77

BARD

FINAL REPORT

PROJECT No. I-166-80

**Factors Determining the Sensitivity of Abscising
Plant Organs to Ethylene, with Special Reference to
Auxin Metabolism and Transport**

R. Goren, J. Riov, P.W. Morgan

1986

Standard BARD Cover Page for Scientific Reports

Date January 28, 1986

BARD
P.O. Box 6
Bet Dagan, ISRAEL

BARD Project No. I-166-80

Title of Research Project: Factors Determining the Sensitivity of Abscising Plant
Organs to Ethylene, with Special Reference to Auxin
Metabolism and Transport.

Names of Investigators

Principal Investigator: Raphael Goren and Joseph Riov

Cooperating Investigator(s): Page W. Morgan

Name and Address of Affiliated Institutions:

Principal Institution: Hebrew University of Jerusalem, Department of Horticulture,
Rehovot, Israel,

Cooperating Institution(s): Department of Plant Sciences, Texas A&M University,
College Station, Texas.

Project's starting date: September 1, 1981

Type of Report: 1st Annual 2nd Annual Final X

Signatures:

Principal Investigator

Raphael Goren
Joseph Riov

Institution's Authorizing Official

A. Barak

Collaborating Scientists

Sections

Aron, Y.

G

Durham, J.I.

E

Huberman, M.

G

Monselise, S.P.

G

Rivera, V.

D,E

Sagee, O.

A,B,C,F

Stipanovic, R.D.

D,E

TABLE OF CONTENTS

	Page
Abstract	1
Objectives and achievements	3
Section A: Effect of ethylene on [14 C]indole-3-acetic acid metabolism in citrus leaf tissues as related to ethylene-induced abscission	4
Section B: IAA metabolism in senescing citrus leaves approaching natural abscission	45
Section C: Comparison of different enzyme immunoassays for measuring IAA in vegetative citrus tissues	53
Section D: Trace level analysis of indole-3-acetic acid from light grown, highly pigmented leaf tissue	71
Section E: Levels of indole-3-acetic acid in leaves of <u>Gossypium</u> <u>hirsutum</u> L.	90
Section F: Levels of indole-3-acetic acid in citrus leaves during ethylene-induced abscission and natural abscission.	102
Section G: Morphactin-induced inhibition of abscission in citrus leaves	111
Benefit to agriculture	121
Description of cooperation	123
Reprints and manuscripts originated from the project	124

ABSTRACT

The effect of ethylene on [^{14}C]indole-3-acetic acid (IAA) metabolism was studied in leaf discs and midrib sections cut from citrus Citrus sinensis (L.) Osbeck var. Shamouti leaves. IAA metabolites were fractionated into 80% ethanol-soluble, H_2O -soluble NaOH-soluble, and insoluble components. Chromatographic analysis indicated that the ethanol and H_2O extracts contained low molecular weight conjugates, whereas the NaOH extract contained high molecular weight conjugates. The major conjugate in untreated citrus leaf tissues was identified by GC-MS as indole-3-acetylaspartic acid. Pretreatment of leaves with 25 $\mu\text{l/l}$ ethylene induced abscission and increased the amount of IAA conjugates in all the above fractions. Ethylene pretreatment particularly enhanced the formation of an IAA-glucose ester and the macromolecular conjugates. A close positive correlation was found between accumulation of these conjugates and abscission of ethylene-treated leaves. Analysis of the macromolecular conjugates indicated that they consist of various cell-wall components. Similar changes in IAA metabolism were found in senescing leaves which produced large amounts of ethylene.

For studying the effect of ethylene on endogenous IAA levels, two methods were developed. Both methods are suitable for analyzing IAA from light-grown, highly pigmented leaf samples. In the Israeli lab, an enzyme immunoassay was perfected. The immunoassay utilized polyclonal antibodies which were raised against IAA conjugated to bovine serum albumin via C_1 of the carboxyl group. Although the method requires some cleanup,

it is fast and allows multiple analyses of the same sample. The second method, developed at the lab in Texas, was an HPLC-based procedure which employs three separate HPLC steps and detection by fluorescence and by an amperometric signal. This method is sensitive and accurate; there is no evidence that it is subject to interference.

These methods were used in initial studies to survey IAA levels in cotton and citrus leaf tissues and to study the effect of ethylene on IAA levels. Cotton leaves contain very low levels of IAA (50 ng/g fr. wt.) and the content of IAA reached a plateau at a very low level, less than 10 ng/g fr. wt., in mature, non-growing leaves. In citrus leaves, the levels of IAA found are much higher. Very young leaves contain about 380 ng/g fr. wt. IAA and its content decreases as leaves mature. The lowest level of IAA was found in senescing leaves approaching natural abscission which produce elevated amounts of ethylene. In most experiments performed, exogenous ethylene did not reduce significantly IAA levels in citrus leaf tissues.

Morphactin inhibited polar auxin transport in midrib sections of citrus leaves, but at the same time it inhibited the stimulatory effect of ethylene on abscission. Inhibition of ethylene-induced abscission by morphactin probably results from suppression of the development of cellulase and polygalacturonase activities in the abscission zone.

OBJECTIVES AND ACHIEVEMENTS

Control of abscission is one of the major aspects of plant physiology which has been extensively studied in both basic and practical levels. The practical use of chemicals for the control of abscission is often confronted with serious problems related to the efficiency of the compounds, timing of application, differential effects on leaves versus fruits, and the wide range of sensitivity exhibited by different varieties.

It is well accepted that auxin is the principal factor which governs the sensitivity of the abscission zone to ethylene, but the specific effects of these two hormones and the interactions between them are still not clear. Our main objective was to study the factors which determine the sensitivity of plant organs to ethylene. Specifically we aimed to study the effect of ethylene on the content of endogenous indole-3-acetic acid (IAA) and the mechanisms by which ethylene affects the endogenous level of IAA.

Effect of Ethylene on IAA Metabolism

It is well accepted that conjugated forms of IAA are an important mechanism for regulating the level of free IAA in plant tissues. We conducted a detailed study on the nature of IAA conjugates in citrus leaf tissues and the effect of ethylene on the formation of these conjugates.

By using several chromatographic procedures we were able to isolate and to identify for the first time the major conjugate in untreated

citrus tissues as indole-3-acetylaspartic acid. Ethylene pretreatment stimulated IAA metabolism and this was reflected by a reduction in free [^{14}C]IAA in the tissues. Stimulation of IAA conjugation was mainly related to increased formation of an IAA glucose ester and macromolecular conjugates localized in the cell-wall. The pattern of formation of these conjugates in ethylene-pretreated tissues was similar to that of abscission. Similar changes in IAA metabolism were observed in senescing citrus leaves approaching natural abscission. These leaves produced high amounts of ethylene. Thus, it has been clearly demonstrated that IAA conjugation is increased in ethylene-pretreated leaves and during natural abscission. Detailed description of these findings appears in Sections A and B.

Developing Methods for Analysis of IAA.

Examining the effect of ethylene on levels of endogenous IAA required reliable methods for assaying IAA. Therefore, one of the objectives of this research proposal was to develop methods for analysis of IAA from light-grown, highly pigmented leaf samples. The difficulty of this task is not commonly recognized or easily explained. Out of necessity, most of the modern, physicochemical data on IAA levels have come from etiolated tissue, usually seeds or seedlings. This approach eliminated the several thousand-fold larger contamination of small molecules found in light-grown, mature plants, and it employed tissue with many fold higher levels of IAA than mature, abscising leaves. While analyzing IAA in germinating corn coleoptiles is much easier than in mature cotton or citrus leaves, the former data are not useful in describing how leaves signal abscission.

The existing data on changes in IAA levels during senescence and abscission and on the effect of exogenous ethylene on IAA levels are surprisingly scant and subject to question. Early studies employing bioassays could have given incorrect results due to the presence of gibberellins or inhibitors. Even the most recent and reliable data were obtained with the α -pyrone fluorescence reaction with IAA which is subject to interference by unknown contaminants in IAA extracts. Thus, the available data on IAA levels suggest that reliable data are difficult to obtain and that many published values may be in error. The overriding conclusion is that reliable means to analyze IAA in mature tissues, rich in contaminating pigments, phenolics, tannins and similar substances are very much needed for progress in understanding plant behavior. In fact, the major theory of abscission assumes that IAA levels decline and that ethylene levels increase during natural senescence. Unfortunately, after 50 years of studying auxins and abscission, scientists do not have definitive proof that IAA levels decline or what the absolute values are.

Physicochemical Methods

General : Our goal was to develop a method which: (a) could be used for a relatively small amount of tissue, (b) was relatively fast, (c) was reproducible, (d) involved recoveries of 30% or more, (e) was relatively inexpensive, (f) included means to verify the identity and quantity of IAA. These objectives seemed to eliminate GC-MS as an approach because the equipment and its use and maintenance are very expensive. Further, GC-MS usually requires extensive prepurification so that yields are low, sample sizes must be increased and the time needed

for analysis is long. Finally, GC-MS requires esterification of the IAA after purification; this procedure adds approximately 8 additional steps, transfers and other operations greatly increasing the opportunity for error or contamination. We decided to assay IAA in the HPLC system using multiple detectors to confirm its identity. This means that we do not have to esterify the IAA and go on to GC or GC-MS, although we have done so on a few occasions in order to get the ultimate verification that we were assaying IAA and only IAA.

Using multiple detectors gives the advantage of assaying the IAA and other materials with different principles of detection. The detector response to IAA differs drastically with wave length or voltage and thus all three detectors are adjusted to the optimum conditions for IAA. The detector response varies for other compounds and so it is almost impossible that a peak containing IAA and some additional compound could pass through both of the quantitative detectors and yield the same values for IAA. Thus, we calculate the IAA value from both the fluorescence and the amperometric detectors, and if the ratio of IAA amount from the two is about 1.0, we can be confident that the peak contains only IAA.

Our identification, validation and quantitation of IAA rests on the following evidence: (a) The putative IAA peak co-chromatographs with standard IAA through three preliminary column steps and three HPLC steps. The final HPLC step produces a single, base line resolved fluorescence or amperometric peaks which corresponds in retention time to authentic IAA and which contain all of the remaining radioactivity from the internal IAA standard. (b) The putative IAA fluoresces at the activation maxima (280 nm) and emission maxima (362 nm) of standard IAA. (c) The putative IAA ionizes at the voltage optima for standard IAA in

the amperometric detector (0.95 v). (d) The putative IAA absorbs UV light at 254 nm as does standard IAA. (e) The putative IAA peak is baseline resolved in all three detectors and is the only compound that is seen by all three detectors. Some fractions of putative IAA were esterified with diazomethane, re-purified by HPLC and subjected to GC on a coated-silica, capillary column yielding a single sharp peak identical to standard IAA methylester. The detection was with a N/P detector which does not sense carbon. The resolving capacity of this long capillary column is in excess of 50,000 theoretical plates and thus the presence of a single peak argues strongly for sample purity. Next, the putative IAA methylester was subjected to GC-MS where the mass fragments characteristic for IAA and identical for the standard IAA were produced. Thus, we have detected the IAA on the basis of retention time, fluorescence, amperometric ionization, UV absorption, presence of N and by mass fragmentation pattern. We are certain that the HPLC procedures produce a pure IAA peak that is sensitively assayed by fluorescence.

Preliminary Clean-up Procedures : This part of our physicochemical procedures represented a serious problem, and it was solved through our BARD collaboration. In our experience, all preliminary clean-up procedures were slow and expensive, in that they required large amounts of organic solvents. We needed a method that could be applied simultaneously to many samples. As Riov & Goren initiated their immunological assay, they realized that they had an even greater need for the same kind of preliminary clean-up procedure. Immunological assays often sacrifice a little in accuracy but make up for that loss by allowing large numbers of replicates and speed in the procedure. Riov & Goren tested a "rapid, mini-column clean-up procedure" which employed no

organic solvents other than methanol. They improved the recovery and reproducibility of the method, and I learned of their success when I visited their lab in January 1984. As a result we adopted their modification of the original procedure, and it has become an important part of our IAA assay method. After some experience, it yields over 50% recovery of the internal standard ^3H -IAA.

Assay Equipment : During the course of this project funds became available to the Texas A&M lab through the Texas Agricultural Experiment Station to update and automate the HPLC facility. As a result of this opportunity, the facility was in a constant state of change during the course of the grant, but the end result was a facility with a remarkable capacity to analyze IAA. Briefly, the facility is completely automated from injection to sample collection and data recording. We can program over-night runs, collect only the fractions of interest from each sample, and record the appropriate data automatically. The system has the capacity to chromatograph up to 20 samples in a 24 hour period; it has the sensitivity to detect as little as 1 pg IAA with the fluorescence detector, but generally the accuracy is much better if the sample contains at least 1 ng IAA at the final step. Obviously, as the amount of IAA per sample increases, the technical problems of working at extreme trace levels of compounds are reduced.

A key aspect of the facility is the detection equipment. We use a single channel UV detector set at 254 nm to monitor the movement of materials through the HPLC columns. This detector senses a wider array of compounds than the subsequent two. We use a Perkin-Elmer Model LS-4 Fluorescence Spectrometer and a BAS amperometer to quantitate IAA. Both are very sensitive and use different principles of detection. If either registers a twin peak, a tailing peak or shoulder on the IAA peak or if

the two do not detect the same amount of IAA (within a selected variability range, i.e. $\pm 10\%$ or $\pm 20\%$), the sample is discarded. This allows us to judge the purity of the final IAA peak with a high level of confidence, based on HPLC data alone. As a verification, IAA from new sources will be esterified and assayed by GC-N/P detector and GC-MS detector. Once the putative IAA peak from a particular plant source has been shown to be IAA and nothing else, we will routinely stop our analysis with the final HPLC step, assuming base line resolved peaks and agreement of the fluorescence and the amperometric detectors.

HPLC Procedures : The rationale of our HPLC procedure was to use ion pair chromatography to attach the IAA to a column and remove most of the contaminating material present at this stage of the procedure. The tetrabutylammonium (TBA)-IAA ion pair is recovered, put on a second column after which it is dissociated, the TBA is removed as a phosphate ion pair and the remaining IAA protonated and eluted in a gradient. The protonated IAA is eluted separately from most of the remaining UV absorbing material. The IAA fraction is then developed on an analytical column.

The HPLC procedure maximizes the purification capacity of the ion pair step by putting it first. We maximize the selectivity of this step by using an ion pair reagent, TBA-acetate, with a pK very near that of IAA. The resultant high affinity of the IAA for the TBA insures that other competing organic acid will not saturate the available ion pair reagent. Others have used ion pair HPLC for IAA, but they used the less selective $TBA-PO_4$ as the reagent and quantitated the IAA as the TBA-IAA complex. Since the fluorescence behavior of compounds is influenced by solvent strength, polarity, and pH and since removing the ion pair complex from a column requires a gradient thus insuring that

quantitation will occur against a background of changing solvent properties, we decided that it would be much more accurate to quantitate the protonated IAA in an isocratic solvent program designed to maximize the detector response and to further promote the separating capacity of a final HPLC column. Although a three step HPLC procedure represents quite a lot of work, the resultant purity of the final IAA peak and the level of confidence in the identity and quantitative data justifies the additional work.

A complete description of our HPLC method for quantitation of IAA is presented in Section D.

Immunoassays

Immunoassays are now in widespread use in animal and plant science. There are four discrete steps in the development of an immunoassay for a low weight antigen such as a plant hormone: (1) immunogen synthesis, (2) immunization, (3) tracer synthesis, and (4) antiserum selection and design of optimal assay conditions. For any of these steps, a large number of possible variations exists, which makes immunoassays a versatile technique adjusted to the specific needs of the research work. When this study was initiated, two methods were already published for analysis of IAA; a radioimmunoassay using antisera raised against IAA linked via the indole nitrogen to protein (IAA-N-BSA) and an enzyme immunoassay using antisera raised against IAA linked via C₁ of the carboxyl group to protein (IAA-C₁-BSA). We prepared both types of antisera, developed an enzyme immunoassay for antisera raised against IAA-N-BSA, and compared this newly developed procedure to enzyme immunoassays utilizing polyclonal and monoclonal (obtained from E.W.

Weiler, W. Germany) antisera raised against IAA-C₁-BSA. Although antibodies to IAA-N-BSA had generally less cross-reactivity to indoles structurally related to IAA, polyclonal antibodies against IAA-C₁-BSA yielded more reliable estimation of IAA in partially purified extracts. The reason for this is undoubtedly the higher affinity of the later antibodies to IAA. By using antibodies to IAA-C₁-BSA it was possible to dilute the extracts up to 100 fold, thus decreasing the level of interfering compound. Monoclonal antibodies also had high affinity to IAA but showed considerable immuno reactivity with unidentified materials present in the extracts.

Our data indicate that the idea that it would be possible to utilize immunoassays for measuring IAA in crude extracts is incorrect. This conclusion has also emerged from other recent studies. However, by employing the clean-up procedure described above and polyclonal antibodies for IAA-C₁-BSA it is possible now to obtain reliable data on IAA levels in citrus leaf tissues. Detailed description of this study is presented in Section C.

Levels of IAA in Leaves and the Effect of Ethylene on the Levels of IAA in Leaves.

The methods developed for analysis of IAA were utilized to determine the effect of ethylene on IAA levels in leaves. We have initiated this study by getting background information on the levels of IAA in cotton and citrus leaves differing in age and senescence.

Our data for cotton leaves show clearly that there is a gradient of IAA decreasing from young to old leaves. However, in this experiment the level of IAA did not decline step-wise from top to bottom of the plant.

The level is highest in the youngest, actively growing leaves. The first fully expanded leaf had about 3 to 4 times less IAA but at this point there was an essentially flat plateau of IAA levels in the mature leaves and cotyledons. Some of the cotyledons had begun to abscise and the mature, basal leaves would begin to abscise soon. However, we did not find a precipitous drop in IAA levels in the basal leaves or the cotyledons. Our detailed findings are presented in Section E. As we conclude this study we will examine IAA levels in populations of leaves at a single position on the plant as they progress through senescence toward abscission and as they respond to ethylene.

In citrus leaves, the levels of IAA found were much higher than those in cotton leaves. Very young leaves and growth tips contained the highest levels of IAA. As leaves mature, IAA content decreases. The lowest levels of IAA were found in senescing leaves approaching natural abscission which produced elevated amounts of ethylene. Although ethylene stimulated the conjugation of IAA, we did not observe in most experiments a decline in IAA levels in ethylene-treated leaves. Our data for citrus leaves seem to agree with the assumption that in leaves approaching natural abscission, the abscission-retarding substances (IAA) decline and the abscission-promoting substances (predominantly ethylene) increase. However, IAA levels did not decline during abscission induced by exogenous ethylene. Further studies are needed to examine whether or not reduction of IAA levels is required before ethylene can induce abscission. Data on IAA levels in citrus leaves appear in Sections C and F.

Effect of Morphactin on Abscission of Citrus Leaves.

One of the objectives of the research proposal was to search for methods and chemicals to enhance the abscission-induction activity of ethylene or ethephon. Morphactins are known to accelerate abscission probably by inhibiting auxin transport. The effect of these compounds on citrus leaf abscission has not been investigated. We examined the effect of morphactin on auxin transport and its interaction with ethylene-induced abscission.

Morphactin inhibited polar auxin transport in midrib sections of citrus leaves as reported for other species. However, the stimulatory effect of ethylene on abscission was inhibited by morphactin. Morphactin also inhibited the activity of cellulase and polygalacturonase in the abscission zone. It seems that morphactin inhibits abscission of citrus leaves by delaying the development of the activity of the cell-wall degrading enzymes, a mechanism which is not related to the inhibition of auxin transport. The data indicate that morphactin can not be used to enhance the abscission-induction activity of ethylene in citrus leaves.

Since the budget of our original proposal was reduced considerably, we were limited in the number of objectives we could work on.

SECTION A

EFFECT OF ETHYLENE ON [14 C] INDOLE-3-ACETIC ACID METABOLISM IN CITRUS
LEAF TISSUES AS RELATED TO ETHYLENE-INDUCED ABSCISSION.

ODED SAGEE, JOSEPH RIOV AND RAPHAËL GOREN

Ethylene has been demonstrated to reduce endogenous IAA levels in different plant tissues (3,13,15) and several investigators have suggested that ethylene regulates various physiological processes through this effect (1,6). Reduction of the IAA level by ethylene may result from increased conjugation and decarboxylation (4,13,18,25), decreased synthesis (13), and reduced transport from the site of synthesis (2). Conjugated forms of IAA are thought to be an important mechanism for regulating the level of free IAA in plant tissues (7). Effects of ethylene on IAA conjugation have been studied by several investigators, but so far no clear cut conclusions have emerged. Beyer and Morgan (5) reported increased IAA conjugation in cotton stem sections pretreated with ethylene. However, they concluded that this was probably not a direct effect of ethylene but rather the result of a greater accumulation of IAA in the ethylene-pretreated sections which no longer transported IAA at a normal rate. In contrast, Ernest and Valdovinus (13) found that ethylene pretreatment of Coleus stem tissue reduced the amount of extractable auxin and at the same time increased the amount of IAA^{sp1}. They suggested that the ethylene-induced reduction in auxin content resulted from increased conjugation. Increased IAA conjugation by ethylene has also been observed in leaf tissues of various species (12,23,25,32) and in potato tubers (17,18). Others reported that ethylene

pretreatment of cowpea leaves increased formation of IAGlu, but decreased formation of IAAsp (14). In Regnellidium diphyllum, a semi-aquatic fern, no effect of ethylene on IAA metabolism could be detected (30).

Previous data from our laboratory indicate that ethylene increased the conjugation of IAA to both low and high molecular weight metabolites (25). However, the nature of the metabolites formed and the kinetics of their formation have not been studied. In the following we describe the results of our study of the above mentioned questions.

¹ Abbreviations: IAAsp, indole-3-acetylaspatic acid; IAGlu, 1-0-(indole-3-acetyl)- β -D-glucose; PVPP, polyvinylpolypyrrolidone.

MATERIALS AND METHODS

Plant Material and Treatments. Leafy shoots or leaves from the current year flush were taken from established trees of Citrus sinensis [L.] Osbeck, var. Shamouti orange. Shoots and leaves were treated with 25 μ l/l ethylene for 24 h at 25°C as previously described (22). IAA metabolism was studied in leaf discs, 6 mm in diameter, and in 1 mm long midrib sections cut from mature leaves.

IAA Metabolism. Three hundred mg of either leaf discs or midrib sections were incubated in 1 ml 20 mM K-phosphate/citric acid buffer (pH 4.5) containing 3.6 μ M [2-¹⁴C]IAA (56 mCi/mmol). Incubation was carried out at 25°C in the dark with constant shaking. At the end of the incubation period, the tissue was rinsed with distilled H₂O and subjected to Davies' method (8) for differential extraction of free and bound IAA with slight modification as follows. Tissue was ground with a

mortar and pestle in 2 ml 80% ethanol. The mortar and pestle were washed with 2 ml 80% ethanol and the wash was combined with the initial extract and centrifuged at 5,000 g for 10 min. The pellet was reextracted successively three times with 80% ethanol and three times with H_2O , using 2 ml for each extraction. Extraction was performed by mixing vigorously for 1 min and centrifuging as above. The pellet obtained after the H_2O extraction was resuspended in 4 ml of 1 N NaOH and extracted overnight at 25°C with constant shaking. The extract was centrifuged at 10,000 g for 10 min. The pellet was resuspended in 2 ml of 1 N NaOH and centrifuged as above. The combined ethanol, H_2O and NaOH extracts were brought to final volumes of 10, 6 and 6 ml, respectively, and aliquots were taken for radioactivity determinations and analysis of IAA metabolites. Radioactivity in the final pellet was also determined. Preliminary experiments indicated that three repeated extractions with 80% ethanol and H_2O as described above removed essentially all of the radioactivity soluble in the corresponding solvent and that the NaOH extraction removed most of the residual radioactivity. Care was taken to ensure complete extraction with each of the solvents used.

Analysis of Free IAA and IAA Metabolites. Quantitative analysis of labeled IAA and IAA metabolites in the 80% ethanol extract was performed by TLC using silica gel HF₂₅₄ plates. The plates were activated at 105°C. Aliquots were applied on 10 x 20 cm plates, 0.5 mm thick. Standards of unlabeled IAA, IAGlu, and IAAsp were spotted together with the extracts for co-chromatography. The plates were developed for 15 cm in chloroform:ethylacetate:formic acid (40:50:10) (Solvent A). After drying the plates were viewed under short UV irradiation for location of standards, and then 10 separate R_F zones were scraped into counting vials for radioactivity determinations. Addition of labeled IAA to the 80% ethanol extracts of untreated tissues and analysis of the extracts by TLC revealed

only one peak of radioactivity which co-chromatographed with authentic IAA, thus demonstrating that the IAA was not conjugated during extraction and remained stable during chromatography. Also, no radioactivity was detected in the pellet after the ethanol extraction, indicating that IAA was not adsorbed to the plant residue.

For qualitative analysis of the IAA metabolites in the ethanol extract additional TLC solvents were used: chloroform:ethyl acetate:formic acid (35:55:10) (solvent B); ethyl acetate:formic acid:H₂O (85:6:9) (solvent C); 1-propanol:methyl acetate:ammonium hydroxide (45:35:20) (solvent D); and ethyl acetate:methyl ethyl ketone:formic acid:H₂O (5:3:1:1) (Solvent E).

GC Analysis . Analysis of methylated IAAsp was performed on a Packard gas chromatograph equipped with a flame ionizing detector. A glass column (2 m x 3.2 mm i.d.) containing 3% OV-1 on Gas Chrom Q, 80-100 mesh, was used. Temperature was programmed from 180 to 260°C at a rate of 3°C/min with injector port at 270°C. The nitrogen carrier gas flow rate was 20 ml/min. Methylated IAAsp was further analyzed by a NP detector. The conjugate was chromatographed on a glass column (2.2 m x 3.2 mm i.d.) containing 3% OV-17 on Gas Chrom Q, 80-100 mesh. Temperatures were 220°C for the injector port, 250°C for the column and 270°C for the detector. The nitrogen carrier gas flow rate was 32 ml/min.

GC-MS Analysis . Combined GC-MS analysis of methylated IAAsp was performed on a Dupont Model 21-940B, CI, with OV-17 column as described above except that the carrier gas was isobutane.

Trimethylsilyl ethers of IAA glucose esters were analyzed by a Finegan Model 4500 MS, EI, with a SE-54 capillary column at 270°C.

Measurement of Radioactivity . All samples were counted with a liquid scintillation spectrometer using a Bray scintillation fluid. Radioactivity data are expressed as dpm after quenching and efficiency corrections.

RESULTS

Nature of IAA conjugates in citrus leaf tissues

Nature of IAA conjugates in untreated citrus leaf tissues .

Chromatography of ethanolic extracts of midrib tissue with solvent system B revealed two distinct radioactive peaks: the first at Rf 0.2 - 0.3 Rf and the second, the major one, at 0.4 - 0.5 (Fig. 1A). The first peak co-chromatographed with IAGlu and the second peak co-chromatographed with IAAsp. Further analysis of the metabolites with solvent systems C and D revealed that the first metabolite is not IAGlu (Figs. 1B and 1C). Similar results were obtained with leaf blade tissue.

Identification of IAAsp . IAAsp was isolated from 180 g of midrib tissue incubated for 8 h in 600 ml of 20 mM K phosphate-citric acid buffer containing 50 μ M cold IAA and 40,000 dpm of labeled IAA. The tissue was extracted three times with 80% ethanol to yield a total volume of 1 liter. The extract was filtered and then centrifuged at 10,000 g for 10 min. The supernatant was reduced to an aqueous solution on a rotary evaporator at 45°C. The aqueous solution was adjusted to pH 8.5 and extracted three times with ethyl acetate. The ethyl acetate fraction was discarded and the aqueous fraction was adjusted to pH 2.5 and extracted with three equal volumes of ethyl acetate. The ethyl acetate fraction containing the IAAsp was reduced to dryness. The gum obtained was dissolved in a small volume of 30 mM K-phosphate-citric acid buffer, pH 4.0, and chromatographed on a PVPP column (1.8 x 16 cm) equilibrated with the same buffer. Fractions containing IAAsp were pooled and after adjusting the pH to 2.5, IAAsp was extracted with three equal volumes of ethyl acetate. The ethyl acetate solution was evaporated to dryness and IAAsp was chromatographed on a 1.2 x 10 cm

Fig. 1 . Radiochromatograms of 80% ethanol extracts of midrib sections cut from untreated citrus leaves following an 8-h incubation period in [14 C]IAA. Analysis was performed by TLC using different solvent systems. The R_f regions of authentic IAAGlu, IAAsp, and IAA run on the same chromatograms are designated by bars. A, solvent system B; B, solvent system C; C, solvent system D.

Sephadex LH-20 using 50% ethanol. Fractions containing IAAsp were evaporated to dryness and IAAsp was methylated with diazomethane (29). The methylated IAAsp was purified on silica gel PF₂₅₄ plates, 1 mm thickness, developed with chloroform:ethyl acetate (2:1) solvent system. After drying, the band corresponding to methylated IAAsp was scraped off and eluted with ethyl acetate. The ethyl acetate solution was reduced to a small volume.

GC analysis of the methylated IAAsp isolated from citrus tissues showed one major peak and several minor ones (Fig. 2B). The major peak had the same retention time as that of authentic methylated IAAsp (Fig. 2A). Further analysis by GC using a NP detector revealed that the peak corresponding to methylated IAAsp is the only peak containing nitrogen (Fig. 2C).

The mass spectra of the isolated methylated IAAsp was identical to that of an authentic compound. The major significant ions were m/e 318 (molecular ion), m/e 286 (loss of 2 hydrogen atoms and 2 methyl groups), and m/e 157 (indole-CH₂CO-H⁺).

Identification of IAA conjugates in ethylene-treated leaves. In a previous report we suggested that the initial conjugation rate reflects more closely the in vivo situation (25). However, in order to obtain sufficient amounts of the conjugates for identification, longer periods of incubation in IAA were required. Figure 3 indicates that prolonging the incubation time did not change the nature of the metabolites found in treated and non-treated tissues, but increased significantly the amounts of the conjugates. Pretreatment with ethylene increased greatly the radioactivity which appeared at R_f 0-0.2 and concomitantly decreased the amount of IAAsp and the second unidentified metabolite (Fig. 4). The metabolite at R_f 0-0.2 was named IAAY.

Midrib sections of ethylene-pretreated and non-treated tissues were

Fig. 2 . GC of methylated IAAsp. IAAsp was isolated from midrib sections of untreated citrus leaves and methylated before analysis. A, analysis of authentic IAAsp; B, analysis of isolated IAAsp; C, analysis of isolated IAAsp by a NP detector.

Fig. 3 . Effect of the duration of incubation in [14 C]IAA on the metabolism of IAA in midrib sections cut from untreated (—) and ethylene-pretreated (---) citrus leaves. Analysis was performed by TLC using solvent system A. Details are as in Figure 1.

Fig. 4 . Radiochromatogram of an 80% ethanol extract of midrib sections cut from ethylene-pretreated (25 μ l/l for 48 h) citrus leaves following an 8-h incubation period in [14 C]IAA. Analysis was performed by TLC using solvent system B. Details are as in Figure 1.

incubated in [^{14}C]IAA and the extracts prepared were subjected to fractionation with different organic solvents at different pH ranges. Extraction with ethyl acetate at pH 2.5 removed IAA, IAAsp and other minor conjugates but not IAAy. This conjugate could be extracted with 1-butanol at pH 8.5 (Fig. 5), indicating that it is a neutral or basic polar metabolite. The level of IAAy in non-treated tissues was very low compared to ethylene-treated tissues (Fig. 5). IAAy co-chromatographed with IAGlu in all acidic solvent systems. However, in solvent system D it appeared in Rf 0.6, whereas IAGlu appeared in Rf 0.9. Extraction of IAAy from TLC plates developed in solvent system D and rechromatography in acidic solvents, indicated that it was not decomposed by the ammonia present in solvent system D.

Isolation and identification of IAAy . Leaves were pretreated with ethylene for 48 h and then 150 g of midrib tissue were excised for isolation of IAAy. Incubation and extraction procedures were similar to those described for IAAsp, except that the tissue was incubated in 100 μM cold IAA, a concentration which gave the highest yield of IAAy. The aqueous solution, obtained after removal of the ethanol used for extraction was adjusted to pH 2.5 and extracted with three equal volumes of diethyl ether. The etheric fraction was discarded and the aqueous fraction was adjusted to pH 8.5 and extracted with three equal volumes of 1-butanol. The butanol fraction containing the IAAy was reduced to dryness. The gum obtained was dissolved in a small volume of methanol and loaded on a 1.5 x 3 cm column of PVPP. The column was eluted with methanol, IAAy being eluted in fractions 18-28 (Fig. 6). The fractions containing IAAy were pooled, reduced to a small volume and further purified on silica gel PF₂₅₄ plates, 1 mm thickness, using ethyl acetate:ethanol:H₂O (85:6:9) and then with chloroform:methanol:H₂O (80:19:1). Final purification was achieved by HPLC using a RP C-18 column

Fig. 5 . Radiochromatograms of IAA metabolites in a butanol fraction, pH 8.5, obtained from 80% ethanol extracts of midrib sections excised from air- and ethylene-pretreated citrus leaves. Leaves were treated either in air or in 25 μ l/l ethylene for 48 h and then midrib sections were excised and incubated in [14 C]IAA for 8 h. Analysis was performed by TLC using solvent system C.

Fig. 6 . Chromatography of IAAy on a PVPP column. A butanol fraction, pH 8.5, obtained from midrib sections incubated in 0.1 mM IAA for 48 h, following a 48 h ethylene-pretreatment, plus 30,000 cpm of IAAy isolated from the same tissue were loaded onto the column. Elution was performed with methanol. (O), absorbance at 254 nm; (Δ), radioactivity.

eluted with 25% methanol in H_2O .

IAAy was hydrolyzed by β -glucosidase and 1 N NaOH yielding free IAA. Other glucosidases such as α -glucosidase and α - and β -galactosidases did not hydrolyze the conjugate. The data suggest that this conjugate is a glucose ester of IAA.

IAAy was subjected to a combined GC-MS analysis (Fig.7). Comparison of the mass spectrum of IAGlu (11) to that of IAAy showed several identical ions, m/e 73, 117, 129, 147, 217, 227, 291, 305, 319 and 450. These are common ions of trimethylsilyl ethers of carbohydrates (11,21). Retention of charge on the glucose ring results in the ion m/e 450, and charge retention on the leaving group gives rise to m/e 246. The ions m/e 103 and 129 result from formation of trimethylsilyl ethers of IAA. The ratio of m/e 204/217 is considered characteristic of the sugar ring size (10). A low ratio of m/e 204/217 is typical for a furanose ring, whereas a high ratio is typical for a pyranose ring. The fact that the spectrum of IAAy shows a low ratio of m/e 204/216-7 suggests the presence of a furanose ring. The existence of the ions m/e 319 and 332 provides additional justification to this assertion (21). The data indicate that the conjugate is indole-3-acetyl-O-glucofuranose. However, additional work is needed for the final identification of this conjugate.

Nature of high molecular weight conjugates . Attempts were made to characterize the macromolecular weight conjugates. Differential centrifugation of the ethanol - and water-insoluble material revealed that most radioactivity was found in the 5,000 g pellet (Table I), indicating that it was associated with the cell-wall. Further studies of the nature of these bound forms of IAA was performed by extracting the ethanol- and water-insoluble material with different solvents according to Scheel and

Fig. 7 . GC-MS analysis of purified IAAy (see text). IAAy was silylated before analysis by treating with bis-(trimethylsilyl)trifluoroacetamide.

Table I. Distribution of ethanol- and water-insoluble radioactivity among subcellular fractions. Leaves were pretreated either with air or with 25 μ l/l ethylene for 24 h and then midrib sections were excised and incubated for 8 h in [14 C]IAA.

Fraction	A i r		Ethylene	
	dpm	%	dpm	%
Ethanol- and water- insoluble	4,190	100	38,994	100
5,000 g	3,270	78	33,873	87
10,000 g	420	10	2,775	7
100,000 g	500	12	2,346	6

Table II. Solubility of ethanol- and water- insoluble conjugates in various solvents. Details are as in Table I, except that only ethylene-pretreated leaves were analyzed.

<u>Solvent</u>	<u>Radioactivity</u> %
SDS	27.2
DMF	19.3
Dioxan	11.8
1 N NaOH	24.6
Insoluble	17.1

Sandermann (28) with some modifications. The procedure utilized successive extractions with sodium dodecylsulphate (SDS), dimethylformamide (DMF), dioxane/water, and 1 N NaOH. SDS has been shown to extract mainly proteins whereas DMF and dioxane are commonly used for extraction of lignin. Significant amounts of radioactivity were detected in all extracts (Table II), suggesting that IAA is probably bound to different cell-wall components.

Chromatography of the NaOH extract on Sephadex G-200 revealed two major peaks; one eluted with the void volume indicating that the molecular weight of the conjugates in this peak is greater than 200 daltons and a second peak at the range of 10 to 15 daltons (Fig. 8). The conjugates in the two peaks behaved similarly on ion exchange columns, suggesting that the conjugates eluted in the first peak are aggregates of those eluted in the second peak.

Changes in IAA conjugation during ethylene-induced abscission

Changes in IAAy formation . Ethylene treatment of citrus leaves resulted in lower levels of [14 C]IAA (Fig. 9). The decline in free IAA level emphasizes the importance of studying changes in IAA metabolites following ethylene treatment as a possible mechanism for removal of free IAA. The data reported above (Figs. 4 and 5) demonstrated that ethylene induced the formation of a glucose ester of IAA (IAAy) with a concomitant decrease in the formation of IAAsp and a second unidentified metabolite. We further investigated the effect of ethylene on the formation of IAAy in relation to its effect on abscission. IAAy formation was assayed by applying [14 C]IAA to midrib tissue and then analyzing IAAy level by TLC, using solvent system D, following extraction with 1-butanol at pH 8.5.

Formation of IAAy in ethylene-pretreated leaves increased with time of incubation in [14 C]IAA up to 5 h and then leveled off (Fig. 10). In air

Fig. 8 . Sephadex G-200 chromatography of NaOH-extractable IAA conjugates of midrib sections excised from ethylene-pretreated citrus leaves (B). The column was calibrated with blue dextran (I) and ribonuclease (II) (A). Prior to chromatography on Sephadex G-200, the conjugates were partially purified on a Sephadex G-25 column. (O), absorbance at 254 nm; (Δ), radioactivity.

Fig. 9 . Effect of ethylene pretreatment (25 μ l/l) on free [14 C]IAA in midrib sections and abscission (ABS) of citrus leaves. Midrib sections were excised at various periods after abscission was recorded and incubated in [14 C]IAA for 1 h. [14 C]IAA was analyzed by TLC. Bars indicate 1 SE.

Fig. 10 . Effect of the duration of incubation in [14 C]IAA on formation of IAAy in midrib sections excised from untreated (□) and ethylene (25 μ l/l for 24 h)-pretreated (○) leaves. IAAy was assayed as described in the text. Bars indicate average SE for each treatment.

control leaves IAAy formation reached maximal level after 1 h of incubation and remained at this level later on. Ethylene increased formation of IAAy by 3 to 4 fold.

The effect of the duration of ethylene pretreatment on the formation of IAAy was studied. Abscission of the same leaves was monitored at different times prior to incubation in [14 C]IAA for analysis of IAAy. A slight, non-significant accumulation of IAAy was observed following an 8 h ethylene pretreatment (Fig. 11). After 24 h of ethylene pretreatment a significant increase in IAAy level was detected. At that time abscission was only 20%. IAAy reached its maximal level after 72 h when 100% abscission was obtained. Level of IAAy in untreated leaves remained constant during the entire experimental period. These leaves did not abscise during this period.

When leaves were subjected to various concentrations of ethylene, a positive correlation between abscission and the increase in ethylene concentration above 1 μ l/liter was observed (Fig. 12). The pattern of IAAy formation in the same leaves, due to increase in ethylene concentration, was similar to that of abscission. Formation of IAAsp decreased with the increase in ethylene concentration above 1 μ l/liter. It should be noted that total uptake of [14 C]IAA was reduced by ethylene.

It is well accepted that formation of IAAsp is adaptive and can be induced by biologically active auxins (7). On the other hand, IAGlu is formed immediately after auxin application. The data described above indicate that IAAy is induced by ethylene. The question whether exogenous IAA will by itself induce formation of IAAy in ethylene-pretreated tissue was studied. Incubation in cold IAA up to 8 h did not induce any additional formation of IAAy (Table III). Furthermore, addition of cycloheximide to the incubation medium had no effect on formation of IAAy (Table IV). On the other hand, IAAsp formation was significantly reduced by CHI in both air- and

Fig. 11 . Effect of ethylene (25 μ l/l) on formation of IAAy in midrib sections and abscission of citrus leaves. Midrib sections were excised at various periods after abscission was recorded and incubated in [14 C]IAA for 8 h. IAAy was assayed as described in the text. Bars indicate 1 SE.

Fig. 12 . Effect of ethylene concentration on total [14 C]IAA uptake (TU) and IAAy formation and abscission (ABS) of citrus leaves. Leaves were pretreated with ethylene for 28 h and after abscission was recorded midrib sections were excised and incubated in [14 C]IAA for 8 h. IAAy was assayed as described in the text. Bars indicate 1 SE.

Table III Effect of preincubation in [^{12}C]IAA on IAA conjugation in midrib sections of ethylene-pretreated (25 $\mu\text{l/l}$ for 24 h) leaves. Midrib sections were incubated either in buffer or in 50 μM [^{12}C]IAA for various periods and then transferred to [^{14}C]IAA for 1 h.

Time of preincubation h	Preincubation	Total uptake dpm	IAA % of	IAAsp total	IAAy uptake
0	-	10,500	33.3 $\pm$ 4.4	10.3 $\pm$ 1.0	23.2 $\pm$ 2.2
4	IAA	11,000	32.7 $\pm$ 3.8	12.9 $\pm$ 1.3	33.1 $\pm$ 2.9
	buffer	15,600	32.0 $\pm$ 3.7	10.9 $\pm$ 1.1	33.1 $\pm$ 3.0
8	IAA	12,000	27.5 $\pm$ 3.0	16.2 $\pm$ 1.6	29.1 $\pm$ 3.1
	buffer	20,000	28.3 $\pm$ 3.1	17.5 $\pm$ 2.2	31.3 $\pm$ 3.4

Table IV Effect of cycloheximide on IAA conjugation in midrib sections of untreated and ethylene (25 $\mu\text{l/l}$ for 48 h)-pretreated leaves. Midrib sections were incubated with or without 50 μM cycloheximide for 1 h and then transferred to [^{14}C]IAA for additional 7 h.

Treatment	Total uptake dpm	IAA % of	IAAsp total	IAAy uptake
Air-CHI	22.400 $\pm$ 3.100	1.3 $\pm$ 0.5	62.5 $\pm$ 7.4	6.3 $\pm$ 0.8
Air+CHI	14.500 $\pm$ 1.620	16.2 $\pm$ 1.8	34.4 $\pm$ 4.2	7.6 $\pm$ 0.8
Ethylene-CHI	14.000 $\pm$ 1.550	10.7 $\pm$ 0.8	20.0 $\pm$ 1.9	42.8 $\pm$ 4.9
Ethylene+CHI	12.800 $\pm$ 1,300	13.2 $\pm$ 1.4	14.0 $\pm$ 1.5	46.8 $\pm$ 4.1

ethylene- pretreated tissues.

Changes in formation of high molecular weight conjugates. Previous studies have shown that ethylene stimulates formation of high molecular weight conjugates in citrus leaf tissues (25). We further studied the effect of ethylene on formation of these conjugates in relation to the abscission process.

Leaves were treated with ethylene for various periods and then midrib tissue was excised and incubated in [^{14}C]IAA for additional 8 h. The distribution of radioactivity in the ethanol and NaOH extracts and in the plant residue is shown in Figure 13. In air control leaves above 80% of the radioactivity was found in the ethanol extract which contained free IAA and low molecular weight conjugates.

Ethylene significantly reduced the amount of radioactivity extracted by ethanol. Substantial amount of radioactivity was detected in the 1 N NaOH extract, which contained macromolecular conjugates, and it was markedly increased by ethylene pretreatment. The highest level of radioactivity, 17% of total uptake, was reached 72 h after ethylene treatment. Prolonging the duration of ethylene pretreatment reduced the formation of macromolecular conjugates. In some experiments the highest level of radioactivity was obtained earlier. The low radioactivity in the NaOH extracts of control leaves was unaffected by the duration of incubation in air.

Formation of macromolecular conjugates was detected after 1 h of incubation in [^{14}C]IAA (Fig. 14). Whereas in air-pretreated leaves level of these conjugates did not change during incubation in [^{14}C]IAA, in ethylene-pretreated leaves it increased continuously with time.

When leaves were pretreated with increasing concentrations of ethylene, a positive correlation was found between ethylene-induced formation of macromolecular conjugates and ethylene-induced abscission (Fig. 15). The

Fig. 13 . Solvent distribution of radioactivity from midrib sections of untreated ($\square$) and ethylene (25 μ l/l)-pretreated ($\circ$) citrus leaves. Midrib sections were excised at various periods and incubated in [14 C]IAA for 8 h.

Fig. 14 . Effect of the duration of incubation in [14 C]IAA on formation of NaOH-extractable conjugates in midrib sections excised from untreated (CON) and ethylene (25 μ l/l for 24 h)-pretreated citrus leaves. Bars indicate 1 SE.

Fig. 15 .Effect of ethylene concentration on NaOH-extractable conjugates (RAD) and abscission (ABS) of citrus leaves. Leaves were pretreated with ethylene for 24 h and after abscission was recorded, midrib sections were excised and incubated in [^{14}C]IAA for 8 h. Bars indicate 1 SE.

threshold level of ethylene for induction of both processes was between 1 to 2 μ l/liter.

Cycloheximide had no effect on formation of macromolecular conjugates.

DISCUSSION

Studies on the effect of ethylene on IAA conjugation in citrus leaf tissues have been performed in our laboratory for several years. However, identification of the metabolites involved was based on co-chromatography with authentic standards using one or two solvent systems (23,25). Based on these studies we initially suggested that IAAsp and IAGlu are the major IAA conjugates in citrus leaf tissues (23). In a later study we recognized that the conjugate identified tentatively as IAGlu might be a different conjugate (25). During the present study we made an effort to isolate and identify the IAA conjugates in air- and ethylene-pretreated citrus leaf tissues.

When air control tissues were incubated in [14 C]IAA, IAA was converted to two metabolites (Fig. 1). TLC (Fig. 1), GC (Fig. 2), and GC-MS analyses indicated that the major metabolite in citrus leaf tissues is IAAsp. The second metabolite has not yet been identified. Although it co-chromatographed with IAGlu in solvent system A, it had different R_f values when analyzed by other solvent systems (Fig. 1). Additional data indicate that it is an acidic conjugate probably with an amino acid. Thus, formation of amide conjugates is the major pathway of IAA metabolism in untreated citrus leaf tissues.

Pretreatment with ethylene reduced formation of the above mentioned amide conjugates and increased formation of a third conjugate (Figs. 4 and 5). Hydrolysis by 1 N NaOH and β -glucosidase indicate that it is a glucose

ester of IAA. From GC-MS analysis we conclude that the conjugate is indole-3-acetyl-O-glucofuranose.

The relationship between formation of amide conjugates and the ester conjugate in ethylene-pretreated tissues is not clear. One may assume that both systems compete with each other for free IAA. According to this assumption two situations are possible; increased formation of IAAy results from inhibition of the formation of amide conjugates or that reduction in the formation of amide conjugates results from increased synthesis of IAAy. The fact that IAAy increased significantly in ethylene-pretreated tissues during the first h of incubation in [14 C]IAA, when level of IAAsp did not change much (Fig. 3) or even increased (25), suggests that there is no direct relationship between the two systems. An additional support for this assumption comes from the experiment with cycloheximide in which inhibition of IAAsp formation did not bring about a concomitant increase in IAAy synthesis (Table IV). The reason for the lack of any direct relationship between the two systems may be due to compartmentation of the enzymes involved. It is concluded that increased formation of IAAy by ethylene results from stimulation of the enzyme system which catalyzes its synthesis and not from suppression of synthesis of amide conjugates. Since ethylene did not reduce formation of IAAsp during the initial period of incubation in [14 C]IAA (Fig. 3), it is reasonable to assume that ethylene suppresses the development of the enzyme system responsible for IAAsp synthesis. Induction of this enzyme system usually occurs a few h after application of IAA to excised tissue (7) and it does not necessarily reflect the situation in the intact tissue. In contrast, the significant increase in IAAy in ethylene-pretreated tissues during the first h of incubation in [14 C]IAA (Fig. 3) indicates that the enzyme system catalyzing the formation of IAAy develops in the intact tissue during the ethylene treatment. This may

explain why synthesis of IAAsp but not that of IAAY is inhibited by cycloheximide (Table IV).

Most studies of the effects of ethylene on IAA conjugation have dealt with the common conjugates such as IAGlu and IAAsp and similar compounds soluble in 80% ethanol. The present study shows that in ethylene-pretreated citrus tissues NaOH-extractable conjugates are clearly quantitatively important products, reaching up to 20% of total [^{14}C]IAA uptake. It has been shown that NaOH-extractable conjugates are macromolecular metabolites (25). The present study indicates that the macromolecular conjugates are associated with the cell-wall (Table I) and probably represent various types of macromolecules (Table II). By using various solvents to extract bound 2,4-D, Scheel and Sandermann (28) reported that the auxin was bound to protein, polysaccharides and lignin. Our data (Table II) suggest that IAA is also bound to these macromolecules. Macromolecular conjugates of IAA have been described in several reports. Bandurski and co-workers (19,20) characterized IAA-glucan esters and a gluco-protein from corn kernels. Davies and Galston (9) reported formation of macromolecular conjugates of IAA which seemed to be polysaccharides. Formation of IAA-protein conjugates has also been suggested (31). Since some of the above mentioned conjugates can be hydrolyzed by 1 N NaOH, the term 'bound auxin' has been used to describe auxin conjugates insoluble in organic solvents which extract IAA and its derivatives but hydrolyzable by NaOH to release IAA (8). Citrus NaOH-extractable conjugates do not correspond to the above definition of 'bound auxin' inasmuch as they are not hydrolyzed by 1 N NaOH. In this respect, citrus NaOH-extractable conjugates are different from those of pea (8) and Nicotiana (16).

The accepted model of ethylene-induced abscission of intact leaves is based on the hypothesis that reduction of IAA content in abscission is a

prerequisite for ethylene action in accelerating abscission (3,6). Reduction of IAA level in the abscission zone has been attributed to the inhibition of auxin transport (2). Whether IAA conjugation is involved in the reduction of IAA level in ethylene-treated leaves is still unclear. Evidence that has emerged over the last several years indicates that conjugate metabolism plays an important role in regulation of IAA levels (1,7). After the major conjugates in citrus tissues had been identified, we examined the relationship between IAA conjugation and abscission in ethylene-treated leaves, using intact leaves which do not abscise when incubated in air for a long period. In a previous report we demonstrated that ethylene increased conjugation of IAA to low and high molecular weight conjugates (25). We were now able to show that increased formation of low molecular weight conjugates was mainly related to the synthesis of an IAA glucose ester. When citrus leaves were treated with increasing concentrations of ethylene, there was a close positive correlation between formation of IAAy and abscission, 1 to 2 μ l/liter ethylene being the threshold level for induction of both systems (Fig. 12). Studies on the effect of the duration of the ethylene pretreatment showed that increased formation of IAAy preceded the abscission process (Fig. 11). Similar relationships were found between accumulation of high molecular weight conjugates and abscission (Fig. 15). Increased IAA conjugation following ethylene treatment was reflected in reduction of free [14 C]IAA during the initial period of incubation in IAA. As mentioned above, we have suggested that when using [14 C]IAA for such studies, the initial period of incubation reflects more closely the in vivo situation.

Although it may be difficult to extrapolate to the in vivo situation from studies with exogenous IAA, data accumulated in the present study as well as in other reports (12,18,23,25,32) suggest that ethylene increases

IAA conjugation. As discussed previously, it is very likely that the stimulation of IAAy synthesis occurs in the intact leaf and unlike the effects of ethylene on IAAsp, it does not result from the specific conditions of the experimental system. Moreover, recent reports demonstrated an increase in endogenous levels of IAA conjugates following ethylene treatment (12,32). It remains now to examine whether increased conjugation of IAA by ethylene will result in lower levels of endogenous IAA.

LITERATURE CITED

1. Bandurski, R.S., Nonhebel, H.M. 1984. In: Advanced Plant Physiology, ed. M.B. Wilkins, Pitman Publishing, London. pp. 1-20.
2. Beyer, E.M.Jr. 1973. Plant Physiol. 52:1-5.
3. Beyer, E.M.Jr. 1975. Plant Physiol. 55:322-327.
4. Beyer, E.M.Jr., Morgan, P.W. 1969. Plant Cell Physiol. 10:787-799.
5. Beyer, E.M.Jr., Morgan, P.W. 1970. Plant Physiol. 46:157-162.
6. Beyer, E.M.Jr., Morgan, P.W., Yang, S.F. 1984. In: Advanced Plant Physiology, ed. M.B. Wilkins, Pitman Publishing, London. pp. 114-120.
7. Cohen, J.D., Bandurski, R.S. 1982. Ann. Rev. Plant Physiol. 33:403-430.
8. Davies, P.J. 1976. Plant Physiol. 57:197-202.
9. Davies, P.J., Galston, A.W. 1974. Plant Physiol. 47:435-441.
10. De Jong, D.C., Radford, T., Hribar, J.D., Hanessian, S., Bieber, M., Dawson, G., Sweeley, C.C. 1967. J. Amer. Chem. Soc. 91:1728-1740.
11. Ehmann, A. 1974. Carbohydr. Res. 34:99-114.
12. Epstein, E. 1982. Physiol Plant. 56:371-373.
13. Ernest, L.C., Valdovinos, J.G. 1971. Plant Physiol. 48:402-406.
14. Goren, R., Bukovac, M.J., Flore, J.A. 1974. Plant Physiol. 53:164-166.

15. Lieberman, M., Knecht, E. 1977. *Plant Physiol.* 60:475-477.
16. Liu, S.T., Gravenet, D., Knight, C.A. 1978. *Plant Physiol.* 61:50-53.
17. Minato, T., Okazawa, Y. 1974. *Proc. Crop. Sci. Soc. Japan* 43:127-128.
18. Minato, T., Okazawa, Y. 1978. *J. Fac. Agric. Hokkaido Univ.* 58:535-547.
19. Percival, F.W., Bandurski, R.S. 1976. *Plant Physiol.* 58:60-67.
20. Piskornik, Z., Bandurski, R.S. 1972. *Plant Physiol.* 50:176-182.
21. Radford, D.C., Jongh, D.C. 1972. In: *Biochemical Applications of Mass Spectrometry*, ed. G.R. Waller, Wiley Interscience. pp. 302-348.
22. Riov, J. 1974. *Plant Physiol.* 53:312-316.
23. Riov, J., Goren, R. 1979. *Plant Cell Envir.* 2:83-89.
24. Riov, J., Gottlieb, H.E. 1980. *Physiol. Plant.* 50:347-352.
25. Riov, J., Dror, N., Goren, R. 1982. *Plant Physiol.* 70:1265-1270.
26. Riov, J., Sagee, O., Goren, R. 1984. 267-276. In: *Biochemical, Physiological and Applied Aspects of Ethylene*, ed. Y. Fuchs, E. Chalutz, Martinus Nijhoff/Dr W. Junk Publishers, The Hague. pp. 267-276.
27. Scheel, D., Sandermann, H.Jr. 1981. *Planta* 152:248-252.

28. Scheel, D., Sandermann, H.Jr. 1981a. *Planta* 152:253-258.
29. Schlenk, H., Gellerman, J.L. 1960. *Anal. Chem.* 32:1412-1414.
30. Walters, J., Osborne, D.J. 1979. *Planta* 146:309-317.
31. Winter, A., Thimann, K.V. 1966. *Plant Physiol.* 41:335-342.
32. Wood, B.W. 1985. *J. Amer. Soc. Hort. Sci.* 110:340-343.

SECTION B

IAA METABOLISM IN SENESCING CITRUS LEAVES APPROACHING NATURAL ABSCISSION

ODED SAGEE, JOSEPH RIOV AND RAPHAEL GOREN

It has been proposed that the balance between IAA and ethylene controls abscission (12). According to this hypothesis, as leaves senesce the abscission-retarding substances (IAA) decline and the abscission-promoting substances (ethylene and probably ABA) increase. Abscission occurs when this balance is shifted in favor of ethylene. Increased production of ethylene in senescing leaves or petioles has been reported in various plants (3). We have presented evidence that ethylene stimulates IAA conjugation in citrus leaves as a result of which free IAA, measured as [^{14}C]IAA, declines (Section A). However, these data were obtained with detached leaves treated with exogenous ethylene. In the present study we examined whether citrus leaves produce elevated amounts of ethylene during senescence and if so whether the hormone stimulates IAA conjugation as it does in detached leaves.

MATERIAL AND METHODS

Plant Material and Treatments . Mature leaves from the current year flush and senescing leaves approaching natural abscission, defined by their yellow-green or yellow color, were taken from established trees of Citrus sinensis L. Osbeck var. Shamouti orange. Mature leaves were treated with 25

$\mu\text{l/l}$ ethylene at 25°C as previously described (4).

IAA metabolism . IAA metabolism in midrib sections was determined as described in Section A.

Ethylene determination . To monitor ethylene production, leaves were transferred to test tubes sealed with a rubber serum cup. After 2 h, a 2-ml gas sample was withdrawn with a hypodermic syringe, and ethylene was assayed by a gas chromatograph equipped with an alumina column and a flame ionization detector.

RESULTS AND DISCUSSION

The initial rate of IAA metabolism in senescing leaves was studied by incubating midrib sections in [^{14}C]IAA for 2 h. In senescing leaves, level of IAA_{sp} and the metabolites at R_f 0-0.2, which consisted mainly of IAA_y (Section A), increased as compared to mature leaves (Fig. 1). The increase in IAA conjugation in senescing leaves was reflected by a significant reduction in free [^{14}C]IAA level. IAA metabolism in senescing leaves was also compared to that in mature leaves pretreated with ethylene. As reported in the previous section, ethylene increased IAA conjugation resulting in a lower level of [^{14}C]IAA (Fig. 1). In contrast to senescing leaves, increased IAA conjugation in ethylene-pretreated leaves was particularly related to formation of IAA_y.

Senescing leaves produced elevated levels of ethylene, whereas mature leaves produced very low levels of ethylene (Fig. 2). The increased production of ethylene in senescing citrus leaves is of a particular

Fig. 1. Radiochromatograms of 80% ethanol extracts of midrib sections excised from mature citrus leaves treated with either air or ethylene and from senescing (old) leaves incubated in air. Leaves were treated with air or 25 $\mu\text{l/l}$ ^{14}C ethylene for 24 h. Excised midrib sections were then incubated in ^{14}C IAA for 2 and 8 h. Analysis was performed by TLC using chloroform:ethyl acetate:formic acid (40:50:10) solvent system. The R_f regions of authentic IAAsp and IAA are designated by bars. Numbers refer to total uptake.

Fig. 2. Ethylene production rate in intact citrus leaves and IAAy formation in midrib sections excised from mature citrus leaves treated with either air or ethylene and from senescing leaves incubated in air. Leaves were treated with air or with 25 μ l/l ethylene for 24 h, and ethylene production rate was then determined. Following ethylene determination, midrib section were excised and incubated in [¹⁴C]IAA for various periods. Bars indicate 1 SE.

Fig. 3. Formation of NaOH-extractable conjugates in midrib sections excised from mature citrus leaves treated with either air or ethylene and from senescing leaves incubated in air. Leaves were treated with air or with 25 μ l/l ethylene for 24 h. Following incubation, midrib sections were excised and incubated in [¹⁴C]IAA for various periods. Bars indicate 1 SE.

interest since it has been reported that citrus leaves possess an effective autoinhibition mechanism (6). The present data suggest that the autoinhibition mechanism does not operate in senescing leaves and therefore these leaves are capable of producing high amounts of ethylene. Increased ethylene production in ethylene-pretreated leaves is explained by autocatalysis which is characteristic for citrus leaves exposed to ethylene for long periods (6).

IAAy formation was significantly higher in senescing leaves as compared to mature leaves during an 8-h incubation period in [^{14}C]IAA (Fig. 2). However, it is clearly evident that exogenous ethylene was much more effective in inducing formation of IAAy.

Senescing leaves produced also high amounts of macromolecular conjugates (Fig. 3). The increase in level of these conjugates in senescing leaves was about 3.5 fold higher than in untreated mature leaves after 8 h of incubation in [^{14}C]IAA. The trend of formation of macromolecular conjugates in senescing leaves was similar to that in ethylene-pretreated leaves, but again the overall amount of these conjugates was much lower than in ethylene-pretreated leaves.

We further studied the relationship between rate of ethylene production, IAA metabolism, and natural abscission in leaves at various senescing stages. A gradual increase in ethylene production was observed during senescence (Table I). The increase was particularly high in leaves approaching natural abscission. Formation of both IAAy and macromolecular conjugates increased substantially when ethylene production rate was above 1.42 nl/g/h.

The data presented in this paper clearly show that when citrus leaves senesce they produce elevated levels of ethylene which are accompanied by increased IAA metabolism. Based on the data with exogenous ethylene (Section

Table I. Ethylene production rate and IAA metabolism at various senescing stages of citrus leaves. Ethylene production rate of intact leaves was determined immediately after the leaves were collected. Following ethylene treatment, midrib sections were excised and incubated in [14 C]IAA for 8 h.

<u>Senescing stage 1</u>	<u>Abscission</u>	<u>Ethylene</u> nl/g/h	<u>IAAy</u> % of total uptake	<u>NaOH-extractable</u> <u>conjugates</u> dpm x 10^{-3}
A	-	0.22 $\pm$ 0.03	8.3 $\pm$ 1.2	5.4 $\pm$ 0.7
B	-	0.38 $\pm$ 0.14	7.3 $\pm$ 1.3	6.1 $\pm$ 0.7
C	-	0.69 $\pm$ 0.18	6.5 $\pm$ 1.1	9.4 $\pm$ 0.8
D	+	1.42 $\pm$ 0.33	10.7 $\pm$ 1.8	11.3 $\pm$ 0.8
E	++	7.73 $\pm$ 1.18	17.2 $\pm$ 2.3	15.4 $\pm$ 1.2
F	+++	22.50 $\pm$ 4.36	13.3 $\pm$ 1.6	11.9 $\pm$ 1.1

1

A, mature; B, yellow-green; C, yellow; D, yellow, detached by a strong pull (+); E, yellow, detached by a light pull (++); F, yellow, detached by a very light pull (+++).

A) it is reasonable to assume that ethylene is responsible for the increase in IAA conjugation in senescing leaves. In both cases we observed increased formation of IAAY and macromolecular conjugates (Figs. 2 and 3). Increased formation of IAAsp was also detected in senescing leaves (Fig. 1) and similar observations have been previously reported for ethylene-pretreated leaves (5). Exogenous ethylene increased IAA metabolism by far compared to the increase in IAA metabolism in senescing leaves (Figs. 2 and 3). However, senescing leaves are exposed to ethylene for a long period through which even a slight increase in IAA metabolism may have a significant effect on level of endogenous IAA.

LITERATURE CITED

1. Morgan, P.W. 1983. In: Agricultural Chemicals of The Future, ed. J.L. Hilton, Rowman and Allanheld Publ., Totowa N.J. pp. 61-74.
2. Morgan, P.W. 1984. In: Ethylene: Biochemical, Physiological and Applied Aspects, ed. Y. Fuchs, E. Chalutz, Martinus Nijhoff/DR W. Junk Publishers, The Hague. pp. 231-240.
3. Osborne, D.J. 1973. In: Shedding of Plant Parts, ed. T.T. Kozlowski, New York/London: Academic. pp. 125-147.
4. Riov, J. 1974. Plant Physiol. 53:312-316.
5. Riov, J., Dror, N., Goren, R. 1982. Plant Physiol. 70:1265-1270.
6. Riov, J., Yang, S.F. 1982. Plant Physiol. 70:136-141.
7. Roberts, J.A., Osborne, D.J. 1981. J. Exp. Bot. 32:875-887.

SECTION C

COMPARISON OF DIFFERENT ENZYME IMMUNOASSAYS FOR MEASURING IAA IN VEGETATIVE CITRUS TISSUES

Oded Sagee, Arie Maoz, Rudiger Mertens, Raphael Goren and Joseph Riov

An enzyme-linked immunosorbent assay (ELISA) using polyclonal antibodies, which were raised against indole-3-acetic acid (IAA) conjugated to bovine serum albumin (BSA) via the indolic nitrogen (IAA-N-BSA), has been developed. The sensitivity and specificity of these antibodies were compared to those of polyclonal and monoclonal antibodies raised against IAA conjugated to BSA via C₁ of the carboxyl group (IAA-C₁-BSA). The sensitivity of the assays improved in the following order: monoclonal antibodies > antibodies to IAA-C₁-BSA > antibodies to IAA-N-BSA. Antibodies against IAA-N-BSA had less cross-reactivity to indoles structurally related to IAA, excluding indole-3-pyruvic acid. A rapid and effective method for purification of IAA in citrus tissues before analysis by ELISA is described. Values of IAA in citrus shoot tips obtained with all three antibodies were similar. However, in leaf tissues which contain lower amounts of IAA compared to shoot tips, monoclonal antibodies gave higher values of IAA than polyclonal antibodies. It is concluded that an additional HPLC purification step is required when using monoclonal antibodies. Estimation of free IAA levels in purified extracts of citrus shoot tips, very young leaves and mature leaves was ca. 380, 248, 74 ng/g fresh weight respectively.

Introduction

Determination of IAA in plants by analytical methods involves special problems because the endogenous IAA is present in trace level amounts and in tissues which are highly pigmented. Several physico-chemical methods such as GC-MS, SICM, indole-pyrone fluorescence method and HPLC have been used (see reviews by Morgan and Durham 1983, Brenner 1981). Despite their advantages in accurate determination, these methods still require costly equipment and do not allow the analysis of many samples simultaneously. The first introduction of immunoassay for plant hormones (Fuchs and Fuchs 1969) opened a new line of research, which has been adopted by RIA of IAA (Pengelly and Meins 1977) and greatly extended by Weiler (1983). EIA with antibodies prepared against IAA-protein conjugates with linkage through C_1 proved to be very sensitive for IAA (Weiler et al. 1981). In contrast, RIA with antiserum to IAA-protein conjugates, in which the indole nitrogen was linked via Manich reaction, showed a high specificity for IAA but had a low titer (Pengelly and Meins 1977). Although both immunoassays have been used for measurements of IAA in plant tissues (Weiler et al. 1981, Weigel et al. 1984, Hirsche et al. 1982), their further application for a wide range of light-grown plants still faces problems associated with high level of pigments and interfering compounds (Pengelly et al. 1981). Recent reports stress the necessity of partial purification of extracts before assaying plant hormones by immunoassays (Sandberg et al. 1985, Oden et al. 1985, Pence and Caruso 1985).

It is well known that citrus tissues are heavy loaded with phenolic compounds, essential oils and other pigments (Sinclair 1984). We previously presented preliminary data on the use of an immunoassay for

analysis of IAA in the abscission zone of citrus leaves using antibodies to IAA-N-BSA (Riov et al. 1984). In this report, we describe detailed methods for extraction and purification of IAA from citrus leaves and growth tips, and its measurement by immunoassays using various antibodies to IAA.

Abbreviations- IAA, indole-3-acetic acid; GC-MS, gas chromatography-mass spectroscopy; SICM, selected ion current monitoring; HPLC, high performance liquid chromatography; RIA, radioimmunoassay; EIA, enzyme-immunoassay; BHT, butylated hydroxytoluene; PVPP, polyvinylpolypyrrolidone (Polyclar AT); BSA, bovine serum albumin; OVA, ovalbumin; AP, alkaline phosphatase; ELISA, enzyme-linked immunosorbent assay; IAAsp, indole-3-acetylaspatic acid; IAGlu, 1-O-(indole-3-acethyl)- β -D-glucose.

Materials and Methods

Plant material

One-year-old shade leaves and 4-5 week old growth tips from the current year flush were taken from established trees of Citrus Sinensis [L.] Osbeck, Var Shamouti orange. Leaf discs or midrib sections were cut immediately after harvesting, frozen and stored in -18°C .

IAA extraction and purification

IAA was extracted and partially purified according to Thompson et al.(1981) with some modifications. Plant tissue corresponding to 1 gr fresh weight was homogenized by a Polytron homogenizer in 5 ml of 80% methanol

containing 0.1 M ammonium acetate, 10 ug/ml BHT and 120 nmol [2-¹⁴C]IAA (2.11 GBq/mmol) as an internal standard. Extraction was performed for 1 min in an ice bath. The homogenized sample was kept in the dark at 4°C. After 30 min. the sample was vigorously mixed, centrifuged at 5000 g for 10 min and the supernatant saved. The pellet was resuspended in 5 ml of distilled water and centrifuged as above. Both supernatants were pooled and IAA was subjected to three open column liquid chromatography steps. Location and recovery of IAA was monitored by the radioactive internal standard. The extract was loaded onto a PVPP column and IAA was eluted with 0.01 M ammonium acetate. Fractions containing radioactivity were then loaded directly onto DEAE-Sephadex column in the acetate form, equilibrated with the same solution. The IAA was eluted from the DEAE-Sephadex column with 1 M acetic acid and applied onto C-18 Sep-Pak. The column was washed with distilled water to remove acetic acid and IAA was eluted with methanol. The methanol solution containing IAA was evaporated to a small volume and dried under a stream of nitrogen.

The partially-purified extract was dissolved in 200 ul methanol and divided into two equal portions. For analysis with anti IAA-C₁-BSA, 100 ul of the extract were methylated with excess of ethereal diazomethane (Schlenk and Gellerman 1960). Both portions were then dried with N₂ and redissolved in 50 ul of ethanol and 950 ul of TBS (50 mM Tris-HCl, 1 mM MgCl₂, 0.1 M NaCl, pH 7.8) and assayed within 12 hours.

Preparation of IAA-specific antibodies

Anti-IAA antibodies were obtained by immunization of rabbits with IAA-C₁-BSA conjugate as described by Weiler (1981) or by immunization with IAA-N-BSA conjugate as described by Pengelly and Meins (1977). The

anti-IAA titer of sera was estimated and immune sera with high titer was fractionated by DEAE-cellulose column and γ -Globulin fractions containing antibodies were pooled and concentrated according to Clark and Adams (1977). Anti IAA monoclonal antibodies were prepared according to Mertens et al.(1985).

Preparation of the enzyme tracer

Coupling IAA to alkaline-phosphatase was done by conjugating IAA through its carboxyl group to amino residues on the enzyme with the carbodiimide method (Weiler et al. 1981). For EIA with antibodies, anti IAA-N-BSA the enzyme tracer was IAA-OVA-AP prepared by coupling IAA to OVA with formaldehyde as described by Ranadive and Sehon (1967) and modified by Pengelly and Meins (1977) except that 300 mg of OVA were used instead of BSA. The IAA-OVA was coupled to AP by the glutaraldehyde method according to Clark and Adams (1977).

Preparation of standards

IAA standard for the EIA using antibody to IAA-C₁-BSA was prepared by methylating 3.5 mg IAA (20 μ mol) with ethereal diazomethane and dissolving the product in methanol. For EIA using antibody to IAA-N-BSA, the same amount of untreated IAA was dissolved in methanol. The stock solutions were stored at -18°C and the desired dilutions were made in distilled water before use.

Standard ELISA procedure

After determining empirically optimal parameters for the ELISA (see Results), the following standard protocol was adopted. The lyophilized immunoglobulins were dissolved in 50 mM NaHCO_3 , pH 9.6, to give a concentration of 10 $\mu\text{g/ml}$ and 200 μl of the coating solution were added to each well of the plate (Nunc-immuno plate 96F). After incubation overnight at 4°C, the wells were emptied and the plate was washed three times with H_2O and stored at 4°C until use. Before the enzyme immunoassay, remaining non-specific protein binding sites were blocked by filling the wells with 200 μl of 0.01% BSA in TBS. After incubation at 4°C for 15 min the surfaces were washed with H_2O and immediately used for the immunoassay. For each type of antibodies, a slightly different procedure was used. To each of the antibody-coated wells, 50 μl TBS and 100 μl of methylated standard of IAA or sample, respectively, were added for antibodies to IAA-N-BSA. The microtiter plates were incubated at 4°C for 30 min. Fifty μl of the appropriately diluted enzyme tracer (1:9000 in TBS-gelatin IAA-AP for antibodies to IAA-C₁-BSA; 1:4000 in TBS IAA-OVA-AP for antibodies to IAA-N-BSA) were subsequently added and the solution was mixed for 1 min and then incubated at 4°C for another 30 min. The plate was then washed three times with water and for the assay of surface-bound AP activity, 200 μl of a freshly-prepared 1 mg/ml solution of P-nitro-phenylphosphate in 50 mM NaHCO_3 , pH 9.6, was added to each well. The enzyme reaction proceeded at 37°C for 1 h and was then stopped with 50 μl of 3M KOH. Procedure for EIA with monoclonal antibodies was exactly as described by Mertens et al. (1985). Enzyme activity was monitored by measuring absorbance at 405 nm with Microelisa Auto Reader

For calculation of IAA quantity the following equation was used: $(Ab-Aub)/(Ao-Aub) \times 100 = \% B/Bo$. "Ab" is the absorbance in the standard with a known amount of IAA or in an unknown sample. "Aub" is the absorbance in the presence of a large excess (250 pmol of antibody to IAA-C₁-BSA or 2000 pmol for antibody to IAA-N-BSA) of IAA, and "Ao" is the absorbance in the absence of IAA. Linear standard curves were obtained by the logit-transformation of the relative binding parameter: $\text{logit}(\% B/Bo) = \text{Ln}[B/Bo(100-B/Bo)]$. Each tissue was assayed in 3 to 4 independent extracts. All measurements for a given extract were carried out in triplicates. Statistical calculations were made with statistical package by DAISY adjusted to ELISA on Apple IIE micro computer.

Results and Discussion

Characterization of antibodies to IAA-BSA

The recent development of several immunoassays for IAA (Pengelly and Meins 1977, Weiler 1981) made it possible to investigate and compare the two methods for the determination of endogenous IAA. Whereas EIA, using antibodies to IAA-C₁-BSA, has been developed and utilized for assaying IAA in plant tissues (Weiler 1984), antibodies for IAA-N-BSA have been used for RIA (Pengelly and Meins 1977). In view of the suggested advantages of EIA over RIA (Weiler 1984), we aimed to develop a new EIA for antibodies to IAA-N-BSA (Riov et al. 1984). Antisera to the two hapten-protein conjugates, IAA-C₁-BSA and IAA-N-BSA, were obtained from rabbits. In order to assay the specific response to IAA of antibodies to IAA-N-BSA an additional hapten-protein conjugate IAA-N-OVA was prepared. The

availability of such a molecule permits cross-reaction only with the hapten portion of the conjugate. The proposed procedure was that the free hapten will inhibit the binding of the antibodies to the IAA-N-OVA coated on ELISA plates. Although antibodies bind specifically to the ELISA plates coated this way, when this approach was utilized for detection of free IAA it was only partially successful. Therefore we tried an alternative approach, using polystyrene plates coated with 10 $\mu\text{g/ml}$ anti-IAA immunoglobulin and with IAA-N-OVA linked to enzyme tracer. Such tracer was prepared using novel conditions (Clark and Adams 1977) which maintained less enzyme activity. Under these conditions a sigmoidal standard curve was obtained (Fig. 1A). The effective standard curve extends from 20 to 150 pmol of IAA with maximum binding of 85% (Fig. 1B). Using lower antibody concentrations (e.g. 5 $\mu\text{g/ml}$) to coat the assay wells resulted in a steeper binding curve which enabled accurate IAA determinations within a narrow concentration range whereas increased antibody concentration produced the opposite effect (results not presented).

An antibody concentration of 10 $\mu\text{g/ml}$ was found to be the most effective also for antibodies to IAA which conjugated to BSA via the carboxylterminus of the molecule (Weiler, 1981). Comparing the standard binding curves of both polyclonal antibodies and monoclonal antibodies shows that the sensitivity of the assay improves in the following order: monoclonal antibodies > Antibodies to IAA-C₁-BSA > antibodies to IAA-N-BSA (Fig. 1). By comparison of the standard curves it could be seen that the assay with antibodies to IAA-C₁-BSA is about 10 fold more sensitive than the assay with antibodies to IAA-N-BSA. Value for 50% inhibition of binding of IAA by the monoclonal antibodies and the antibodies to IAA-C₁-BSA is 3.2 pmol and 5.1 pmol methyl IAA respectively. In order to get the same inhibition for antibodies to

Fig. 1. Comparison of standard curves for IAA enzyme immunoassay using polyclonal antibodies raised against IAA-N-BSA (Δ) and polyclonal ($\bullet$) and monoclonal ($\circ$) antibodies raised against IAA-Cl-BSA, (A). Logit transformation of A, (B). Bars represent SD of triplicate standards. "B"= binding of IAA-AP or IAA-OVA-AP in the presence of methylated IAA or IAA, respectively. "Bo"= binding in the absence of antigen. Logit $B/B_0 = \ln[(B/B_0) - (100 - B/B_0)]$.

IAA-N-BSA. 46 pmol of IAA were required. The greater sensitivity of the assay with antibodies to IAA-C₁-BSA presumably results from higher affinity of the antibodies, and possibly because of the stability of the IAA methyl ester which is used in this assay (Weiler et al. 1981).

Assay specificity

Cross reactivity studies with indoles structurally related to IAA demonstrated that antibodies to IAA-N-BSA are in general more specific than antibodies to IAA-C₁-BSA (Tab. 1). These results confirm earlier observations (Pengelly and Meins 1977, Weiler 1981, Weiler et al. 1981). Antibodies to IAA-C₁-BSA exhibited cross-reactivity of about 6 to 12% with a number of indoles with some modifications in the side chain such as indole-3-butyric acid and indole-3-acetaldehyde. Other indoles and L-tryptophan were cross-reactive to a much lesser extent or not at all. Similar results were reported by Weiler (1981). Antibodies to IAA-N-BSA showed a relatively high cross-reactivity (ca. 8%) only with indole-3-pyruvic acid. IAA conjugates, e.g. IAAsp and IAGlu, which are present in plant tissues have been found to show less than 1% cross-reaction with antibodies to IAA (Pengelly and Meins 1977, Weiler 1981, Weiler et al. 1981). Nevertheless during purification these conjugates were removed from the extracts; IAGlu was not retained on the DEAE-Sephadex column and IAAsp did not elute from that column with 5% acetic acid used for elution of IAA. Other neutral indoles such as indole-3-acetaldehyde which may be present in plant tissues are also removed during purification of IAA.

Determination of IAA levels in citrus tissues

Tab. 1. Cross reactivities of anti-IAA antisera on a molar basis.

Compound	Cross Reactivity (%)	
	Antibodies to IAA-N-BSA	Antibodies to IAA-Cl-BSA
Indole-3-acetic acid	100	100
Indole-3-pyruvic acid	8.1	2.1
Indole-3-butyric acid	4.2	11.4
5-Hydroxyindole-3-acetic acid	2.1	0.4
Indole-3-aldehyde	0.9	0.9
Indole-3-acetamide	0.6	7.8
Indole-3-acetaldehyde	0.6	6.0
Indole	0.6	0
L-Tryptophan	0.2	0

Tab. 2. Endogenous IAA content in vegetative tissues of citrus, determined by EIA using different types of antibodies. Each value represents mean $\pm$ S.E. for 3-4 independent experiments, 1 gr. of plant tissue each.

Tissue	IAA content, ng (gr. FW)		
	Monoclonal antibodies to IAA-Cl-BSA	Polyclonal antibodies to IAA-Cl-BSA	Polyclonal antibodies to IAA-N-BSA
Shoot tips	405.6 $\pm$ 47.6	360.1 $\pm$ 51.8	380.5 $\pm$ 39.5
Very young leaves	-	-	394.8 $\pm$ 48.6
Leaf blad of young leaf	-	184.3 $\pm$ 44.9	248.0 $\pm$ 42.5
Midrib of young leaf	-	-	220.0 $\pm$ 29.5
Leaf blad of mature leaf	112.4 $\pm$ 28.8	54.2 $\pm$ 12.7	74.3 $\pm$ 16.2
Midrib of mature leaf	101.4 $\pm$ 26.3	62.4 $\pm$ 15.7	79.6 $\pm$ 18.4

The necessity of purifying plant extracts before analyzing IAA by immunoassays has been demonstrated (Sandberg et al. 1985, Pence and Caruso 1985, Riov et al. 1984). In the present work we employed a purification procedure which involved PVPP and DEAE-Sephadex column chromatography. The procedure is rapid and recovery of IAA in citrus extracts is 65-85%. We examined the efficiency of the purification procedure in removing substances which might interfere or cross-react with the antibodies. Since antibodies to IAA-N-BSA appear to have a relatively low affinity for IAA, the study was performed with polyclonal and monoclonal antibodies to IAA-C₁-BSA. Purified citrus leaf extracts were further chromatographed on a HPLC column and all fractions collected were analyzed for IAA (Fig. 2). It is evident that when IAA was assayed by polyclonal antibodies, the majority of the immunoreactive material co-chromatographed with authentic IAA methyl ester (Fig. 2). In contrast, monoclonal antibodies demonstrated considerable immuno reactivity with additional substances which did not correspond to the authentic IAA fraction. Similar results were obtained for polyclonal antibodies when extracts were analyzed by TLC (Fig. 3). The data clearly show that the purification procedure utilized is adequate for assaying IAA in citrus leaf tissues by polyclonal antibodies to IAA-C₁-BSA. The reason why monoclonal antibodies show less specificity is not yet clear. The presence of interfering compounds in plant extracts has been documented (Pengelly 1985). Interference may be caused by competitive inhibitors, or by non-specific interactions with substances which interfere with the immune reaction. The data indicates that for measuring IAA in citrus tissues, by monoclonal antibodies, an additional step of HPLC purification is required.

This is further demonstrated by comparing the levels of free IAA in

Fig. 2. Differential specificity of polyclonal and monoclonal antibodies raised against IAA-Cl-BSA. A, distribution of immunoreactive materials purified from citrus leaf extract (containing 10,000 dpm ^3H -IAA) on HPLC. Polyclonal antibodies, ($\square$); monoclonal antibodies, (Δ). B, distribution of radioactivity, (O); absorbance at 280 nm, (—). The fraction corresponding to cold standard IAA is designated by bar. HPLC (waters), stainless steel column (4 x 250 nm) packed with LICROSORB RP-8 was used. Starting eluent was acetonitrile/water (20:80, v/v) and programmed to 100% acetonitrile at 20 min. Flow rate was 12 ml/min at 22°C. Fractions of 0.6 ml were collected and analyzed for IAA.

Fig. 3. Distribution of radioactive (—) and immunoreactive (--) materials in purified citrus leaf extract on a thin layer chromatogram. The R_f region of IAA run on the same chromatogram is designated by a bar.

citrus tissues assayed by the different antibodies (Tab. 2). Values of IAA obtained with antibodies to IAA-C₁-BSA were usually lower than those estimated by antibodies to IAA-N-BSA. These findings are contrary to those reported by Pence and Caruso (1985) showing that anti IAA-N-BSA antibodies gave readings which averaged 30% less than those made with anti IAA-C₁-BSA antibodies. On the other hand, monoclonal antibodies to IAA-C₁-BSA showed higher values as compared to both polyclonal antibodies. In the growth tips which contained the highest amount of IAA the results obtained by all antibodies were similar (No significant difference at $P=0.05$). The differences in IAA levels in tissues containing a relatively low level of IAA could be explained by the difference in titers and affinities of both antibodies. By using antibodies to IAA-C₁-BSA it was possible to dilute plant samples 50-100 fold thereby significantly reducing the level of cross-reaction with these antibodies. This was not possible with antibodies to IAA-N-BSA which had a lower affinity and hence a higher detection limit for IAA (Fig. 1A). Under these conditions monoclonal antibodies also gave significantly higher values of IAA compared to polyclonal antibodies to IAA-C₁-BSA (Tab. 2). The reason for this is undoubtedly the lower specificity of these antibodies as demonstrated in Fig. 2. When assaying tissues which contained high amounts of IAA it was possible to dilute the extracts before analysis up to 100 fold, thus decreasing interference in the immunoassay. Therefore, all of the assay formats yielded similar values of IAA in shoot tips (Tab. 2).

A significant difference was found in the levels of endogenous IAA content during leaf development (Tab. 2). The highest IAA content (350-400 ng/g) was found in the growth tips and in the very young leaves. Goldschmidt et al. (1971) identified native auxin in citrus tissues. By using a bioassay, IAA level in growth tips was estimated to be 250 ng/g

(Goldschmidt 1976). During maturation extractable IAA content declined and levels of IAA in mature leaves were about one fifth of that in young leaves (Tab. 2). Quantitative data on free IAA levels in vegetative tissues are very limited and often based on bioassays. Similar to our observations relatively high levels of IAA have been reported to occur in young vegetative tissues, which later on declined during maturation (Roberts and Osborne 1981, Morgan unpublished data).

In view of these findings we conclude that antibodies to IAA-C₁-BSA enable an accurate determination of endogenous IAA in small samples of citrus tissues. When plant samples with higher levels of endogenous IAA are assayed, antibodies to IAA-N-BSA are also suitable for use in IAA determinations. Citrus tissues which contain high levels of IAA, can be assayed at high dilution and therefore can be assayed with the monoclonal antibodies with only 10-20 % cross-reaction. For citrus tissues with lower levels of IAA (below 60 ng/g), further HPLC purification is essential when using monoclonal antibodies.

Acknowledgements-The authors wish to thank Mrs. A. Arnscheidt and Mrs. V. Lachman for their technical assistance. This research was supported by a BARD grant (No. I-166-80) to R.G and J.R.

References

- Bandurski, R.S. 1980. Homeostatic control of concentrations of indole-3-acetic acid. -In: 10th inter. conf. on Plant Growth Substances. (F. Skoog, ed.), pp. 38-49, Springer Verlag, New York.
- Brenner, M.L. 1981. Modern methods for plant growth substance analysis. -Ann. Rev. Plant Physiol. 32:511-38.
- Clark, M.F. & Adams, A.N. 1977. Characteristics of microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. -J. Gen. Virol. 34:475-483.
- Fuchs, S. & Fuchs, Y. 1969. Immunological assay for plant hormones using specific antibodies to indoleacetic acid and gibberellic acid. -Biochim. Biophys. Acta 192:528-30.
- Goldschmidt, E.E. 1976. Endogenous growth substances of citrus tissues. -HortScience 11:95-99.
- Goldschmidt, E.E., Monselise, S.P. & Goren R. 1971. On the identification of native auxins in citrus tissues. -Canad. J. Bot. 49:241-245.
- Hirsch, A.M., Pengelly, W.L. & Torrey, J.G. 1982. Endogenous IAA levels in boron-deficient and control root tips of sunflower. -Bot. Gaz. 143:15-19.
- Mertens, R., Edberle, J., Arnscheidt, A. & Weiler, H.W. 1985. (In press).
- Morgan, P.W. & Durham, J.I. 1983. Strategies for extracting purifying and assaying auxins from plant tissues. -Bot. Gaz. 144:20-31.
- Oden, P.C., Weiler, E.W., Schwenen, L. & Graebe, J.E. 1985. Comparison of gas-chromatography-Mass spectrometry, radioimmunoassay and bioassay for the quantitation of gibberellin Ag in Norway spruce (Picea Abies). -12th Int. Confer. Plant Growth Sub. Heidelberg 73:18 (Abs.).
- Pence, V.C. & Caruso, J.L. Comparison of two enzyme-linked immunosorbent assays for indole-3-acetic acid. -12th Int. Confer. Plant Growth Sub. Heidelberg 51:68 (Abs.).
- Pengelly, W.L. 1985. Validation of immunoassays. -12th Int. Confer. Plant Growth Sub. Heidelberg 3:140 (Abs.).
- Pengelly, W.L., Bandurski, R.S. & Schulze, A. 1981. Validation of a radioimmunoassay for indole-3-acetic acid using gas chromatography-selected on monitoring mass spectrometry. -Plant Physiol. 68:96-98.
- Pengelly, W.L. & Meins, F. Jr. 1977. A specific radioimmunoassay for nanogram quantities of the auxin, indole-3-acetic acid. -Planta 136:173-180.
- Ranadive, N.S. & Senon, A.H. 1967. Antibodies to serotonin. -Can. J. Biochem. 45:1701-1710.

- Riov, J., Sagee, C. & Goren, R. 1984. Effect of ethylene on indole-3-acetic acid transport, metabolism, and level in leaf tissues of woody plants during abscission. -In Ethylene: Biochemical, Physiological and Applied Aspects. (Y. Fuchs and E. Chalutz, eds.), pp. 267-275, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague. ISBN 90-247-2984.
- Roberts, J.A. & Osborne, D.J. 1981. Auxin and the control of ethylene production during the development and senescence of leaves and fruits. -J. Exp. Bot. 32:875-887.
- Sandberg, G., Ljung, K. & Alm, P. 1985. Precision and accuracy of radioimmunoassay in the analysis of endogenous indole-3-acetic acid from needles of scots pine. -Physiol. Plant. 64:109 (Abs.).
- Schlenk, H. & Gellerman, J.L. 1960. Esterification of fatty acids with diazomethane on a small scale. -Anal. Chem. 32:1412-1414.
- Sinclair, W.R. 1984. The biochemistry and physiology of lemon and other citrus fruits. -University of California, pp. 946.
- Thompson, D.G., Morris, J.W., Morris, R.C. & Zaerr, J.B. 1981. Rapid isolation and quantitation of indole-3-acetic acid. -Plant Physiol. 67 (Suppl.):98.
- Wang, T.L. 1985. Immunoassays for plant growth regulators - a help or a hindrance? -12th Int. Confer. Plant Growth Sub. Heidelberg 2:140 (Abs.).
- Weigel, V., Horn, W. & Hock, B. 1984. Endogenous auxin levels in stem cuttings of Chrysanthemum morifolium during adventitious rooting. -Physiol. Plant. 61:422-428.
- Weiler, E.W. 1981. Radioimmunoassay for pmol-quantities of indole-3-acetic acid for use with highly stable ^{125}I - and ^3H IAA derivatives as radiotracers. -Planta 153:319-325.
- Weiler, E.W. 1983. Immunoassay of plant constituents. -Biochem. Soc. Trans. 11:485-495.
- Weiler, E.W. 1984. Immunoassay of plant growth regulators. -Ann. Rev. Plant Physiol. 35:85-95.
- Weiler, E.W., Jourdan, P.S. & Conrad, W. 1981. Levels of indole-3-acetic acid in intact and decapitated coleoptiles as determined by a specific and sensitive solid-phase enzyme immunoassay. -Planta 153:561-571.

TRACE LEVEL ANALYSIS OF INDOLE-3-ACETIC ACID FROM LIGHT-GROWN, HIGHLY
PIGMENTED LEAF TISSUE

V. G. Rivera/P. W. Morgan/R. D. Stipanovic

Abstract

A new chromatographic method has been developed for the isolation and quantitation of trace levels of the plant hormone, indole-3-acetic acid (IAA). The individual steps in the method were selected and adjusted specifically to remove the large amounts of interfering substances present in highly pigmented, light grown plant tissue. This method employs a rapid extraction and preparative cleanup by open column chromatography, followed by preparative reverse phase ion pair HPLC (RPIPC) to remove the bulk of the interfering substances. The RPIPC step maximizes the resolution of the IAA from its native matrix by interaction with the tetrabutylammonium acetate ion pair at the ideal pH for the quantitative formation of the TBA-IAA ion pair. Two additional HPLC separations ultimately produce a baseline resolved IAA peak, which is quantitated by fluorescence and amperometric detection. Identity of the IAA peak was verified by capillary GLC with N/P detection and by GC-MS analysis. The method is illustrated here by the quantitation of IAA from 1 g fresh weight of highly-pigmented, light grown cotton leaf tissue. The limit of detection is as low as 1 ng IAA from 1 g fresh weight with average recoveries of not less than 50% at the quantitation step. Although the method was designed to quantitate IAA, the extraction and HPLC steps can be used to prepare purified IAA samples for esterification and GC-MS analysis.

Introduction

The analysis of plant hormones by physicochemical methods involves special problems because the active compounds are present in trace amounts in a highly complex matrix, and they are often chemically labile (1,2,3). The methods of choice are GC-MS and HPLC (1,4). The mass spectrometer offers advantages in the accuracy of identification and quantitation, while HPLC offers advantages in cost effectiveness and speed. Recent advances in column materials (5,6,7) and detectors (8,9,10,11,12) have added to the versatility of HPLC for both the purification and the analysis of plant hormones.

Despite the rapid evolution of analytical technology, the analysis of plant hormones, and particularly the growth promoting substance indole-3-acetic acid, remains difficult. Surveys of the present literature in the plant hormone field (1,2,3,4,13,14) reveal that most of the data are still obtained from tissues that have relatively low levels of interfering compounds, such as seeds and etiolated tissue. Thus the inherent problems associated with the analysis of low levels of IAA in the presence of high levels of pigments and other interfering compounds found in light-grown plants are avoided. The method described in this paper has allowed us to remove the high levels of interfering compounds found in leaves of light-grown cotton plants leaving an IAA peak pure enough to be quantitated by paired fluorescence and amperometric detectors. The identity of the IAA peak was confirmed by GLC and GC-MS.

Experimental

Materials

All solvents were HPLC grade. Water was type I water oxidized by ultraviolet irradiation to remove the last traces of organic contaminants. BHT was used as antioxidant. ^3H -IAA was used as radiotracer.

Precautions

During the extraction and subsequent analysis, all samples and fractions were protected from overhead fluorescent lights. BHT was replaced after every separation to inhibit oxidation. All glassware was silylated and clean of contaminants.

HPLC Apparatus

The HPLC system consisted of: two Waters model 6000A pumps, WISP model 710 autosampler, Waters system controller and data module, ISCO fraction collector with an Angar isolation valve (P/N 368-3-12), Perkin-Elmer LS-4 fluorescence spectrometer, Waters model 440 UV monitor, and a Bioanalytical Systems model LC-4B amperometric detector.

Scintillation Counting

Recovery of internal standard was monitored with a Beckman LS-1801 liquid scintillation counter. Aliquots of 0.1 ml were counted and corrected for quenching with curves developed from plant extracts.

Capillary GLC and GC-MS Apparatus

The capillary GLC instrument was a Varian Model 3400 with the capillary on-column cryogenically cooled injector, electron capture detector, and N/P detector.

The GC-MS instrument consisted of a Varian model 6000 capillary GC interfaced to a V.G. Industries model 70-70 double focusing mass spectrometer.

Extraction

Tissue samples to be studied are harvested, immediately frozen in liquid nitrogen, and ground to a fine powder in liquid nitrogen with a mortar and pestle. One gram (fresh weight) samples are placed in cooled, pre-tared test tubes and stored at -70°C until extracted.

Our extraction procedure is similar to that developed by Thompson et al. (15) and employed by Law and Hamilton (12) and by Riov, Goren and Sagee (personal communication, 1984). One gram samples are suspended in 5 ml of 80% MeOH/H₂O, 0.1 M NH₄OAc, 10 µg/ml BHT; an appropriate amount of radioactive tracer is added, and the mixture is homogenized with a Brinkmann (7 mm diameter probe) Polytron at full speed for 30 sec in an ice bath. Each homogenized sample is refrigerated in the dark (30 min), centrifuged, and the supernatant is saved. The pellet is resuspended in 5 ml of water, centrifuged, and the supernatant is pooled with the supernatant from the initial centrifugation.

Preparative Cleanup

The highly pigmented extracts are subjected to three open-column liquid chromatography steps, yielding virtually colorless, partially purified extracts. Location and recovery of the IAA is monitored by the radioactive internal standard.

As described by Thompson et al. (15), the extracts are loaded on a polyvinylpyrrolidone (PVP) column developed with 0.01 M NH_4OAc . The active fraction is placed directly on a DEAE Sephadex (A-25-120) column developed with 0.01 M NH_4OAc . The active fraction is eluted from this column with 1 M AcOH and applied to a Sep-pak (Waters Associates) prewashed with MeOH . The excess acetic acid is eliminated by washing with 5 ml water, and the active fraction is eluted with 5 ml HPLC grade methanol. This fraction is brought to dryness with a Buchler Evaporonix test tube evaporator and later is dissolved in 4 ml of 5% $\text{MeOH}/\text{H}_2\text{O}$, 0.1% BHT for subsequent HPLC analysis.

HPLC Analysis

Two ml aliquots of the samples are first purified on a C-8 column (3 μm , Dupont Golden Series, 6.2 mm x 8.0 cm, with a 5 μm Supelguard LC-8, 4.6 mm x 2.0 cm precolumn) by preparative reverse phase ion pair chromatography (RPIPC) using the tetrabutylammonium cation (TBA). A gradient, as shown in Fig. 1, is employed with H_2O and 90% $\text{MeOH}/\text{H}_2\text{O}$ each containing 100 mM TBA, 100 mM TRIS and adjusted to pH 8.0 with acetic acid (flow rate = 1.0 ml/min). The tetrabutylammonium acetate ion pair is formed in situ in each solvent by titrating tetrabutylammonium hydroxide with AcOH . The eluent is monitored by UV detection at 254 nm and by fluorescence

Fig. 1 Chromatogram of plant extract analyzed by RPIPC on a Dupont Golden Series 3 μ m C-8 column. Retention time for TBA-IAA was 31.8 min. UV at 254 nm x 0.2 AU full scale, FD at Ex²⁸⁰/Em³⁶² x 1 full scale. Dotted line is the gradient profile; full scale indicated by maximum traces at upper right. HPLC conditions described in text.

detection at $\text{Ex}^{280}/\text{Em}^{362}$. The TBA-IAA ion pair elutes at 31.8 min and is collected and reduced to 1 ml with the test tube evaporator. If samples are stored between steps the BHT is replaced, capped vials are covered with foil and placed in a 0° freezer.

A 0.9 ml aliquot of the TBA-IAA fraction is rechromatographed on a C-8 column (3 μm Supelcosil LC-8 4.6 mm x 15.0 cm, with a 5 μm Supelguard LC-8 precolumn, 4.6 mm x 2.0 cm). The gradient shown in Fig. 2 is employed with H_2O and 50% $\text{MeOH}/\text{H}_2\text{O}$ each adjusted to pH 2.1 with H_3PO_4 (flow rate = 0.7 ml/min). The eluent is monitored as above showing a retention time for IAA of 22.1 min. The IAA fraction is collected and reduced to 1 ml with the test tube evaporator.

A 0.9 ml aliquot of the IAA fraction is rechromatographed on a C-18 column (3 μm Supelcosil LC-18, 4.6 mm x 15.0 cm, with a 5 μm Supelguard LC-18 precolumn, 4.6 mm x 2.0 cm). The mobile phase is 22% Acetonitrile/Water (u/v) with the pH adjusted to 3.0 with AcOH . The flow rate is 1.0 ml/min. IAA quantitated at this step by fluorescence (as above) and by amperometric detection (0.95V with a glassy carbon electrode) has a retention time of 11.67 min (Fig.3).

Quantitation

The baseline resolved peaks obtained from the last HPLC step are quantitated against authentic standards and normalized to reflect the total sample composition. This is done by taking into account the percentage of the recovered radioactivity, the amount of labeled IAA present at the quantitation step, and all the dilution factors.

Methylation

Fig. 2 Chromatogram of IAA containing fraction collected from RPIPC step (see Fig. 1) on a 3 μ m Supelcosil LC-8 column. Retention time for IAA was 22.1 min. UV at 254 nm x 0.1 AU full scale, FD at Ex²⁸⁰/Em³⁶² x 1 full scale. Dotted line is the gradient profile; major UV peak reaches full scale. HPLC conditions described in text.

Fig. 3 Quantitation step for IAA. Chromatogram of IAA containing fraction collected from second HPLC step (see Fig. 2) on 3 μ m Supelcosil LC-18 column. Retention time for IAA was 11.67 min. Detector sensitivities for full scale set at 200 nA (AD), 0.005 AU (UV) and $\times 5$ (FD). HPLC conditions described in text.

The pure endogenous IAA fractions obtained from the HPLC analysis were partitioned three times against ethyl ether and methylated by the dropwise addition of freshly generated diazomethane in ether (19). With frequent stirring the reaction usually was complete after 20 min at 30°C. An aliquot was taken for liquid scintillation counting (LSC), the fractions were brought to dryness and dissolved in 50% MeOH/H₂O for subsequent HPLC purification. This was done by gradient elution from 50% MeOH/H₂O to 100% MeOH in 5 min with a linear program on the C-18 column used in step 3 (Fig. 3). The purified MeIAA was collected, an aliquot was taken for LSC, and the methylation yield was calculated based on the amount of radioactivity recovered. Each sample was brought to dryness under an N₂ stream and resuspended in hexane (Burdick & Jackson) for capillary GLC and GC-MS analysis.

Capillary GLC and GC-MS

The MeIAA was subjected to capillary GLC on an SGE BP-10 Bonded Phase cyanopropyl silicone capillary column, 0.33 mm ID x 30 m, 1.0 μ m film thickness. The column flowrate was 1.2 ml/min of He, and the makeup flowrate was 28.8 ml/min He. A thermionic specific detector for nitrogen and phosphorous was used at 280°C. Injection (2 L) was isothermal at 190°C. The oven program was: initial at 100°C (hold for 10 min), increase at 5°C/min up to 240°C, then hold for 10 min. The retention time for MeIAA was 38.7 min (Fig. 4).

The same column/program combination was used for the GC-MS analysis, which gave only one total ion peak at 38.7 min, which had the appropriate fragmentation pattern for authentic MeIAA, showing a parent peak at 189 and a base peak at 130 m/z (Fig. 5).

Fig. 4 Chromatogram of capillary GLC analysis of MeIAA from reagent (standard) or plant (endogenous) source. The retention time observed was 38.7 min. See text for conditions.

Fig. 5 GC-MS trace of fragmentation pattern of MeIAA, reagent standard (above) and plant sample (below). See text for conditions.

Results and Discussion

After preparative cleanup by open-column chromatography the extracts are subjected to three HPLC separations at the end of which the total baseline resolution of IAA is accomplished for FD and UV detection, Fig. 3. The bulk of this purification is done in the first HPLC step by the use of RPIPC. In this step the tetrabutylammonium acetate ion pair is formed in situ by mixing the tetrabutylammonium hydroxide reagent with an appropriate amount of acetic acid in the mobile phase. Acetic acid is used since it has approximately the same pKa as IAA, therefore assuring quantitative TBA-IAA ion pair formation, on-column retention and subsequent separation by programmed gradient elution. The separation of TBA-IAA from an extract of cotton leaf tissue is shown in Fig. 1. TBA-IAA elutes at 31.8 min followed by the bulk of the remaining material after 40.0 min. We estimate that about 90% of the remaining interfering material is eliminated at this step. The TBA-IAA-containing fraction is collected and reduced in volume for the next HPLC step.

The second HPLC step is designed to dissociate the TBA-IAA ion pair upon injection into a second reverse phase C-8 column running at a low pH at which IAA is not ionized. This can be accomplished quantitatively only if the acid chosen in this mobile phase has a lower pKa than that of IAA. For that reason we chose to use H_3PO_4 which has a pK_{a1} around 2.1; this allows the H_2PO_4^- to displace the IAA from its ion pair, protonating it and retaining it in the column for subsequent elution by gradient programming. A typical separation is shown in Fig. 2; the new TBA- H_2PO_4^- ion pair

elutes soon after the void volume, followed by the elution of a series of protonated acids. IAA elutes around 22.1 min and is collected, reduced in volume and subjected to a third HPLC separation.

The last HPLC step serves a dual purpose. First, it separates the IAA from the last traces of contaminants, yielding a baseline resolved peak in two of the three detectors. Second, since it is an isocratic separation, it helps to achieve a more accurate quantitation by providing gaussian shaped peaks at different concentration ranges. Also, it optimizes the fluorescence detection (FD) by keeping the solvent conditions constant, allowing the detector to be set at the maximum excitation and emission wavelengths at that particular solvent composition. Furthermore, it allows the use of amperometric detection (AD) downstream of the previous detector. With the present technology amperometric detection can be used only under isocratic conditions. Our recovery of the radioactive internal standard is routinely above 50% at this third HPLC step.

The excitation and emission wave lengths ($\text{Ex}^{280}/\text{Em}^{362}$), employed are the maxima for IAA under these conditions and when supplied as narrow bands give a degree of specificity to the FD. The AD is operated at 0.95 V, the optimum voltage for IAA. By using dual detection we can use detector response ratios as a qualitative tool. If the IAA peak did happen to be contaminated at this stage, it is highly unlikely that the contribution of the contaminant to the fluorescence signal at the chosen wavelengths would equal its contribution to the signal in the amperometric detector at the selected potential. Law et al. (16) have shown that AD sensitivity

for IAA and contaminating substances is not parallel with voltage and varies considerably at selected voltages. Therefore, the detector response ratio comparison becomes an effective tool to verify the purity of the IAA peak (Table 1).

Table 1 shows an example of data from cotton leaf tissue; a detector response ratio of 1.002 was obtained for samples containing 38 ng/g fr. wt., while a ratio of 0.817 was obtained for samples in the 7 to 9 ng/g fr. wt. range. We usually observe a sloping base line with the amperometric detector (Fig. 3) which makes quantitation of the peak less accurate, especially with low amounts of IAA. This error accounts for the detector ratio deviation shown in Table 1; reagent IAA samples give linear responses and detector ratios of 1 over the range from 1 ng to several hundred ng per sample. Because of this situation, extreme sample purity and careful chromatography of the quantitation step is especially desirable for samples with low amounts of IAA (1 to 2 ng/sample as for intermediate leaves, Table 1). Therefore, as a quality control standard we have adopted a minimum response ratio of 0.8 for samples containing small amounts of IAA. This criteria, plus co-chromatography authentic standards in all three HPLC steps, plus confirmation by GC-NP detection and GC-MS analysis, demonstrates that a high degree of confidence can be placed in our qualitative and quantitative analysis of IAA.

This method has several advantages: first, the high level of confidence for the correct identification and quantitation after extensive HPLC purification allows for the analysis of IAA without having to perform a GC-MS confirmation for each sample, therefore, allowing scientists without routine access to GC-MS instrumentation to accurately and confidently analyze IAA from plant tissue. This

Table 1. Levels of IAA detected by fluorescence and amperometric detectors from 1 g fr. wt. samples leaf blades of 38-day-old cotton plants.^{a/}

	F.D.	A.D.	RATIO
<u>TISSUE</u>	<u>IAA (ng/g)</u>	<u>IAA (ng/g)</u>	<u>F.D./A.D.</u>
youngest leaf, partially unfolded	38.02	37.94	1.002
intermediate leaf, fully expanded	7.46	9.13	0.82

^{a/} Actual amounts quantitated by the detectors can be calculated by dividing data by 4.4 for this experiment.

statement assumes an initial unequivocal identification of the IAA by MS and sufficient chromatographic purity to allow use of the FD/AD detector response ratio to verify the purity of the putative IAA peak (Fig. 3). Secondly, this method maximizes the resolving power of HPLC to remove the high levels of interfering compounds found in extracts of leaves and other highly pigmented plant organs. Even if GC-MS is used to quantitate IAA, the present HPLC procedures represent an effective means to clean up highly complex extracts so as to allow analysis of uncontaminated MeIAA in the MS detector.

Using the automated HPLC system described, 12 to 20 samples can be purified and assayed in a 3 to 4 day cycle. Extraction and the initial open-column chromatography clean up requires one person about 1 day for 8 samples; thus, the procedure could handle roughly 16 samples per week. Chromatographic conditions, flow rates and steepness of gradients, have been chosen to optimize chromatography, and in our hands changes to speed up the method resulted in less pure samples and greater losses.

RPIPC has been employed previously (17,18) for this type of analysis, but in these previous cases the ion pair used is the tetrabutylammonium phosphate, which in our experience is less efficient in the quantitative ion pair formation with IAA than the tetrabutylammonium acetate. Also, quantitation has been done by measuring the peak intensity of the TBA-IAA ion pair as it is eluted by gradient programming (18); however, this approach is more subject to error for reasons mentioned before.

REFERENCES

- [1] M. L. Brenner, *Ann. Rev. Plant Physiol.* 32, 511 (1981).
- [2] R. Horgan, *Phytochem.* 7, 137 (1981).
- [3] D. R. Reeve, A. Crozier, in J. MacMillan, ed., *Molecular and Sub-cellular Aspects of Hormonal Regulation in Plants*, Springer, Berlin Vol. 1, page 1 (1980).
- [4] P. W. Morgan, J. I. Durham, *Bot. Gaz.* 144, 20 (1983).
- [5] R. Mayors, *J. Chromatog.* 18, 488 (1980).
- [6] A. Goldberg, *Anal. Chem.* 54, 342 (1982).
- [7] F. Rabel, *J. Chromatog.* 18, 394 (1980).
- [8] P. B. Sweetser, D. G. Swartzfager, *Plant Physiol.* 61, 254 (1978).
- [9] A. Crozier, P. K. Loferski, J. B. Zaerr, R. O. Morris, *Planta* 150, 366 (1980).
- [10] G. Sandberg, A. Dunberg, *Physiol. Plant.* 55:315 (1982).
- [11] D. Richards, *J. Chromatog.* 175:293 (1975).
- [12] D. Law, R. Hamilton, *Bioch. and Biophys. Res. Comm.* 106(3), 1035 (1982).
- [13] J. D. Cohen and R. S. Bandurski, *Ann. Rev. Plant Physiol.* 33, 403 (1982).
- [14] E. A. Schneider, F. Wightman. In D. S. Letham, P. B. Goodwin, T. J. V. Higgins, etc., *Phytohormones and Related Compounds: A Comprehensive Treatise*, Elsevier/North-Holland, vol. 1, page 29 (1978).
- [15] D. G. Thompson, J. W. Morris, R. O. Morris, J. B. Zaerr, *Plant Physiol.* 67(Supplement), 98 (1981).

- [16] D. M. Law, D. V. Basile, M. R. Basile, *Plant Physiol.* 77, 926 (1985).
- [17] D. Mousdale, *J. Chromatog.* 209, 489 (1981).
- [18] E. Tillberg, *Plant Physiol.* 76, 84 (1984).
- [19] V. Magnus, R. Bandurski, A. Schulze. *Plant Physiol.* 66, 775 (1980).

SECTION E

Levels of Indole-3-Acetic Acid in Leaves of Gossypium hirsutum L.

Page W. Morgan, Victor Rivera, James I. Durham and Robert Stipanovic

Soon after the discovery of auxin it was recognized to prevent the abscission of debladed petioles (1,2). Although a multiple hormone concept of abscission control has developed more recently (3,4,5,6,7), IAA is still accorded a major role in regulating this process. Hypothetically, the abscission-preventing substances, predominately IAA, decline with leaf age, and the senescence- and abscission-promoting substances, ethylene, and possibly ABA, increase (6,7.) At some time the hormone balance is shifted to the point that abscission is initiated.

The accepted decline in auxin levels with leaf age is based on data from surprisingly few studies. Bioassays first revealed that extractable and diffusible auxin levels declined in leaves with age (8,9). More recently, the α -pyrone fluorescence reaction for IAA was used to determine that IAA levels declined with leaf age in two species and remained the same in a third (10). On the other hand, there are scattered reports that auxin levels increase in leaves during senescence (11,12,13,14,15,16). In addition, since auxin promotes ethylene production (17), and ethylene production rates are often relatively high in young, auxin-rich tissue (18,19), some have concluded that the ethylene production rate of a tissue predicts its auxin content (20,21). Since there is evidence for increasing rates of ethylene release during leaf senescence and abscission (19,22,23), it might follow that

senescing leaves could be expected to contain high levels of auxin. The dilemma this interpretation holds for the hormone balance hypothesis of abscission is obvious. It seems desirable to confirm or clarify the trends in IAA levels in leaves using the most reliable physicochemical methods currently available.

We report here data on the IAA levels in leaves of cotton (Gossypium hirsutum L.), a species whose abscission behavior and ethylene physiology have been extensively studied. The isolation and assay methods were developed specifically for light-grown plants rich in pigments and other interfering substances (24).

MATERIALS AND METHODS

Plant Culture and Sampling

Cotton plants (Gossypium hirsutum L. cultivar Stoneville 825) were grown in a controlled environment room with a combination of cool white fluorescent and incandescent light sources ($250-300 \mu\text{E M}^{-2} \text{ sec}^{-1}$ at plant level, Lambda LI-185 quantum photometer) which operated with 12 h photoperiod (30°C) and 21°C during darkness. Plants were cultured in 1-gal pots in a rooting media of peat moss, perlite and vermiculite (2:1:1 by volume) which was supplemented with 225g Osmotcote (14-14-14 slow release fertilizer, Sierra Chemical Co., Milpitas, CA.), dolomite, 70g superphosphate, 195g gypsum, trace elements and fertilized weekly with soluble fertilizer (Peters Peat-Lite Special 15-16-17, Peters Fertilizer Products, W. R. Grace and Company, Fogelsville, PA).

Plants were harvested at 38 days; there were 7 true leaves; the youngest fully opened leaf was 14 to 25 mm wide. Numbering from the bottom of the plant, leaves 1, 4 and 6 plus 7 (combined) were harvested, bulked leaf blades from many plants were ground to a powder with a mortar and pestle with liquid N_2 , 1 g samples were weighed into tared, prechilled 30 ml test tubes which were quickly placed in a -70°C freezer until extracted.

Samples were homogenized in a Polytron (Brinkmann) with 5 ml 80% methanol / H_2O , 0.1 M NH_4O acetate, 10 $\mu\text{g/ml}$ BHT and internal standard IAA- C^{14} or H^3 as described previously (24).

Initial purification (24) was adapted from Thompson et al. (25); samples were passed successively through small columns of PVP and DEAE Sephadex into a C-18 Sep-pak (Waters Associates) from which IAA was eluted with HPLC methanol.

HPLC Purification. The HPLC purification involves three steps (24). In the first step ion-pair chromatography is performed with tetrabutyl ammonium acetate (TBA) reagent and the TBA-IAA ion pair is eluted separately from over 90% of the dry material in the sample. The ion pair is separated on the next column, TBA-phosphate eluted followed by the protonated IAA. Both of these steps require solvent gradients (24). The final step is isocratic chromatography on a C-18 column in 22% acetonitrile/H₂O; the IAA peak is baseline resolved from any contaminant as it passes through successively a UV detector at 254nm, a fluorescence detector at 280nm excitation and 362nm emission (5nm band widths), and an amperometric detector at 0.95 V. Each detector condition optimizes sensitivity to IAA, but the detector response ratio to IAA is much different for the latter two detectors.

Confirmation of Identity. That the method measured only IAA was indicated by the HPLC data (see Results). To further confirm that the putative IAA from plant samples was IAA, we prepared the methyl ester of IAA with diazomethane, re-chromatographed the esterified IAA on the third HPLC step, collected that fraction, and subjected it to capillary column GC with N/P detection (24). We then subjected the esterified -IAA fraction to GC-MS.

RESULTS AND DISCUSSION

The value of this study rests upon the absolute accuracy of the data, that the analytical procedure quantitates IAA and nothing else. The analytical procedure and the identity of the IAA were verified by (a) identical retention time of putative plant IAA and standard IAA at the third HPLC step, (b) co-chromatography of the internal standard ¹⁴C-

Table I. Analysis of putative IAA levels in cotton leaf samples by fluorescence (FD) and amperometric (AD) detectors.

Tissue	Rep	FD	AD	Ratio/FD/AD
IAA, ng/g				
Leaves 6 and 7				
(youngest, partially				
unfolded)				
	A	39.02	37.19	1.05
	B	39.02	37.94	1.00
Leaf 4				
(intermediate, fully				
expanded)				
	A	7.46	9.22	0.81
	B	7.05	9.13	0.77

or ^3H IAA with the putative IAA at the third HPLC step, (c) detection by three different HPLC detectors optimized for IAA and using different principles of detection. We then esterified the putative the putative IAA and standard IAA; both produced methyl esters which co-chromatographed as single peaks with ^{14}C - or ^3H -labeled internal standard or HPLC and capillary GC with N/P detection (24). Subsequently, these preparations produced identical mass fragmentation patterns, further confirming that the putative IAA was indeed IAA (24).

The quantitative agreement of the fluorescence and amperometric detectors is illustrated in Table 1. At higher IAA levels, the detector response ratio was essentially 1; for the lower levels of IAA the amperometric detector overestimated the fluorescence detector so that the ratio was approximately 0.8. The ng/g values are corrected for recovery of the internal standard plus removal of aliquots for counting, transfers and others operations; thus, in the young leaf sample the FD saw only an average of 14.42 ng IAA, and in the intermediate leaf sample the FD saw only an average of 2.74 ng IAA. The failures of the detector response ratio to equal 1 with the intermediate aged leaves (Table 1) probably reflects inaccuracies in integration of the small IAA peak from the amperometric detector rather than from the presence of another compound in the peak. It is highly unlikely that the IAA peak could be significantly contaminated and produce a detector response ratio of 1 or close to 1 because the fluorescence detector limits the excitation energy given and the emission energy sensed to narrow, 5nm bands at the specific maximum for IAA. Compounds that require excitation at other wavelengths or emit light at different wave lengths will not be seen by the fluorescence detector at these conditions. Similarly, the

amperometric detector gives very different detector responses for different compounds and the detector response is very different at different conditions such as the operating voltage. Also, as the detector voltage changes, the detector response to different compounds is not parallel. Thus, the likelihood that a contaminant of the putative IAA peak could produce the same or similar signal for both the fluorescence and amperometric detector appears so small that the quantitative agreement in Table 1 represents a form of verification of IAA peak identity and purity.

The complementary interplay of the three HPLC detectors is illustrated by a typical plant sample analysis: the fluorescence detector saw only one significant peak at retention time (RT) 11.67 min, the UV detector sensed several intermediate sized peaks prior to the 11.67 min RT IAA peak and a large peak immediately after it. Although this large peak absorbed UV at 280nm, it neither fluoresced nor ionized at 0.95 V in the amperometric detector; the amperometric detector, third in sequence, produced an IAA peak at RT 11.68 min, several large peaks preceded the IAA but none followed it. Under these conditions only the IAA peak appeared, baseline resolved, in all three detectors, and it was the only significant peak in the fluorescence detector (data not given, see Fig. 3 in Ref. 24). While some contaminants remain in the IAA fraction, based on Table 1 and the GC and GC-MS data (24), none of them co-chromatograph with the IAA peak and none of them fluoresce. Thus the evidence verifies that the procedure purifies and assays IAA accurately. The data in Table 1 were obtained, and then later the GC and GC-MS verifications were accomplished. The amperometric detector is partially destructive; therefore, quite a few samples were processed

through the fluorescence detector without passing through the amperometric detector in order to accumulate IAA for esterification for GC and GC-MS (Table 2).

The levels of IAA declined with age or from apex to base on a fresh weight basis (Table 2). The youngest opened or unfolded but only partially expanded leaves had the highest level of IAA, about 35 ng g^{-1} fr. wt. The first fully expanded, non-growing or slowly growing leaf, number 4 from the base of the plant (the youngest leaves were numbers 1 to 7), contained only about 8 ng g^{-1} fr. wt. The level of IAA appeared to plateau or level off in the older tissue because we observed about 8 or ng g^{-1} fr. wt. in the oldest leaf and about 5.4 ng g^{-1} fr. wt. in the cotyledons. The cotyledons and some of the oldest leaves had begun to senesce before the tissue was collected. It is not clear whether the IAA levels decline to a relatively low level after which ethylene induces leaf abscission or whether individual leaves drop in auxin content rather drastically after which existing ethylene levels initiate abscission. A treatment which induces rapid abscission might reveal whether auxin levels drop significantly.

Table 2. Levels of IAA in cotton leaf blades of different ages and stages of development.

Tissue	Avg. % of Recovery	ng/g IAA, FD
Top leaves <u>1</u> / youngest leaves, opened, partially expanded	42.49%	34.98 $\pm$ 3.75
Intermediate Leaf <u>2</u> / fully expanded, no. 4 from bottom	32.43%	7.67 $\pm$ 1.42
Oldest leaf, <u>3</u> / fully expanded, no. 1 from bottom	37.6%	7.85 $\pm$ 1.56
Cotyledons <u>4</u> /	42.51%	5.40 $\pm$

1/ Average of separate determinations of six 1 gm samples extracted as pairs on three different dates.

2/ Average of separate determinations of four 1 gm samples extracted as pairs on two different dates.

3/ Average of separate determinations of five 1 gm samples extracted on three different dates.

4/ Average of separate determinations of four 1 gm samples extracted on three different dates.

LITERATURE CITED

1. Mai, G. 1934. Korrelationsuntersuchungen an entspreiteten Blattstielen mittels lebender Orchideenpollinien also Wuchsstoffquelle. Jahrb. Wiss. Bot. 79: 681-713.
2. La Rue, C. D. 1936. The effect of auxin on the abscission of petioles. Proc. Natl. Acad. Sci. U.S.A. 22: 254-259.
3. Hall, W. C. 1952. Evidence on the auxin-ethylene balance hypothesis of foliar abscission. Bot. Gaz. 713: 310-322.
4. Abeles, F. B. 1968. Role of RNA and protein synthesis in abscission. Plant Physiol. 43: 1577-1586.
5. Beyer, E. M., Jr. and P. W. Morgan. 1971. Abscission: The role of ethylene modification of auxin transport. Plant Physiol. 48: 208-212.
6. Morgan, P. W. 1983. Chemical manipulation of abscission and desiccation. In: J. L. Hilton, ed., Agricultural Chemicals of the Future. Rowman and Allanheld, Publ., Totowa, N.J. pp 61-74.
7. Morgan, P. W. 1984. Is ethylene the natural regulator of abscission? In: Y. Fuchs and E. Chalutz, eds, Ethylene: Biochemical, Physiological and Applied Aspects, Martinus Nijhoff/Dr. W. Junk, Publ., The Hague. pp 231-240.
8. Shoji, K., F. T. Addicott, and W. A. Swets. 1951. Auxin in relation to leaf blade abscission. Plant Physiol. 26: 189-191.
9. Wetmore, R. H. and W. P. Jacobs. 1953. Studies on abscission: the inhibiting effect of auxin. Am. J. Bot. 40: 272-276.

10. Roberts, J. A. and D. J. Osborne. Auxin and the control of ethylene production during the development and senescence of leaves and fruits. *J. Expt. Bot.* 32: 875-887.
11. Wheeler, A. W. 1968. Changes in auxins in expanding and senescent primary leaves of dwarf french bean (*Phaseolus vulgaris*). *J. Expt. Bot.* 19: 102-107.
12. Dorffling, K. 1983. Über das wuchsstoff-Hemmstoffsystem von *Acer pseudoplatanus* L. i. Der Jahresgang der Wuchs-und Hemmstoffe in Knospen, Blatten und im Kambium. *Planta* 60: 390-412.
13. Sheldrake, A. R. and D. H. Northcote. 1968. Production of auxin by detached leaves. *Nature* 217: 195.
14. Atsumi, S. and T. Hayashi. 1979. Examination of the pronounced increase in auxin content of senescent leaves. *Pl. Cell Physiol.* 20: 861-865.
15. Kaska, N. 1970. Changes in the levels of growth promoters and inhibitors in apple leaves, buds, fruits and seeds during the growing period. *Univ. of Ankara, Yb. Fac. Agric.* 1969, 67-83.
16. Kaska, N. 1972. Fluctuations of growth substances in different stages of leaves, buds, fruits and seeds of sour cherry. In, H. Kaldewey and Y. Vardar, eds, *Hormonal Regulation in Plant Growth and Development*. Verlag Chemie, Weinheim, pp. 431-438.
17. Morgan, P. W. and W. C. Hall. 1964. Accelerated release of ethylene by cotton following application of indolyl-3-acetic acid. *Nature* 201: 99.
18. Burg, S. P. and E. A. Burgy. 1968. Ethylene production in pea seedlings; its relation to the inhibition of bud growth caused by indole-3-acetic acid. *Plant Physiol.* 43: 1069-1074.

19. Jackson, M. B. and D. J. Osborne. 1970. Ethylene, the natural regulator of leaf abscission. *Nature* 225: 1019-1022.
20. Burg, S. P. 19068. Ethylene, senescence, and abscission. *Plant Physiol.* 43: 1503-1511.
21. Abeles, F. B. 1973. *Ethylene in Plant Biology*. Academic Press, New York. 302 pp.
22. Beyer, E. M., Jr. and P. W. Morgan. 1971. Abscission: The role of ethylene modification of auxin transport. *Plant Physiol.* 48: 208-212.
23. Morgan, P. W. and J. I. Durham. 1980. Ethylene production and leaflet abscission in Melia azedarach L. *Plant Physiol.* 66: 8-92.
24. Rivera, V. G., P. W. Morgan, R. D. Stipanovic. 1985. Trace analysis of indole-3-acetic acid from light-grown, highly pigmented leaf tissue. *J. Chromatography* (to be submitted).
25. Thompson, D. G., J. W. Morris, R. O. Morris, J. B. Zaerr. 1981. rapid isolation and quantitation of indole-3-acetic acid. *Plant Physiol* (Supplement) 67: 98.

SECTION F

LEVELS OF INDOLE-3-ACETIC ACID IN CITRUS LEAVES DURING ETHYLENE-INDUCED
ABSCISSION AND NATURAL ABSCISSION.

ODED SAGEE, JOSEPH RIOV AND RAPHAEL GOREN

The ability of exogenously supplied ethylene to promote abscission of plant organs is a well-known phenomenon. Beyer and Morgan (6) proposed a model for the role of ethylene in the regulation of abscission of intact leaves. The proposed regulatory system first involves a modification of the hormonal balance in the abscission zone achieved as ethylene reduces indole-3-acetic acid (IAA) transport capacity of the petioles. After the reduction of auxin levels, ethylene exerts direct action in the abscission zone such as stimulating synthesis of cell-wall degrading enzymes (1,3) and secretion of these enzymes into the cell-wall (2). Also, ethylene has been demonstrated to reduce endogenous IAA level (5,10,13). Beyer (5) demonstrated that the leaf blade is the initial target tissue of exogenously supplied ethylene, where some essential function of the hormone must first be performed before abscission can occur. There are data which indicate that this essential function of ethylene is to reduce the amount of auxin transported out of the blade, possibly by reducing auxin levels and inhibiting auxin transport in the veinal tissues (5).

Conjugated forms of IAA are thought to be an important mechanism for regulating the level of free IAA in plant tissues (7). We have clearly demonstrated that IAA conjugation increased significantly during ethylene-induced abscission (25 and Section A) and during natural abscission (Section B). The development and the adaptation of immunoassays for

measuring IAA levels in citrus leaf tissues (Section C), enabled us to examine whether the increased IAA conjugation results in a reduction of IAA levels before abscission occurs. We report here data on IAA levels in citrus leaves during ethylene-induced abscission and natural abscission.

MATERIAL AND METHODS

Plant Material and Treatments . Mature leaves from the current year flush and senescing leaves approaching natural abscission (defined by their yellow-green color) were taken from established trees of Citrus sinensis L. Osbeck var. Shamouti orange. Mature leaves were treated with 25 μ l/l ethylene at 25°C as previously described (16).

Extraction and Assay of IAA . IAA was extracted and partially purified by the procedure described in Section C. IAA was assayed by a solid-phase enzyme immunoassay according to Weiler et al . (21). Detailed description of the preparation of antibodies and assay conditions appears in Section C.

RESULTS

Effect of Ethylene on IAA Levels . Citrus leaves were treated with ethylene for 24 h and then leaf blade, midrib, and abscission zone tissues were excised and assayed for IAA. IAA levels in all tissues of leaves incubated in air tend to increase during incubation (Table I). Ethylene treatment eliminated this increase. IAA levels in tissues of ethylene-treated leaves were similar to those in tissues of freshly cut leaves, except for the abscission zone in which IAA level decreased following ethylene treatment. We further studied the effect of the duration of ethylene treatment on IAA levels in midrib tissue. Again, there was a

Table I. Effect of ethylene on endogenous IAA levels in citrus leaf tissues. Leaves were treated with 25 μ l/l ethylene for 24 h. IAA was assayed by an enzyme immunoassay.

<u>Tissue</u>	<u>Zero time</u> ng / g / f r e s h	<u>Air</u> w e i g h t	<u>Ethylene</u>
Leaf blade	36.1 $\pm$ 12.9	49.4 $\pm$ 16.5	41.3 $\pm$ 13.8
Midrib	37.2 $\pm$ 13.6	50.5 $\pm$ 18.2	36.1 $\pm$ 13.7
Abscission zone	48.1 $\pm$ 17.8	62.2 $\pm$ 17.5	34.1 $\pm$ 11.5

Table II. Effect of the duration of ethylene treatment on endogenous IAA levels in midrib tissue. Ethylene concentration was 25 μ l/l. IAA was assayed by an enzyme immunoassay.

<u>Time</u> h	<u>Air</u> ng/g/fresh weight	<u>Ethylene</u>
0	58.6 $\pm$ 17.8	-
12	63.4 $\pm$ 13.4	58.0 $\pm$ 12.3
24	64.0 $\pm$ 16.5	57.5 $\pm$ 13.9
32	67.3 $\pm$ 16.9	51.4 $\pm$ 13.9

Table III. Effect of ethylene on endogenous IAA levels in isolated abscission zones of citrus leaves. Abscission zones were excised from mature leaves and then treated with 25 μ l/l ethylene for various periods. IAA was assayed by an enzyme immunoassay.

<u>Time</u> h	<u>Air</u> ng/g/fresh weight	<u>Ethylene</u> ng/g/fresh weight
0	79.2 $\pm$ 12.6	-
24	63.3 $\pm$ 14.8	70.0 $\pm$ 13.8
48	59.9 $\pm$ 15.0	62.7 $\pm$ 13.2

Table IV. Ethylene production rates and endogenous IAA levels in midrib tissue of mature and senescing citrus leaves. IAA was assayed by an enzyme immunoassay.

<u>Leaf</u>	<u>Ethylene</u> nl/g/h	<u>IAA</u> ng/g/ fresh weight
Mature	1.0 $\pm$ 0.04	74.6 $\pm$ 14.9
Senescing	9.2 $\pm$ 2.3	32.8 $\pm$ 8.1

tendency for IAA levels to increase in air control leaves during incubation and this increase was inhibited by ethylene (Table II).

Using a bioassay for measuring IAA, Gaspar et al. (11) reported that ethylene markedly decreased IAA levels in isolated abscission zones of citrus leaves. We repeated this experiment employing an immunoassay for measuring IAA. In contrast to intact leaves, IAA levels in isolated abscission zones declined during incubation in air (Table III). Treatment with ethylene did not cause any significant changes in IAA levels.

IAA Level in Senescing Leaves. IAA level in senescing leaves approaching abscission was determined and compared to mature leaves. The data show that level of IAA in the distal side of the abscission zone of senescing leaves was lower by about 55% compared to mature leaves (Table IV). Ethylene production in senescing leaves was 7-fold higher than in mature leaves (Table IV).

DISCUSSION

The success in employing an immunoassay for measuring IAA in citrus leaf tissues (Section C) enabled us to examine changes in IAA levels during ethylene-induced abscission and during natural abscission. The results achieved are preliminary in nature and lay the basis for further studies. Using [^{14}C]IAA, it has been clearly demonstrated that ethylene reduces the level of free IAA in citrus leaf tissues and concomitantly increases the conjugation of the hormone (Section A). However, when levels of endogenous IAA were determined, we could not find in most experiments a significant reduction in IAA levels following ethylene treatment (Tables I and II). The only effect of ethylene on IAA levels was the elimination of the increase in

IAA content that occurred during incubation.

One may question whether studies with [^{14}C]IAA reflect the in vivo situation. Nevertheless, two recent studies which measured changes in endogenous levels of IAA and IAA conjugates in ethylene-treated tissues reported similar results i.e. increased levels of IAA conjugates and no changes in free IAA levels (9,24). At present we cannot offer any reasonable explanation for this phenomenon. Stimulation of IAA biosynthesis is ruled out since there are data showing that ethylene suppresses this process (10). Also, it is not clear why Gaspar et al . (11), using a bioassay, observed a significant decrease in IAA levels in ethylene-treated abscission zone sections, whereas we could not detect any effect of ethylene on IAA content in the same tissue (Table III). It is, however, noteworthy that reduction of endogenous IAA levels by ethylene has been reported by several investigators (5,10,13). Further studies are needed to examine whether reduction of IAA levels is required before ethylene can induce abscission.

It has been suggested that in leaves approaching natural abscission, the abscission-retarding substances (IAA) decline and the abscission-promoting substances (ethylene and probably ABA) increase (14,15). Our data agree with this concept. Senescing citrus leaves produced elevated amounts of ethylene (Section B) and their IAA content was significantly below that of mature leaves (Table IV). There is a discrepancy in the literature concerning changes in IAA levels during senescence of leaves. Some investigators reported that IAA levels declined or did not change with age (18,20,22), whereas others reported that IAA levels increased during senescence (4,8,12,19,23). As noted above, citrus leaves showed a significant decline in IAA levels during senescence (Table IV) and this may be attributed to the increase in IAA conjugation that was observed in these leaves (Section B). If this is true we are still left with the question why IAA level in

ethylene-treated leaves did not decline although their ability to conjugate IAA increased (Section A). Based on the data presented for senescing citrus leaves approaching abscission, it may be suggested that natural abscission will be initiated when the balance between auxin and ethylene will be shifted in favor of ethylene.

Low levels of IAA and elevated production of ethylene seem to be contrary to the many correlations that have been made indicating that a high level of ethylene production reflects high levels of endogenous auxin. However, Roberts and Osborne (18) have shown that a correlation between auxin content and ethylene production can be made for immature tissues but no such relationship exists in senescing tissues where ethylene production appears to be independent of the level of endogenous auxin.

The interaction between ethylene and IAA in the control of abscission seems to be more complex than is usually believed. In nature, the level of ethylene is relatively low, therefore the decrease of IAA level may be critical in order for ethylene to induce abscission. In experiments with exogenous ethylene, apparently the relatively high concentration of ethylene applied induces abscission, although IAA content does not decline.

LITERATURE CITED

1. Abeles, F.B., ed. 1973. Ethylene in Plant Biology. New York/London:Academic.
2. Abeles, F.B., Leather, G.R. 1971. *Planta* 97:87-91.
3. Addicot, F.T. 1982. Abscission. University of California Press, Berkeley.
4. Atsumi, S., Hayashi, T. 1979. *Pl. Cell Physiol.* 20:861-865.
5. Beyer, E.M.Jr. 1975. *Plant Physiol.* 55:322-327.
6. Beyer, E.M.Jr., Morgan, P.W. 1971. *Plant Physiol.* 48:208-212.
7. Cohen, J.D., Bandurski, R.S. 1982. *Ann. Rev. Plant Physiol.* 33:403-430.
8. Dorffling, K. 1983. *Planta* 60:390-412.
9. Epstein, E. 1982. *Physiol Plant.* 56:371-373.
10. Ernest, L.C., Valdovinos, J.G. 1971. *Plant Physiol.* 48:402-406.
11. Gaspar, T., Goren, R., Huberman, M., Dubucq, M. 1978. *Plant Cell Envir.* 1:225-230.
12. Kaska, N. 1972. In: *Hormonal Regulation in Plant Growth and Development*, ed. H. Kaldewey, Y. Vardar, Verlag Chemie, Weinheim. pp.431-438.
13. Lieberman, M., Knecht, E. 1977. *Plant Physiol.* 60:475-477.
14. Morgan, P.W. 1983. In: *Agricultural Chemicals of The Future*, ed. J.L. Hilton, Rowman and Allanheld Publ., Totowa N.J. pp. 61-74.

15. Morgan, P.W. 1984. In: Ethylene: Biochemical, Physiological and Applied Aspects, ed. Y. Fuchs, E. Chalutz, Martinus Nijhoff/DR W. Junk Publishers, The Hague. pp. 231-240.
16. Riov, J. 1974. Plant Physiol. 53:312-316.
17. Riov, J., Dror, N., Goren, R. 1982. Plant Physiol. 70:1265-1270.
18. Roberts, J.A., Osborne, D.J. 1981. J. Exp. Bot. 32:875-887.
19. Shelldrake, A.R., Northcote, D.H. 1968. Nature. Land. 217:195.
20. Shoji, K., Addicott, F.T., Swets, W.A. 1951. Plant Physiol. 26:189-191.
21. Weiler, E.W., Jourdan, P.S., Conrad, W. 1981. Planta 153:561-572.
22. Wetmore, R.H., Jacobs, W.P. 1953. Am. J. Bot. 40:272-276.
23. Wheeler, A.W. 1968. J. Exp. Bot. 19:102-107.
24. Wood, B.W. 1985. J. Amer. Soc. Hort. Sci. 110:340-343.

MORPHACTIN-INDUCED INHIBITION OF ABSCISSION IN CITRUS LEAVES

R. Goren, Y. Aron, S.P. Monselise and M. Huberman

ABSTRACT

Morphactin (chlorflurecol-methylester, $10^{-6}M$ to $10^{-3}M$) inhibited polar auxin transport in midrib sections of citrus leaves, but not as a result of lack of free auxin in the absorbing apical segment of the midrib. Morphactin also induced the conjugation of IAA to aspartic acid and additional unidentified amino acid and delayed abscission. However, at the level of $10^{-3}M$ this delaying effect was much lower, probably due to the presence of ethylene which is induced by morphactin at this concentration. The stimulatory effect of ethylene on abscission was inhibited by morphactin during the first 10 to 16 hours after excision and exposure of leaves to ethylene. Morphactin also inhibited the activity of cellulase and polygalacturonase at the abscission zone. It is suggested that morphactin inhibits abscission by delaying the activity of cell-wall degrading enzymes, a mechanism which is not related to morphactin's inhibition of auxin transport to the abscission zone.

INTRODUCTION

Morphactins (derivatives of fluorene-9-carboxylic acid) have a wide spectrum of influence on plants (Schneider, 1970). One of the most known effects concerns its inhibitory action on polar auxin transport (Kreele and Liebert, 1968). There are also some indications that morphactin accelerates abscission (Schneider, 1970; Beyer, 1972; Morgan and Durham, 1973; Beyer, 1975). This effect may be related to the ability of morphactin to inhibit

auxin transport, since it has been shown that abscission of intact cotton leaves, mediated by native and exogenous ethylene, was induced when auxin transport was inhibited by morphactin (Beyer, 1972; Morgan and Durham, 1973; and Beyer, 1975).

The effect reported by Morgan and Durham (1973), led us to the hypothesis that the addition of morphactin may allow us to reduce the concentration of ethephon used for inducing citrus fruit abscission for mechanical harvesting, and thus preventing, or at least reducing the accompanied leaf drop (Wilson et al, 1977).

The aim of the study was to investigate the role of morphactin in the regulation of auxin transport in a model system of leaf explants, and to study its interaction with ethylene-induced abscission.

MATERIAL AND METHODS

The experiments involved 8 to 10 months-old leaves of *Citrus sinensis* (L.). Osbeck var. Shamouti, picked from 30-year-old trees. Unless otherwise indicated, treatment was applied for 48 h at $25 \pm 1^\circ\text{C}$ in the dark. At the end of incubation period, the number of leaf blades that had abscised, or which abscised due to a gentle touch, was determined (Ratner et al, 1969). Experiments were run twice, with 4 replications of 20 to 30 leaves each.

The morphactin used was methyl-2-chloro-9-hydroxyfluorene-(9)-carboxylic acid; synthesized by Celamerck GMBH and Co. KG, Darmstadt W. Germany; code No. EMD-7301W or CME-10905 EC 112; common name: chlorfluorecol-methylester; in 0.025% Tween-80 in water. It was applied by dipping samples of 20 to 30 leaves for 15 min in the appropriate solutions, which were aerated during application. Treated leaf groups were air-dried, closed in nylon-net pockets and exposed again to ethylene or to air.

Ethylene treatments were applied to samples of 30 leaves, placed in containers on plastic screens. Ethylene (25 μ l) was supplied in 12-liter jars with a continuous stream of humid air at the rate of 200 ml/min. In some cases each group of 20 leaves was placed in 18-liter container, to minimize the possible effects of endogenous ethylene. The jars contained thin water layer - to maintain a saturated atmosphere, and vials with 40% with KOH - to absorb CO_2 . Ethylene (2-1000 μ l/l) was injected with a hypodermic syringe.

^{14}C -IAA transport was measured in 6 mm long leaf midrib sections by the agar donor-receiver block method, as described by Riov and Goren (1979). The same system was used to study IAA metabolism, analyzing the endogenous levels of free IAA, IAA-aspartate (IAA_{sp}) and IAA metabolites (Riov and Goren, 1979; Riov et al, 1985). Ethylene measurements were carried out by gas chromatography on an alumina column at room temperature (Sagee et al, 1980).

The extraction of total cellulase and total polygalacturonase (PG) from laminar abscission zone was performed according to Huberman et al, (1975). Extraction of exo-cellular enzymes was carried out by the diffusion method and the enzymic activity in the extracts was assayed by measuring changes in viscosity of 1% Na-carboxymethyl cellulose in water for cellulase and the release of reducing sugars from D-galacturonic acid for PG (Huberman and Goren, 1979).

RESULTS

Effects on auxin transport and metabolism. In treatments with 10^{-6}M to 10^{-4}M morphactin, as in control air-treated leaves, only negligible amount of ethylene was produced in the system. When the level of morphactin was 10^{-3}M , ethylene formation was increased 5 to 10 fold (up to 10 to 32 $\mu\text{l.g}^{-1}.\text{h}^{-1}$). At higher levels of morphactin polar auxin transport capacity was reduced - from about 40% in the control sections to about 15% at 10^{-4}M (Fig. 1). At

10^{-3} M morphactin auxin transport capacity was reduced to about 5%, but inhibition may have involved ethylene (Riov and Goren, 1979), which is induced by the high concentration of morphactin.

Figure 1 - Morphactin-induced inhibition of polar IAA transport capacity in midrib sections. Leaves were dipped in the appropriate concentration of morphactin for 15 min and then left in air for 24 h. Six mm midrib sections were excised and used for the determination of auxin transport capacity over a period of 4 h.

When leaves were pretreated with 10^{-4} M morphactin, significant changes in auxin conjugation could be observed in the whole midrib section 4 h after auxin transport. Morphactin caused more radioactivity to accumulate in the upper part of the midrib section, below the agar-donor-disc. In the lower parts of the midrib section, less radioactivity was detected in morphactin-treated sections. Only traces of radioactivity were found in the receiver block. During the first 2 h of ^{14}C -IAA transport, more IAA metabolites were

detected at the apical segment of morphactin-treated midrib sections. These differences almost disappeared 2 h later, and only an IAA-amide remained higher in the morphactin-treated sections. Two and four hours after the beginning of ^{14}C -IAA transport, more free IAA was found in air control tissue.

Effect on abscission. Morphactin at the range of 10^{-6}M to 10^{-4}M , delayed abscission significantly, whereas 10^{-3}M was much more similar to the air control treatment, apparently due to the fact that at this concentration morphactin induces ethylene formation. Leaves treated with 10^{-4}M morphactin reached 60% abscission 120 h after the beginning of the experiment (Fig. 2). Pretreatment with morphactin partly abolished the acceleration effects of ethylene on the process. At morphactin concentrations of 10^{-5}M to 10^{-4}M no abscission was detected up to 48 h. The inhibition of abscission gradually disappeared when the interaction of each morphactin concentration was tested with increasing levels of ethylene, from 2 to 1000 $\mu\text{l/l}$. The inhibition was found to be non-competitive.

Figure 2 - Morphactin-induced inhibition of abscission in the presence or absence of ethylene.

Leaves were dipped for 15 min in either water or morphactin (10^{-4}M) and then incubated in a flow system consisting of either air or ethylene ($25\mu\text{l/l}$). Treatments were: water and further exposure to air (O); morphactin and further exposure to air (■); water and further exposure to ethylene (Δ); morphactin and further exposure to ethylene (▲).

In a similar way 10^{-4}M morphactin inhibited ethylene ($25\mu\text{l/l}$)-induced abscission when applied either at excision time or 10 h after excision and exposure to ethylene. Later on, the inhibitory effect of morphactin on the acceleration of abscission by ethylene disappeared in a way similar to that found with cycloheximide (Ratner et al, 1969) and 2,5-norbornadiene (Sisler et al, 1985), which are specific inhibitors of protein synthesis and ethylene binding to specific receptors, respectively.

Effects on cellulase and polygalacturonase. Morphactin (10^{-4}M) delayed the increase of both total cellulase and PG activity at the abscission zone. Ethylene ($25\mu\text{l/l}$)-induced activity of both enzymes was slightly delayed by pretreatment with morphactin. In both cases the effect on cellulase was more significant.

The interaction of increasing concentrations of morphactin (from 10^{-5}M to 10^{-4}M) with increasing concentration of ethylene (from $2\mu\text{l/l}$ to $100\mu\text{l/l}$) on exo-cellular activity of cellulase and PG was studied. It was found that with the increase in ethylene concentration the activity of both enzymes increased at all the levels of morphactin used. However, ethylene-induced activity of both enzymes was reduced when higher concentrations of morphactin were used. Again, the interactions were found to be non-competitive.

DISCUSSION

The most surprising result of the present investigation was that in citrus, the inhibition of auxin transport caused by morphactin was not accompanied by acceleration of abscission. Ethylene, which is known to induce abscission (Addicott, 1982), is also known to inhibit polar auxin transport to the abscission zone (Beyer, 1972 and 1975; Riov and Goren, 1979). It is well established that auxin level at the abscission zone should be reduced before ethylene is able to induce the synthesis and activity of the cell-wall hydrolyzing enzymes and abscission (Abeles, 1969; Ratner et al, 1969; Riov, 1974; Addicott, 1982).

Morgan and Durham (1972) discovered that specific inhibitors of auxin transport: 2,3,5-triiodobenzoic acid (TIBA), N-1-naphthylphthalamic acid (Naptalam, NPA) and morphactin (DPX-1840), promoted ethylene-induced leaf abscission in cotton. The fact that ethylene-induced inhibition of auxin transport is responsible for the induction of abscission was also found in *C i t r u s*, *P o p u l u s* and *E u c a l i p t u s* (Riov and Goren, 1979). The data of the present study indicate that no such correlation exists when morphactin is used, since the delay in polar auxin transport was accompanied by a delay, rather than by an acceleration, of abscission. The mechanism by which morphactin inhibits auxin transport is not yet understood.

The metabolism of IAA during the inhibition of its polar transport resembles results obtained earlier, when ethylene-induced inhibition of auxin transport was studied with the same plant system (Riov and Goren, 1979). Morphactin-induced IAA conjugation was significantly higher than air control, and the level of free IAA at the absorbing segment of the midrib tissue was lower compared with control. The relatively high level of free auxin found in the absorbing segments of the morphactin treated midrib section indicates that

the inhibition of IAA transport caused by morphactin did not result from a lack in free IAA. Whether IAA conjugation was responsible for the inhibition of the polar transport of auxin needs further study, especially since it was suggested (Beyer and Morgan, 1970; Riov and Goren, 1979) that ethylene-induced inhibition of auxin transport is not a result of IAA conjugation to aspartic acid and glucose.

The finding that morphactin is mostly active in the delay of ethylene-induced abscission during the first 10 h after excision, indicates that it may act at the level of protein synthesis (Schneider, 1970) when inhibiting the synthesis and activity of both cellulase and PG. This assumption is based on previous report (Ratner et al, 1969), that the main synthesis of cellulase occurs during the first hours after excision and exposure to ethylene. It is suggested that this may explain why the inhibition of auxin transport was not found to induce abscission.

Schneider (1970) suggested that morphactins should be considered as "polyvalent disturbing substances" acting for example, by affecting protein and enzymes synthesis rather than as competitive "antiregulators". This suggestion is strongly supported by the present study, in which morphactin was found to be non-competitive with ethylene in its effect on abscission and on the activity of cellulase and PG. It is suggested that morphactin inhibits abscission by delaying the activity of cell-wall degrading enzymes, a mechanism which is not related to morphactin's inhibition of auxin transport to the abscission zone.

ACKNOWLEDGEMENT

We are very grateful to Dr. G. Schneider of the Celmerck GmbH and C. KG. Darmstadt, Federal Republic of Germany, who kindly provided us with morphactin EMD-7301W and CME-10905 EC 112, as a free gift.

REFERENCES

- Abeles, F.B., 1969. Abscission: role of cellulase. *Plant Physiol.* 44:447-452.
- Addicott, F.T., 1982. Abscission. University of California Press. Berkeley, Los-Angeles, London. pp 369.
- Beyer, E.M. Jr., 1972. Auxin transport: A new synthetic inhibitor. *Plant Physiol.* 50:322-327.
- Beyer, E.M., Jr., and Morgan, P.W., 1970. Effect of ethylene on the uptake, distribution, and metabolism of indoleacetic acid-1-¹⁴C and 2-¹⁴C and naphthaleneacetic acid-1-¹⁴C. *Plant Physiol.* 46:157-162.
- Huberman, M. and Goren, R. 1979. Exo- and endo-cellular cellulase and polygalacturonase in abscission zones of developing orange fruits. *Physiol. Plant.* 45:189-196.
- Morgan, P.W., and Durham, J.I., 1972. Abscission: potentiating action of auxin transport inhibitors. *Plant Physiol.* 50:313-318.
- Morgan, P.W., and Durham, J.I. 1973. Morphactins enhance ethylene-induced leaf abscission. *Planta (Berl.)*. 110:91-93.
- Kreele, E., and Libbert, E., 1968. Inhibition of the polar auxin transport by a morphactin. *Plant (Berl.)*. 80:317-320.
- Ratner, A., Goren, R., and Monselise, S.P., 1969. Activity of pectin esterase and cellulase in the abscission zone of *C i t r u s* leaf explants. *Plant Physiol.* 44:1717-1723.
- Riov, J., 1974. A polygalacturonase from *C i t r u s* leaf explants. Role in Abscission. *Plant Physiol.* 53:312-316.
- Riov, J., and Goren, R. 1979. Effect of ethylene on auxin transport and metabolism in midrib sections in relation to leaf abscission of woody plants. *Plant Cell Environ.* 2:83-89.

- Riov, J., Sagee, O., and Goren, R., 1985. Ethylene-induced changes in indol-3-acetic. Acid metabolism in citrus leaf tissue during abscission and senescence. 5th Int. Symp. Growth Regulators in Fruit Production, Rimini, Italy. Acta Hort. (In press).
- Sagee, O., Goren, R. and Riov, J., 1980. Abscission of C i t r u s leaf explants: Interrelationships of abscisic acid, ethylene, and hydrolytic enzymes. Plant Physiol. 66:750-753.
- Schneider, G., 1970. Morphactins. Physiology and performance. Ann. Rev. Plant Physiol. 21:499-536.
- Wilson, W.C., Holm, R., and Clark, R.K., 1977. Abscission chemicals - aid to C i t r u s fruit removal. Proc. Int. Soc. Citriculture. 2:404-406.

BENEFIT TO AGRICULTURE

The natural plant auxin, indole-3-acetic Acid, is a vital component of the hormone balance influencing all sorts of developmental/behavioral processes in plants. Leaf abscission is only one of these processes; others include seed dormancy, root initiation, bud dormancy, flower bud and fruit abscission, tropisms, all aspects of general growth and specific differentiation, translocation, and senescence. Most of the studies of natural roles of IAA on plant development have been limited to tropisms and early seedling growth restricted to etiolated plants. The methods developed here will increase the means to study the entire array of auxin-influenced, developmental/behavioral processes. This capacity should be beneficial to agriculture in a wide array of crops and post harvest systems.

In addition, the development of two centers for IAA analysis, the one at the lab in Israel and the other in Texas, will significantly increase the output of data on this hormone's status in a number of systems.

The publication of these methods and their application on a routine basis in our laboratories should help establish the legitimacy of detectors other than the mass spectrometer for quantitation of IAA. For the past almost 20 years plant physiology has struggled with the two extremes of, on the one hand, equipment and procedural requirements which made data prohibitive to obtain except in a few GC-MS laboratories in the entire world and; on the other hand, superficial and inexact methods which left legitimate doubts about the identification and quantitation of the putative IAA. HPLC and immunological techniques are much more readily available now. We have employed them in conditions where there can be

little doubt about the identification and quantitation of the putative IAA. This represents a significant contribution to agriculture.

The physiological data generated, though incomplete, suggest certain new approaches in abscission control. If the level of IAA does indeed plateau in mature cotton leaves and does not drop precipitiously before abscission, the role of ethylene will be even more important in that crop. A similar conclusion stems from the observations that ethylene levels do not decline in ethylene-treated citrus leaves. This, however, does not rule out the possibility to influence abscission by inhibiting IAA synthesis or promoting IAA conjugation, and thus to enhance the effectiveness of ethylene-releasing chemicals as defoliants.

DESCRIPTION AND RESULTS OF COOPERATION

Cooperation during the work done on this proposal has been effective and helpful. Plans for our initial work were developed in detail as a result of R. Goren's visit to the laboratory in Texas in 1982. Subsequently, P.W. Morgan visited Israel twice in 1984, once under sponsorship of the BARD grant. During these visits Morgan was able to learn the mini-column clean-up procedure for IAA which was subsequently tested and adopted for use in the laboratory in Texas. Morgan also had the opportunity to observe the immunoassay procedure and to analyze some samples of cotton leaf tissue by the immunological procedure. Subsequently Riov and Goren visited the lab in the U.S.A. and learned the techniques necessary for a multicolumn, HPLC assay procedure. The Israeli scientists brought samples of citrus tissues which were analyzed and proved to contain much more IAA than cotton leaf samples. The exchange of samples has been helpful.

A second benefit of the cooperation has been the opportunity to have exhaustive discussions and to make plans for future collaborations. As the result of these discussions we believe we have greatly improved our focus on how to resolve some of the more difficult problems remaining in abscission. These ideas plus the IAA-analyzing programs developed in this proposal has been put forward to BARD in a new grant proposal. The ideas in this grant proposal will guide our continuing studies of the hormonal regulation of abscission.

REPRINTS AND MANUSCRIPTS ORIGINATED FROM THE PROJECT

USA

1. Morgan, P. W. and J. I. Durhan. 1982. Strategies for extracting, purifying, and assaying auxins from plant tissues. *Botanical Gazette* 144:20-31.
2. Beyer, E. M., Jr., P. W. Morgan and S. F. Yang. 1984. Ethylene. In: *Advanced Plant Physiology*, M.B. Wilkins, editor, Pitman Books Limited, London, pp. 111-126.
3. Morgan, P. W. 1984. Is ethylene the natural regulator of abscission? In *Biochemical, Physiological and Applied Aspects of Ethylene*, Y. Fuchs and E. Chalutz, eds, Martinus Nijhoff Publishers, The Netherlands. pp. 231-240.
4. Morgan, P. W. 1985. Chemical manipulation of abscission and desiccation. In: *Agricultural Chemicals of the Future*. (Beltsville Symposia on Agricultural Research #8). James L. Hilton, editor, Rowman and Allanheld, Publishers; Totown, New Jersey. pp.61-74.
5. Rivera, V. G., P. W. Morgan & R. D. Stipanovic. 1985. Trace-level analysis of indole-3-acetic acid from light-grown, highly pigmented leaf tissue. *Plant Physiol.* 77 (Supplement): 127.
6. Morgan, P. W., V. G. Rivera and J. I. Durham. 1985. Changes in levels of IAA in cotton leaves with age. *Plant Physiol.* 77(Supplement): 127.
7. Rivera, V. G., P. W. Morgan, and R. D. Stipanonic. Trace levels analysis of indole-3-acetic acid from light-grown, highly pigmented leaf tissue. To be submitted, *J. Chromatography*, summer 1985. (copy attached).
8. Morgan, P. W., V. G. Rivera, J. I. Durham & R. D. Stripanonic. Levels of indole-3-acetic acid in leaves of Gossypium hirsutum L. To be submitted, *Plant Physiology*, summer 1985 (copy attached).

Israel

1. Riov, J., O. Sagee, and R. Goren. 1984. Effect of ethylene on indole-3-acetic acid transport, metabolism, and level in leaf tissues of woody plants. In *Biochemical, Physiological and Applied Aspects of Ethylene*, Y. Fuchs and E. Chalutz, eds., Martinus Nijhoff/Dr. W. Junk Publishers, The Netherlands pp. 267-276.
2. Riov, J., O. Sagee, and R. Goren. 1986. Ethylene-induced changes in indole-3-acetic acid metabolism in citrus leaf tissues during abscission and senescence. *Acta Hort.* In press.
3. Goren, R., Y. Aron, S.P. Monselise and M. Huberman. 1986. Morphactin-induced inhibition of abscission in citrus leaves. *Acta Hort.* In press.
4. Sagee, O., A. Maoz, R. Mertens, R. Goren, and J. Riov. 1986. Comparison of differential enzyme immunoassays for measuring IAA in vegetative citrus tissues. To be submitted, *Physiol. Plant.*
5. Sagee, O., J. Riov and R. Goren. 1986. Effect of ethylene on [^{14}C] indole-3-acetic acid metabolism in citrus leaf tissues. In preparation.
6. Sagee, O., J. Riov and R. Goren. 1986. Indole-3-acetic acid metabolism and levels in senescing citrus leaves approaching natural abscission. In preparation.
7. Sagee, O., J. Riov and R. Goren. 1986. Effect of ethylene on levels of endogenous indole-3-acetic acid in citrus leaves. In preparation.