	0.1	67.7 ± 7.96	60.1 ± 4.90	94.7 ± 9.53
	0.5	89.5 ± 8.76	63.6 ± 7.04	89.7 ± 5.97
	1	75.6 ± 9.32	88.1 ± 8.88	76.5 ± 4.90
deionized water only control, no STS or ACC		46.5 ± 6.25	69.9 ± 3.65	55.4 ± 5.56

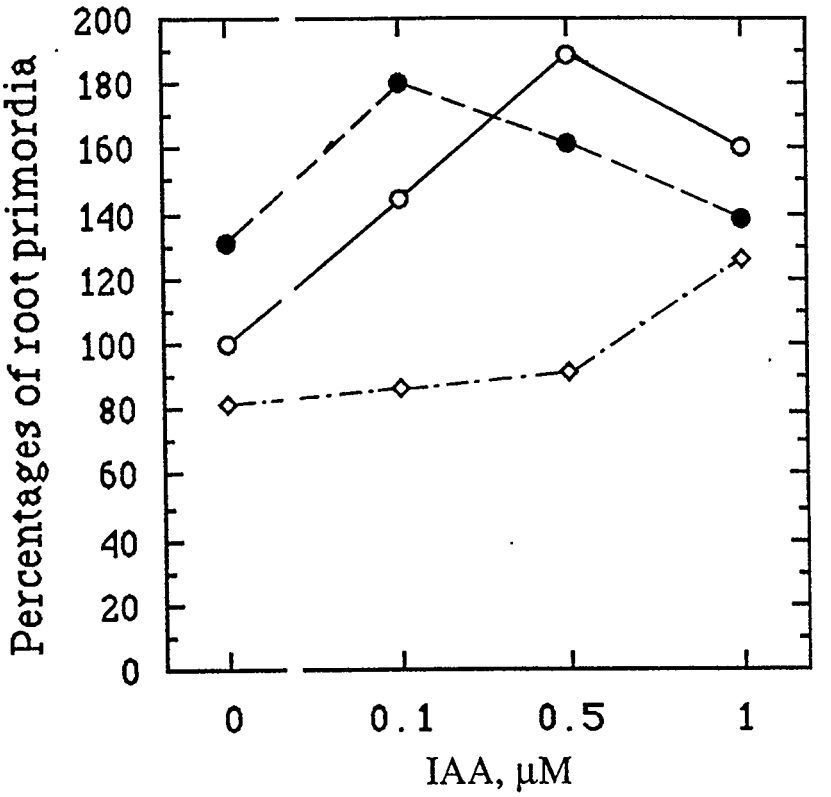


Fig. 32. Effect of ACC and STS on rooting response of hypocotyls to exogenous IAA. The compounds were applied to the base of the hypocotyls. Figure shows 3 separate experiments each containing a deionized water only control. Data are the percentage of the deionized water only control. Deionized water, ○; 50 µM ACC, •; and 10 µM STS, ◊.

Influence of ACC, ethephon and STS on adventitious rooting in auxin-deprived hypocotyls

I then decided to see how might auxin-depleted hypocotyls respond to ethylene-releasing compounds and ethylene inhibitor. Table 12 shows original data from 4 experiments carried out on different occasions. It can be seen that the number of root primordia decreased as the concentration of NPA increased irrespective of the treatment with either ethylene-releasing compounds or inhibitor. In order to compare the response curves of the treatment of ethylene-releasing compounds or inhibitor from separate experiments the number of root primordia was again converted into the percentage the response for distilled water only control. Then NPA concentrations were plotted against relative rate of root primordia (Fig. 33). Response curves shifted as the result of the application of ethephon, ACC or STS. Results indicated that ethylene enhanced the sensitivity of tissue to endogenous auxin.

Tab. 12. Effect of ACC, ethephon and STS, in combination with NPA, on the number of root primordia per hypocotyl (means ± SE, n = 15). NPA was applied to the hypocotyls below cotyledons and others to the base of hypocotyls. Table shows 4 separate treatments each containing a deionized water only control.

		deionized water	STS,10 µM	ethephon,6.9 µM	ACC, 50 µM
NPA, µM	0	58.8 ± 8.11	57.3 ± 4.10	82.7 ± 5.34	72.8 ± 9.82
	5	39.4 ± 4.52	32.2 ± 3.08	–	64.8 ± 6.58
	10	33.5 ± 4.95	35.0 ± 3.59	48.1 ± 5.86	51.5 ± 7.67
	50	27.0 ± 2.83	25.8 ± 1.28	51.3 ± 7.24	39.9 ± 4.67
deionized water only control, no STS, ethephon or ACC		58.8 ± 8.11	69.9 ± 3.65	64.1 ± 4.98	55.4 ± 5.56

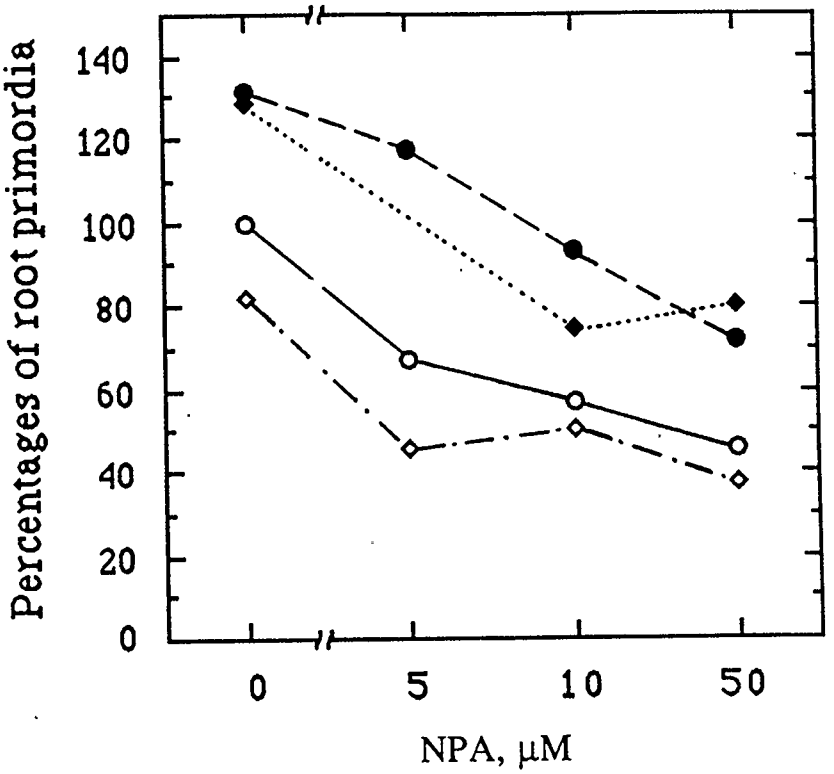


Fig. 33. Effect of ethylene-releasing compounds and one inhibitor of ethylene action on rooting response of hypocotyls to the inhibitor of auxin transport NPA. NPA was applied to the hypocotyls just below cotyledons, while other compounds were supplied to the base of the hypocotyls. Figure shows 4 separate experiments each containing a deionized water only control. Data are the percentage of deionized water only control. Deionized water, ∘ ; 50 μM ACC, • ; 6.9 μM ethephon, ♦ ; and 10 μM STS, ◊ .

DISCUSSION

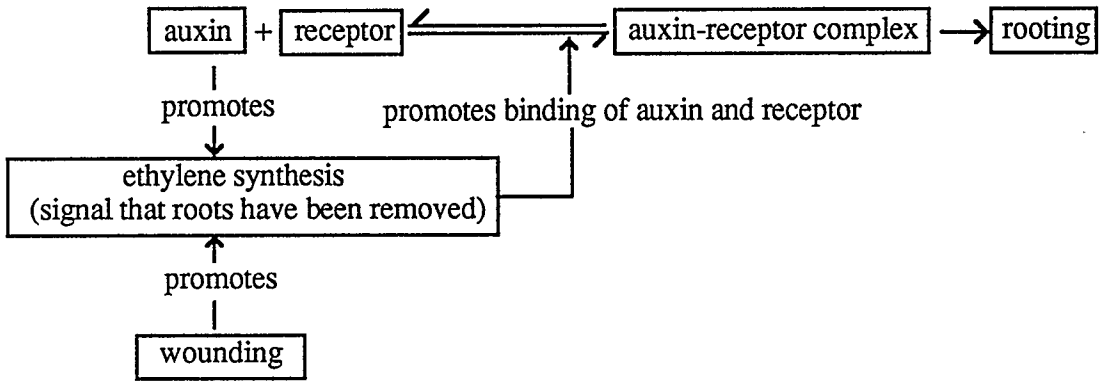
The key findings of the experiments described in this Chapter are: (i) An inhibitor of ethylene biosynthesis, and an inhibitor of ethylene action showed no inhibitory effect on [^{3}H]-IAA transport; (ii) These same inhibitors appeared to have no effect on the rates and types of [^{3}H]-IAA metabolism; (iii) Ethylene-releasing compounds enhanced the rooting response of hypocotyls to exogenous IAA and decreased the inhibition of rooting by IAA transport inhibitor NPA. The inhibitor of ethylene action STS, reduced the rooting response of hypocotyls to exogenous IAA and increased the inhibition of rooting by NPA.

Apelbaum & Burg (1972) proposed that the effect of ethylene on rooting may be due to alteration of the distribution of auxin in the hypocotyl by ethylene. The findings presented here do not support that hypothesis, since ethylene did not affect [^{3}H]-IAA transport in the hypocotyls of sunflower seedlings. The evidence showing that ethylene inhibits auxin transport is mainly found in older literature (Morgan & Gausman 1966, Burg & Burg 1967, Osborne & Mullins 1969, Beyer & Morgan 1969, 1971). These authors showed that ethylene pretreatment of intact plants or excised sections prior to transport is inhibitory to IAA transport, but ethylene supplied to excised sections along with auxin during a test of auxin transport is ineffective (Abeles 1966, Burg & Burg 1967, Osborne & Mullins 1969). Furthermore, the response to ethylene varied among the species, with highly susceptible cotton and okra and insensitive sunflower which showed little response after a prolonged pretreatment of 15 h (Abeles 1972). In either case, the response to ethylene is relatively slow. Therefore it is not surprising that ethylene was ineffective on IAA transport in the investigation here in which sunflower hypocotyls was tested with 3 h ethylene pretreatment or even without any pretreatment. If the pretreatment of ethylene prolonged it might inhibit basipetal auxin transport, however, this would not likely affect rooting because a short time

exposure to ethylene was critical in promoting adventitious rooting (Chapter 5). It was also found that ACC applied to apical portion of hypocotyls did not inhibit ARF (data not shown) but NPA did (see Chapter 4).

Neither ethephon nor inhibitors of ethylene action/synthesis influenced [^{3}H]-IAA metabolism even though at these concentrations they could either promote or inhibit rooting. The result was consistent with earlier findings (Chapter 4) that the types of [^{3}H]-IAA metabolites in the hypocotyl of intact and derooted seedlings were very similar, although wounding by excision caused an increase in ethylene production in derooted seedlings (Chapter 5).

Thus, there was no evidence that ethylene altered the level of auxin in the RZ of hypocotyls either by inhibiting auxin transport or the rate of auxin metabolism. However, ethylene changed the rooting response to endogenous (basipetally transported) or exogenously applied auxin (Figs 32 & 33). With the presence of ethephon or ACC the responsiveness of hypocotyls to endogenous and exogenous auxin was enhanced. In contrast, the inhibitor of ethylene action STS reduced the response of tissue to auxin. How might sensitivity to auxin change in response to ethylene? The writer proposes that ethylene may change the affinity of auxin to its receptor or alter the response capacity of receptor to auxin at a molecular level. The possible relationship of auxin and ethylene in the control of ARF is illustrated as following:



Chapter 8. ROOTING-PROMOTIVE EFFECTS OF ACIDIC pH AND PIPERAZINE IN RELATION TO PLANT HORMONES

INTRODUCTION

The experiments described in Chapter 2 were designed to examine the effect of pH on ARF. In these experiments acidic pH and a buffer component, piperazine, greatly stimulated ARF. How acidic pH and piperazine achieve this effect is unclear. As endogenous auxin and ethylene appear to be involved in ARF in sunflower hypocotyls (Chapters 3 & 5), I investigated the possible relationship between acidic pH or piperazine and these plant hormones on ARF.

According to the Rubery chemiosmotic hypothesis of polar auxin transport, the pH on the exterior side of the cell membrane has an effect on auxin transport (Rubery 1988). Since the membrane permeability coefficient to lipophilic IAAH is in the order of 10^3 times that of IAA^-, lower pH values, which inhibit dissociation of IAAH, foster greater diffusional uptake. Thus the effect of acidic pH on basipetal transport of auxin in the sunflower hypocotyls was investigated. In intact (non-decapitated) cuttings, there appears to be sufficient auxin to form many adventitious roots. The major sources of auxin are the cotyledons and the apical bud (Chapter 3, Katsumi et al. 1969, Fabijan et al. 1981a). Thus the interaction of acidic pH and auxin in auxin-depleted hypocotyls was investigated. In one experiment, decapitated sunflower hypocotyls were used, in which the main source of auxin was removed. In another experiment NPA was applied to inhibit auxin transport. The number of root primordia was then observed. The ethylene production in buffer- and piperazine-treated sunflower hypocotyls was also measured in the early stage of root initiation.

Benzyladenine inhibits ARF in sunflower hypocotyls (Chapter 3 and Fabijan et al. 1981b). At the concentration of 10 μM BA, ARF was completely inhibited. Since piperazine was the most effective substance in promoting rooting in my study so far, it was of interest to know if piperazine counteracts for BA effect on rooting. As far as I know, piperazine is not a naturally occurring compound in higher plants. I have been unable to find any reports on the effect of piperazine or its structural related compounds on ARF. Thus the effect of piperazine and some of its chemical analogues on ARF in species other than sunflower was also examined.

MATERIALS AND METHODS

The effect of piperazine on ARF was tested in derooted mung bean (*Vigna radiata*), bean (*Phaseolus vulgaris* L.) and peas (*Pisum sativum* L.) seedlings. Growth conditions for them were identical to those for sunflower described in Chapter 2. Cuttings were taken 7 day after sowing for mung bean, 6 day for bean and 10 day for pea. Production of cuttings for mung bean and bean was as described for sunflower hypocotyls (Chapter 2). For pea seedlings the base of cuttings were trimmed above the second scale leaf. The modified rooting system was used for all cuttings. Hoagland's solution (1/20 strength) was applied to derooted seedlings of pea throughout the experiment. Application of test solutions, ethylene measurement and estimation of number of root primordia were as described in Chapter 2. Root primordia were counted 3 days after derooting for mung bean and bean and 7 days for pea.

[^{3}H]-IAA transport experiment was conducted (with minor modifications) as described in Chapter 3. Briefly, the solutions of DMGA buffer at pH 4.5 and 6.5 were applied from the base of the sunflower hypocotyls. The basal 2 cm of the hypocotyls were submerged

under the solutions. [^{3}H]-IAA was applied to one cotyledon immediately after the initiation of buffer treatments. Four h later whole hypocotyl (about 7 cm in length) were harvested and further excised into 4 equal divided sections. Hypocotyl sections were analyzed for radioactivity with an oxidizer and scintillation counting.

Production of the decapitated hypocotyls and the application of NPA were as described in Chapter 2.

The effect of chemical analogues of piperazine, morpholine, thiamorpholine, 2,5-piperazinedione, 1-formylpiperazine (FP), N-2-hydroxyethylpiperazine (HEP), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) (Sigma Chemical Co., St. Louis, MO, USA) on ARF was tested in sunflower hypocotyls. Their formula are shown in Tab. 18. All these compounds are water soluble. HCl and NaOH were used for pH adjustment.

RESULTS

Effect of external pH on [^{3}H]-IAA transport in sunflower hypocotyls

When DMGA buffer was used to test the effect of external pH on [^{3}H]-IAA transport, it was observed that total radioactivity that moved to the hypocotyls did not change regardless of pH (Fig. 34). Distribution of radioactivity in different portions of hypocotyls treated with DMGA buffer at pH 6.5 followed the same pattern as that found in deionized water controls. DMGA buffer at pH 4.5 altered the distribution of radioactivity in hypocotyls. In the basal portions of hypocotyls (section 1), significantly more radioactivity was found at pH 4.5 than at pH 6.5, indicating acidic pH may have promoted IAA movement.

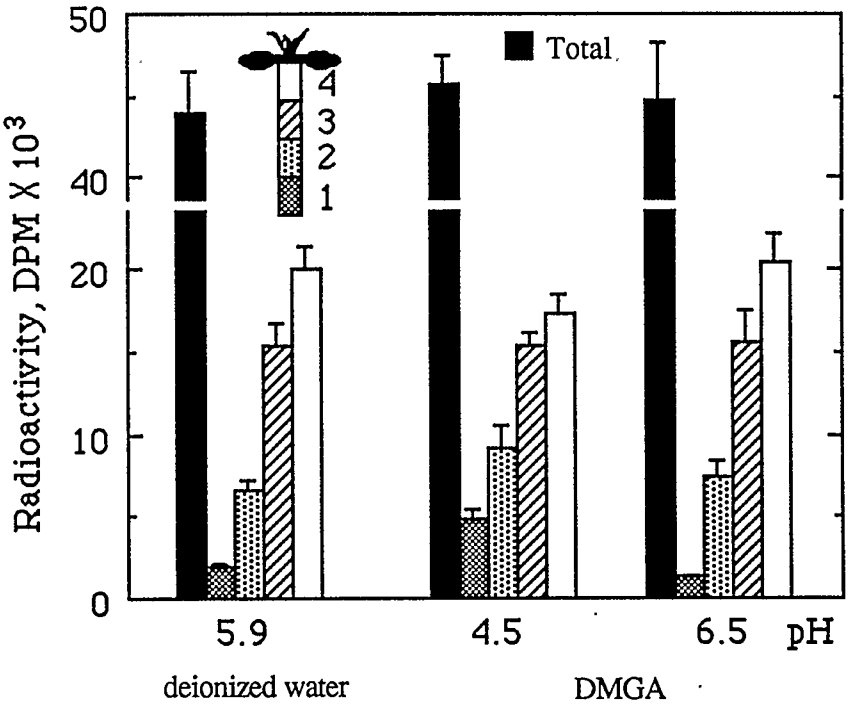


Fig. 34. Effect of pH (DMGA buffer) on the distribution of radioactivity in different portions of sunflower hypocotyls 4 h after [^{3}H]-IAA was applied to one cotyledon. The solution of DMGA or deionized water was supplied to the base of the hypocotyls throughout the experiment. Means ± SE (n = 10).

Effect of external pH on ARF in auxin-depleted hypocotyls of sunflower seedlings

Table 13 shows that pH had a small promotive influence on rooting in decapitated hypocotyls. IAA also promoted rooting in these decapitated hypocotyls as was shown earlier (Chapter 3). The combined effect of IAA and acidic pH was approximately additive. It also can be seen that no difference could be found between P-GG and DMGA buffers in their ability to stimulate rooting if cotyledons and apical bud were removed. P-GG buffer at pH above 6.5 had no effect on rooting. This result suggests that the effect of piperazine, a component of P-GG buffer, depends on the existence of cotyledons and shoot apex. It seems that there was no interaction of the buffer component of P-GG and exogenous IAA in these decapitated hypocotyls as any possible interaction would have produced a synergistic effect. In auxin-depleted hypocotyls, produced by NPA treatment, the number of root primordia was significantly reduced (Fig. 35). Acidic pH simulated rooting in these auxin-depleted hypocotyls. This result also suggests that there is an additive effect of endogenously produced auxin and acidic pH.

External pH, piperazine and ethylene production in sunflower hypocotyls

Table 14 shows the effects of 3 buffers, supplied from 0 - 5 h, on the production of ethylene after 3 - 10 hin the RZ of sunflower hypocotyls. With all the three buffers, acidic conditions promoted ethylene evolution at both 3 and 10 h in the bottom of the hypocotyls. Small stimulations of ethylene production in acidic conditions was also reported by Arny and Pell (1985) in potato, radish and soybean and by Leslie and Romani (1988) in pear cell suspension culture. Ethylene production at 3 h by cuttings treated with the P-GG buffer at all three pH values was the highest of all treatments. DMGA buffer was intermediate in its promotive effect on ethylene evolution and the Na_2HPO_4-citrate was the least effective.

Tab. 13. Effect of pH of two buffers, DMGA and P-GG, on the number of root primordia in decapitated hypocotyls. Buffer (5 mM) or IAA solution (50 µM in half-strength Hoagland's solution or buffers) was applied from 0-5 h. Decapitated hypocotyls were then placed in half-strength Hoagland's solution containing 10 mM sucrose.

		root primordia (hypocotyl)$^{-1}$	
buffers	pH	IAA 0	IAA 50 mM
Hoagland's solution	5.5	9.5 ± 1.50	15.2 ± 1.85
DMGA	4.5	16.8 ± 3.92	32.2 ± 3.84
	5.5	14.0 ± 2.75	27.4 ± 3.20
	7.0	10.4 ± 1.47	17.2 ± 2.72
P-GG	5.5	13.5 ± 2.63	22.2 ± 4.55
	6.5	10.6 ± 0.51	19.8 ± 1.59
	9.0	9.8 ± 1.5	14.6 ± 1.86

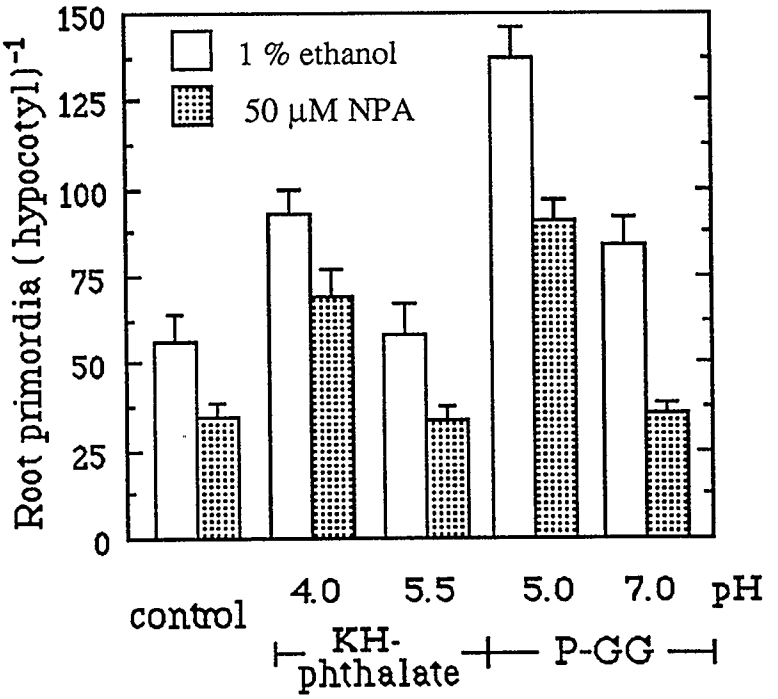


Fig. 35. Effect of two buffers on the number of root primordia in sunflower hypocotyls which have been treated with NPA. Buffers were supplied to the base of hypocotyls from 0 - 5 h. NPA was applied to the hypocotyls just below the cotyledons. For control hypocotyls 1 % ethanol was applied instead of NPA solution. Means ± SE (n = 20).

Generally, there was a strong positive correlation between increased ethylene production and enhanced rooting (compare Tab. 14 with Fig. 3. B). Figure 36 shows the effect of piperazine on ARF and ethylene production. Similarly, a positive correlation between was observed piperazine concentration, increased ethylene production and enhanced rooting.

Tab.14. Effect of three buffers, supplied from 0-5 h, on the production of endogenous ethylene in the RZ of hypocotyls. Measurements were made 3 and 10 h after cuttings were made.

		ethylene, p mol(gFW)$^{-1}$ h^{-1} ± SE (n = 3)	
buffers	pH	3 h	10 h
deionized water control	5.9	26.9 ± 0.49	11.8 ± 1.67
Na_2HPO_4 -citrate	4.0	29.8 ± 2.28	27.7 ± 4.32
	6.0	21.6 ± 2.48	16.3 ± 2.04
DMGA	4.5	32.6 ± 2.77	41.2 ± 2.12
	5.5	26.9 ± 1.67	19.2 ± 3.34
	7.0	30.2 ± 2.98	12.2 ± 1.43
P-GG	5.0	63.6 ± 3.34	37.9 ± 4.73
	6.5	51.4 ± 5.06	14.7 ± 1.07
	10.0	37.1 ± 6.81	16.3 ± 2.04

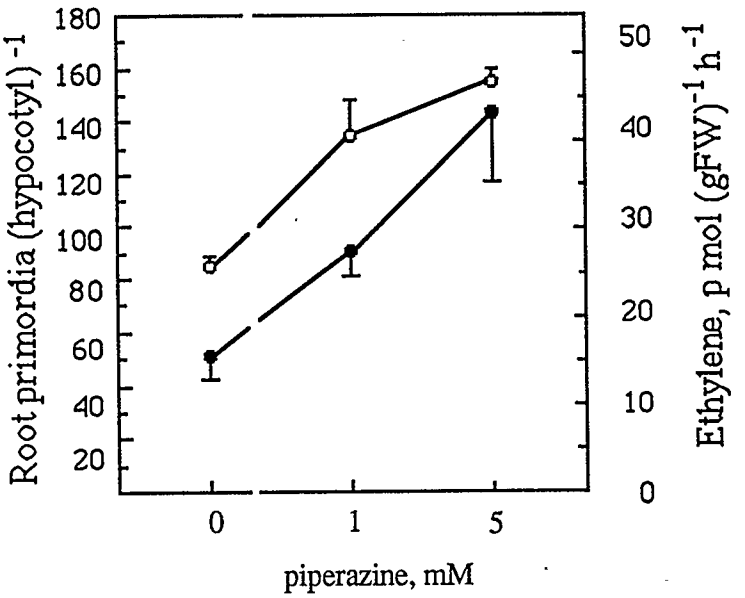


Fig. 36. Effect of piperazine on the number of root primordia (•, means ± SE, n = 20) and ethylene production (o, means ± SE, n = 3) in sunflower hypocotyls at 3 h after the initiation of the treatment. Piperazine solution at pH 5.5 was applied from 0 - 5 h.

Interaction of piperazine and BA on ARF in sunflower hypocotyls

Table 15 shows the effect of piperazine, in combination with BA, on ARF in sunflower hypocotyls. At 5 μM, BA almost completely inhibited ARF in piperazine-treated and untreated hypocotyls. The results indicated that piperazine was able to overcome the inhibitory effect of 0.1 to 1.0 μM BA.

Tab.15. Effect of piperazine, in combination with BA, on the number of root primordia per hypocotyl (means ± SE, n = 15). Chemicals were applied to the base of hypocotyls from 0 - 5 h. pH of the solutions was 7.

		piperazine, mM	
		0	5
BA, μM	0	61.8 ± 7.3	143.5 ± 14.5
	0.1	52.5 ± 7.0	114.3 ± 16.0
	0.5	30.8 ± 3.8	69.8 ± 14.8
	1.0	22.1 ± 2.4	51.6 ± 4.4
	5.0	4.5 ± 2.6	2.6 ± 1.4

Effect of piperazine on ARF in derooted pea, mung bean and bean seedlings

In Tab. 16 it can been seen that the number of root primordia in 5 mM piperazine-treated mung bean hypocotyls was almost twice of the control hypocotyls. The promotive effect of piperazine on rooting was more pronounced in pea cuttings. At the concentration of 5 mM the number of root primordia was triple of the control stem of the cuttings. The effect of piperazine on rooting in bean hypocotyls was small but still significant. Table 17 shows the effect of piperazine on ethylene production in the RZ of NRZ of pea stem. Similarly to sunflower hypocotyls wound ethylene was also observed in the cut-end of the stem of this species 3 h after the excision of original roots. Piperazine significantly promoted ethylene production in the RZ of stem and had small effect in the NRZ.

Tab. 16. Effect of piperazine on the number of root primordia (means ± SE, n = 15) in cuttings of pea (*Pisum sativum*), mung bean (*Vigna radiata*) and bean (*Phaseolus vulgaris*). The solutions of piperazine at pH 7.0 were applied to the base of hypocotyls from 0 - 5 h.

plant material	piperazine, mM			
	0	1	5	10
pea	5.8 ± 1.5	11.8 ± 1.4	17.1 ± 3.0	14.8 ± 2.0
mung bean	12.8 ± 0.8	22.9 ± 3.2	23.2 ± 3.7	21.3 ± 2.9
bean	67.8 ± 5.6	—	66.0 ± 2.8	81.0 ± 3.0

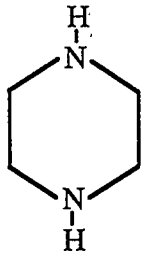
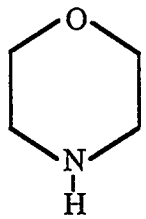
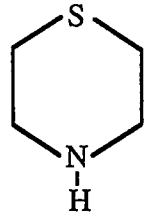
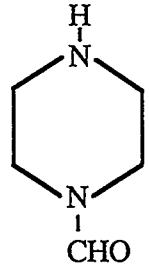
Tab. 17. Effect of piperazine on ethylene production [p mol $(g\ FW)^{-1}\ h^{-1}$, means ± SE, n = 3) in the RZ (1 cm from the bottom of cutting) and the NRZ of pea stems (1 cm from second internode). The solution of piperazine at pH 7.0 was applied to the base of hypocotyls from 0 - 5 h. Ethylene was measured at the time cuttings were made (0 h) and 3 h later.

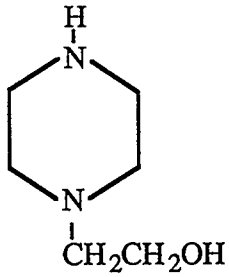
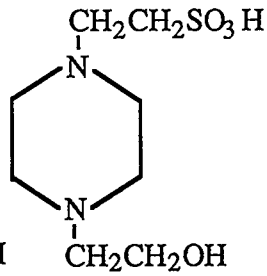
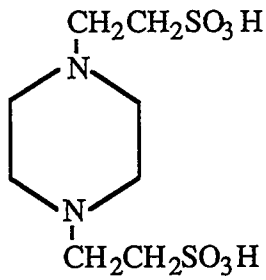
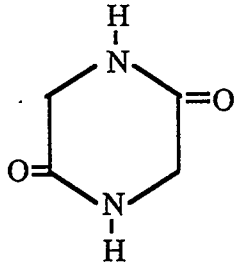
time, h	0	3	3	3
piperazine, mM	0	0	1	5
RZ	72.6 ± 13.5	195.0 ± 8.57	282.7 ± 10.22	297.8 ± 16.2
NRZ	63.2 ± 3.06	114.2 ± 7.10	93.8 ± 22.70	140.8 ± 9.4

Effect of piperazine analogues on ARF in the hypocotyls of sunflower seedlings

Table 18 shows the chemical structures of piperazine analogues investigated for their effect on the formation of root primordia. Morpholine, thiamorpholine and FP promoted ARF, but their effect was smaller in comparison to those of piperazine. HEP showed no effect on rooting, while HEPES, PIPES and 2,5-piperazinedione inhibited rooting.

Tab. 18. Effect of piperazine and its analogues at pH 7.0 on the number of root primordia per sunflower hypocotyl. Means ± SE (n = 15 - 20).

	 piperazine	 morpholine	 thiamorpholine	 FP
0	51.6 ± 8.0	65.9 ±7.9	77.1 ± 5.1	66.2 ±5.7
1	90.5 ± 9.9	72.0 ± 8.7	80.1 ± 8.7	75.6 ± 8.2
mM 5	143.3 ± 26.6	76.2 ± 9.1	94.5 ± 10.2	85.0 ± 6.7
10	—	95.2 ± 11.4	104.5 ± 11.1	94.3 ± 8.2

	 HEP	 HEPES	 PIPES	 2,5-piperazindione
0	66.6 ± 6.8	77.7 ± 6.9	77.7 ± 6.9	77.7 ± 6.9
1	64.7 ± 9.1	54.7 ± 7.7	58.1 ± 5.1	74.3 ± 6.9
mM 5	63.4 ± 8.6	51.7 ± 4.5	65.5 ± 4.2	60.0 ± 9.4
10	56.5 ± 7.3	55.9 ± 6.1	43.9 ± 3.7	50.7 ± 5.8

DISCUSSION

This Chapter mainly deal with the possible interactions of acidic pH, piperazine and plant hormones. The results suggest that the action of acidic pH and piperazine on rooting was, in part, related to auxin and ethylene. A drop in the pH of the buffer facilitated basipetal transport of endogenous auxin. This could then cause auxin accumulation at the target site responsible for rooting. Acidic pH has been reported to enhance IAA-induced lateral root formation in radish seedling roots (Blakely et al. 1986). They reasoned that the effect of acidic pH in lateral root formation was due to enhanced auxin uptake into the tissues. The additive effect of acidic pH and auxin on ARF in decapitated hypocotyls or with NPA treatment supports the idea that acidic pH stimulated rooting via the promotion of exogenous IAA uptake, or endogenous auxin transport. In addition to this, when the levels of endogenous auxin were reduced by decapitation, pH alone had a small promotive effect on rooting. This might indicate that acidic pH itself has an effect on rooting independent of auxin. Furthermore, part of the effect of acidic pH on rooting could be due to its ability to stimulate ethylene production. This ethylene enhances the responsiveness of tissues to auxin. The mode of action of piperazine is unknown, however it might be significant that piperazine treatment stimulated greater ethylene production than does low pH.

From the experiment with decapitated sunflower hypocotyls (Tab. 13), it is also concluded that the effect of piperazine on rooting is independent of auxin, but depends on some unknown translocable factors. The reasoning here is (i) P-GG buffer showed no promotive effect on rooting in decapitated hypocotyls at pH above 6.5; (ii) However there is no interaction between P-GG buffer and exogenous IAA. Piperazine stimulated ethylene production in the RZ of both sunflower and pea cuttings, but the effect of piperazine on the

formation of root primordia was much greater than that obtained by wound ethylene and the treatments of ethylene-releasing compounds (Chapter 5). Perhaps piperazine influences adventitious rooting only partially through a control over ethylene production.

In this Chapter I also included some results which are less related to plant hormones, but are nevertheless of interest. Piperazine promoted ARF in cuttings of several species. Its effect on rooting are greater than those of any substances I studied so far. I believe that the small effect of this compound on rooting in bean hypocotyls is due to the distinct pattern of adventitious root development in this species. In bean hypocotyls adventitious roots form in four well-defined and distinct longitudinal rows (Friedman et al. 1979), while distribution of roots in the hypocotyls of sunflower and in stem of pea seedlings is irregular. I observed that there were a large number of root primordia even in control bean hypocotyls. These root primordia were close to each other longitudinally. In other words, most of the potential root formation sites developed root primordia without any treatment of exogenous substances. Thus only a small effect of piperazine in bean hypocotyls was seen. Adventitious roots appear to have a more random formation in sunflower hypocotyls and pea stems. Many potential root formation sites may not produce root primordia in a control cuttings of these species. For example, I observed that in the control sunflower hypocotyls, root primordia usually formed from interfascicular parenchyma cells adjunct to phloem. When piperazine was applied interfascicular parenchyma cells away from phloem (in between 2 fascicular bundles) could also develop into root primordia (data not shown). It was always observed in the cuttings of sunflower, mung bean and pea that when the number of root primordia was promoted by piperazine, not only did additional roots develop in a more acropetal position, but also the density of root primordia in the very base of the cuttings increased. Thus great increase in the number of root primordia by piperazine was seen in sunflower, mung bean and pea cuttings.

The relationship of rooting ability of piperazine and its chemical structure was studied. The key findings were: (i) If one nitrogen in the molecule of piperazine is substituted by other elements, rooting ability of the chemical is reduced. (ii) Substitution of hydrogen in "N" position by a small group (-CHO) does not inactivate the chemical on rooting, but the activity of the chemical decreases. (iii) If the hydrogen in this position is replaced by a large group (i.e. $-CH_2CH_2OH$, or $-CH_2CH_2SO_3H$), the rooting ability of the chemical is lost or even becomes negative. (iv) If two hydrogens on a ring C are substituted by an oxygen, the chemical becomes inhibitory in rooting. In summary, chemical in the form of piperazine is most effective in promoting ARF. Modifications of the structure appear to make the chemical less effective, or even inhibitory.

Despite the present research, little is known concerning the physiological effect of piperazine in plants. Being a non-naturally occurring compound, piperazine may undergo little metabolism and little interaction with other substances and might be a promising tool in the study of cell growth and differentiation.

Chapter 9. GENERAL DISCUSSION AND CONCLUDING COMMENTS

This work was conducted to investigate some of the hormonal controls of ARF in derooted sunflower seedlings. My interest was focused on exploring the regulatory roles of endogenous auxin and ethylene.

In this study, the original experimental system of Fabijan et al (1981a) was carefully evaluated and consequently modified. One important finding was that gravistimulation of seedlings was a potential problem that might lead to a misinterpretation of experimental results. Gravistimulation of any part of hypocotyl induced ethylene production and reduced the number of root primordia in the base of hypocotyls. One might argue that treatment 2 and 4 in Fig. 1 would mimic some of the treatments that increase ethylene production and ARF (such as ACC treatment, wound ethylene production). Thus treatment 2 and 4 should promote rooting. Why then did gravistimulated cuttings produce fewer roots? I envisage various possibilities: (i) The gravistimulation of the hypocotyl produces elevated ethylene levels for longer time than formed in vertical hypocotyls (see Tab. 1) and this may somehow be inhibitory to root formation. However, this seems unlikely because STS was ineffective (Fig. 27) on treatment 2 where gravistimulation of the RZ increase ethylene production. Thus although gravistimulation increased ethylene levels do not promote rooting, in this case they are nevertheless, useful and an easy to measure, signal that the cuttings have been gravistimulated. (ii) Gravistimulation might interfere with some other factor involved in ARF such as auxin. While the mechanism of the inhibition of ARF by gravistimulation is unknown, it is clear that it is an unwanted complication in the study of ARF. However, it is interesting that, for whatever reason, gravistimulation affects rooting and this deserves further study.

Another factor influencing the outcome of the experiments was the pH of solutions used to treat the cuttings. Many plant growth substances are weak acids. Not only did the pH of the solutions affect rooting, but there was also complicated interaction of pH and some growth substances, for example, additive effect of acidic pH and IAA on ARF. In laboratory and in practice unbuffered auxin solutions are usually used to treat cuttings. These solutions are naturally in acidic pH. Understanding of the effect of acidic pH on rooting may aid in understanding the mechanism of auxin action in cellular and molecular levels.

An overall strategy in this study was to compare what happens in the RZ of hypocotyl to those in the NRZ. A rigorous method of estimating the endogenous concentration of IAA was used through the application of GC-MS. A improved ethylene sampling method was used. Short incubation time for tissue samples (10 min) excluded wound ethylene production which was usually seen about 25 min after the excision of tissues (Saltveit & Dilley 1978). The syringes for incubating tissue samples were first flushed with ethylene-free air before use. This procedure eliminated the possibility of contamination from ethylene air pollution. In summary, the results strongly suggest that auxin is the primary effector of ARF in sunflower hypocotyls, while the action of ethylene is mediated by one or more substances. Auxin appears to be one such substance, as ethylene enhances tissue sensitivity to auxin. Exogenous auxin stimulated ethylene production. This ethylene may have some positive feedback effect on auxin in promoting rooting. I also found that promotive effect of acidic pH on adventitious rooting in sunflower hypocotyls was partially due to the promotion of basipetal transport of auxin and enhanced ethylene production rate. Stimulation of endogenous ethylene production at least partially accounted for piperazine promotion of adventitious rooting.

Since little work has been done concerning piperazine and rooting, it is too early to propose any mode of action for piperazine on adventitious rooting. In view of weak

similarity in molecular structures between piperazine and some vitamins, i.e. thiamine, nicotinic acid and pyridoxine, which are known to have hormone like properties on root growth (Bonner 1940, Bonner & Galston 1952), it is tempting to suggest that piperazine might be a analogue of these vitamins. However, (i) in these experiments piperazine was applied for a short period immediately after the excision of roots, thus it is likely that piperazine was involved in early stage of root initiation rather than root growth; (ii) Root growth requires thiamine and nicotinic acid at very low concentration (below 1 μM, Bonner 1940), while piperazine promoted rooting at much higher concentration (above 1000 μM).

In the propagation of cuttings in forestry and horticulture, enhancement of endogenous ethylene in many cases may be naturally achieved by manipulation of the cuttings, e.g. the excision of cuttings and application of exogenous auxin. Therefore the application of ethylene-releasing compounds to enhance rooting may not be necessary. While the treatment with acidic buffer, especially P-GG buffer, might be of potential commercial importance in stimulating ARF in cuttings; field experiment should be conducted to test these buffers.

The developmental sequence of adventitious roots has been studied in sunflower hypocotyls (Fabijan et al. 1981a). Cytological changes could be observed in the interfascicular parenchyma cells near to the phloem tissue at 24 h after original roots were removed; the nuclei of these cells appeared to be larger than non-initial cells and the cells had a dense cytoplasm. By the second day, small primordia could be observed. The results presented here show that changes of hormone (auxin and ethylene) levels in the RZ of hypocotyls were evident in early few hours after the excision of original roots. In this study exogenous substances (e.g. auxins, inhibitors of auxin transport, ethylene-releasing compounds and ethylene inhibitors et al) were applied to hypocotyls for this short period. Thus, auxin and ethylene commit their actions during the early few hours after excision of

roots; possibly the time period before any biochemical differentiation occurs.

Based on the information available in the literature and the results presented here, I propose a tentative scheme of the control of ARF in the hypocotyls of derooted sunflower seedlings (Fig. 37). The balance of endogenous hormones in the base of hypocotyls change immediately following the excision of roots (rooting inducing treatment). The inhibitors of ARF, i.e. CK and GA, decrease as original roots, the sources of these hormones, are removed. As the ARF promoter IAA is continuously supplied by cotyledons, the ratio of auxin / CK increase. Carbohydrates and recycling nutrients, possibly some unknown rooting promoters, move to the base of hypocotyls. Among these substances auxin is a primary trigger. Possibly a reduction in CK level enables auxin to activate the gene that is responsible for induction of cell division and differentiation. Wounding by the excision of roots caused an increase in ethylene production. This ethylene enhances the sensitivity of tissue to auxin, possibly by enhancing auxin binding to its receptor. Carbohydrates and nutrients, especially boron, are needed for primordium growth. The assumption for the existence of unknown translocatable substance from cotyledons and/or shoot apex is based on two observations: (i) Exogenous auxin is unable to fully overcome the inhibitory effect of decapitation even though it was applied in combination with sucrose and Hoagland's solution; (ii) Piperazine, a potent promoter of ARF in non-decapitated cuttings, was unable to stimulate ARF in decapitated hypocotyls. It seems that some unknown factors, but not IAA are needed for the action of piperazine. However, we do not know if exogenous auxin enters the target cells in the same way as does the natural auxin. We do not know if the exogenous sucrose works in the way identical to endogenous sucrose.

Further research is also needed to identify initial cells of root primordia, and to learn how auxin and other rooting promoters enter these cells and the localization of these

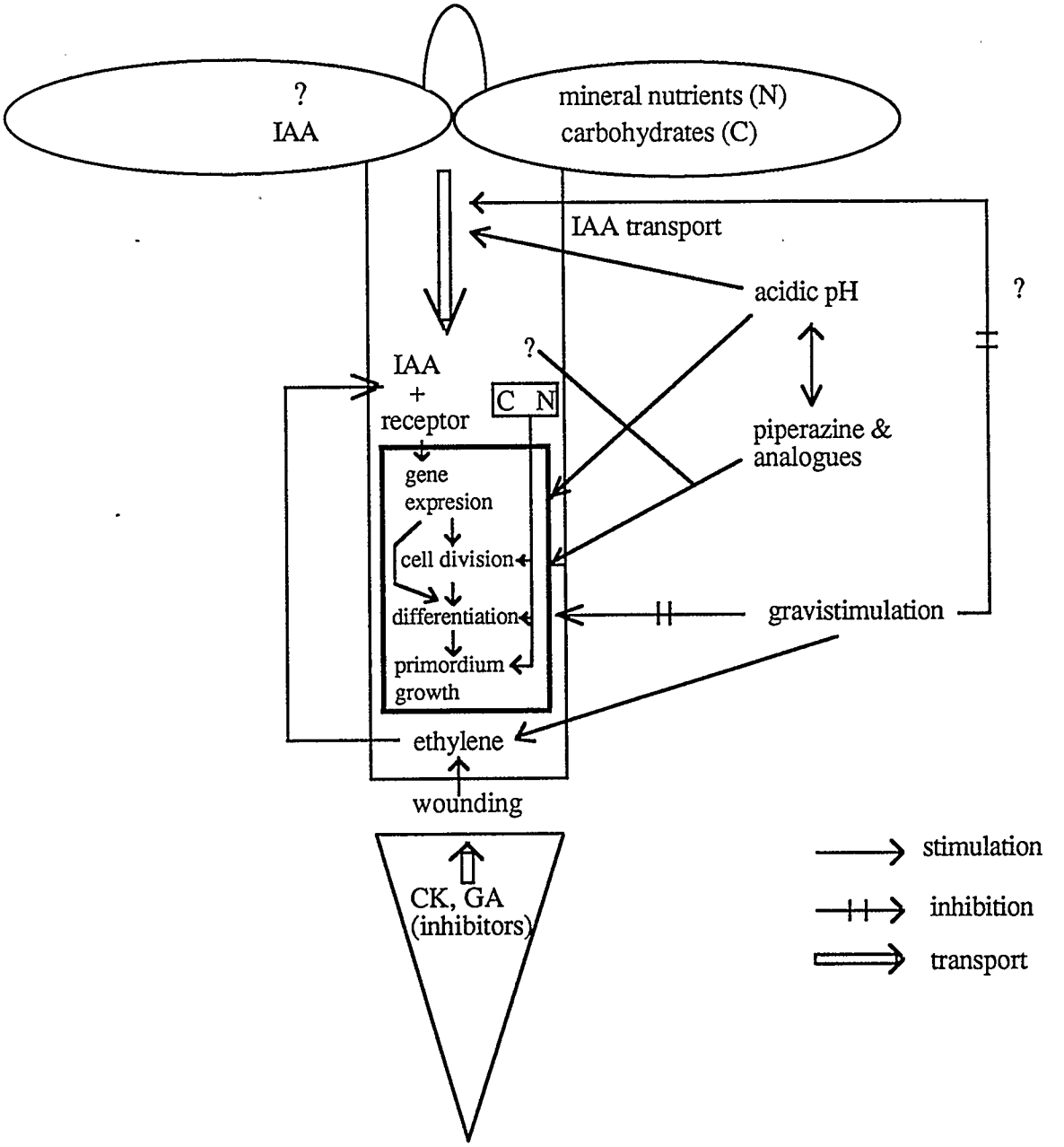


Fig. 37. A proposed scheme of the control of ARF in derooted sunflower seedlings.

promoter and their receptors at the subcellular level. The better anatomical methods are needed to easily and accurately identify preprimordial cells, while microautoradiography offers promise for studying some translocation of various radioactively labeled compounds.

This scheme of the control of ARF may apply to root formation in other analogous situations, for instances, ARF in leafy cuttings of other species, ARF induced by flooding, as well as lateral root formation induced by pruning of root tips.

It appears that auxin and CK are directly involved in the control of ARF with auxin being a promoter, while CK an inhibitor. Little is known concerning the interaction of these hormones in the formation of adventitious roots. By studying the changes of protein upon the treatment of hormones MacIsaac & Sawhney (1990) have suggested that the mechanisms of auxin-stimulation and CK-inhibition of lateral root initiation probably differ. Similar research should also be directed to study the possible interaction of auxin and CK in adventitious rooting.

Root pruning removes the source of inhibitors CK and GA. A high ratio of auxin/CK and promotion of ethylene production are expected to occur in the roots near the cuts and thus lateral roots form in these sites.

In the case of flood-induced ARF, anoxia of roots inhibits the biosynthesis and acropetal transport of CK and GA. It has been shown anoxia of the roots reduced the quantities of CK and GA in xylem sap (Burrows & Carr 1969, Reid & Crozier 1971). In contrast, anoxia of roots caused an increase in the levels of rooting promoters, e.g. auxin (Phillips 1964, Wample & Reid 1979) and ethylene (Kawase 1972, El-Beltagy & Hall 1974, Jackson & Campbell 1975, Wample & Reid 1979) in the base of a plant.

In conclusion, the findings reported here support the following hypothesis: In the rooting of derooted sunflower seedlings, endogenous auxin is the primary effector of ARF.

Wound ethylene, due to the excision of original roots, promotes rooting via enhancing the action of endogenous auxin. I have presented the following supporting evidence: (i) At the time the original roots were removed endogenous IAA level was higher in the RZ of the hypocotyls than that in the NRZ. (ii) Reduction in endogenous auxin level by a number of treatments decreased the number of root primordia in the hypocotyls. (iii) Exogenous auxins promoted the initiation of root primordia and could substitute for endogenous auxin in promoting rooting. (iv) An increase in endogenous ethylene in the RZ promoted the initiation of ARF if endogenous auxin was not limiting. (v) Ethylene inhibitors reduced the number of root primordia in hypocotyls. (vi) Endogenous ethylene enhanced the responsiveness of hypocotyls to endogenously produced and exogenously applied auxin.

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