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**Indole-3-Acetic Acid Metabolism in Relation
to Root Initiation**

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C: ABSTRACT

Indole metabolism in a variety of plant systems was studied using isotope tracking methods. These studies indicated that IBA conjugates were a better source of free auxin than IAA conjugates, however the transport and metabolism of pure IAA and IBA were similar. Extensive studies of both applied IAA and IBA metabolism were also made using tobacco leaf discs, mung bean, olive and other plant materials. IBA, usually considered a synthetic auxin, was identified as a naturally occurring auxin in several plant species by GC-MS techniques. Conjugates of IBA were shown to be excellent compounds for root promotion in several difficult-to-root species and cultivars and, in addition, treatment on plant material with paclobutrazol improved the rate of auxin induced rooting.

Finally, an enzyme from carrot showing activity against amino acid conjugates was described. This activity was relatively stable and was found in the soluble fraction. Work was initiated on understanding auxin metabolism during differentiation of carrot tissue cultures.

D: OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL

- 1) Measure the uptake and movement of applied IAA, IBA and conjugates in plant tissues (See Ref. 1,2,3,4).
- 2) Measure conversion of IBA to IAA (See Ref. 16,17).
- 3) Isolate and identify IBA metabolites See Ref. 13,14,16,17,19,21).
- 4) Determine time course for metabolic conversions (See Ref. 1,2,3,5,6).
- 5) Isolate enzymes important for IBA and conjugate metabolism (See Ref. 7).
- 6) Test IBA conjugates for rooting (See Ref. 11,18,20 and Appendix I).

E: DESCRIPTION OF THE RESEARCH RESULTS

1) Indole metabolism: We began our investigations by developing methods for the quantitative evaluation of the metabolism of exogenous indoles applied to plants. First, because it has been a well studied system, we used tobacco leaf disks to study the metabolic fate of IAA applied at two different levels (1,2,3). The metabolism of IAA in tobacco leaves was studied by incubation of leaves for 48 h with 10 or 100 μM [$1\text{-}^{14}\text{C}$]-IAA. At both 10 and 100 μM , IAA-aspartate was found by GC-MS analysis to be a major metabolite (60 and 40% of the ^{14}C , respectively). However, with the other metabolites, different compounds were obtained depending on the IAA level. Increasing the IAA concentration from 10 to 100 μM increased the percentage of acidic metabolites from 56% to 90%. Although IAA-aspartate was the major metabolite, it had only weak activity in biological tests for ethylene induction. Two fractions isolated by chromatography on Sephadex LH-20 markedly stimulated ethylene production when applied to tobacco leaf disks. These results suggest that a novel acidic metabolite contributed to the stimulation of ethylene production in IAA-fed tobacco leaves.

Neutral metabolites, washed from an initial fractionation on a DEAE-Sephacel column, were further purified by HPLC. Extracts of 100 μM IAA-fed tissue contained radiolabeled IAA-glucose, as determined by GC-MS analysis, and this compound accounted for 50% of the neutral metabolites. Because of the large fraction for acidic metabolites, however, IAA-glucose accounted for only 5% of the total radioactivity in the tissue. The relative amounts of IAA-glucose in 10 μM IAA-fed tissue was only 15% of the neutral ^{14}C metabolites and 2% of the total radioactivity. Extracts on 100 μM IAA-fed tissue contained radiolabeled IAA-myo-inositol at low levels; only 1% of the total radioactivity. However, 10 μM IAA-fed tissue contained IAA-inositol but without detectable radioactivity. These results indicate that IAA-inositol is an endogenous component of the tobacco leaf. In addition to these two conjugates, two esterified ^{14}C containing conjugates were found only in the 10 μM IAA-fed tissue. These conjugates accounted for 25% of the neutral ^{14}C metabolites and 12% of the total radioactivity. HPLC of these conjugates revealed two closely running compounds, both of which released IAA upon 1 N NaOH hydrolysis. GC-MS and MS-MS analysis of the acylated derivatives confirmed two similar compounds, possibly interconvertible, with apparent molecular ions both at 303 and base peaks at 228 and 245, respectively. A preliminary characterization was possible based on high resolution mass spectral analysis.

As a follow-up to these studies the interaction between IAA and sucrose as effecting auxin metabolism and the rate of ethylene production was studied in leaf disks. The disks were pre-aged for 24 h in darkness to reduce internal sugar pools, and then incubated for an additional 48 h with radioactive IAA. Following ethanol extraction about 80% of the radioactivity was in IAA-aspartate. Following the addition of 100 mM sucrose, which increased ethylene production from the leaf disks by six fold, the percentage of IAA-aspartate was reduced to only 30% and

the rest of the radioactivity was found associated with polar metabolites with chromatographic properties similar to known conjugates. IAA-lysine, IAA-alanine, IAA-glucose, which chromatographed similar to the isolated metabolites, and IAA-inositol stimulated ethylene production when applied to sucrose treated leaf discs even at very low concentrations (1 μ M). Increasing the concentrations of the applied conjugates up to 100 μ M increased markedly the amount of ethylene production with IAA-inositol being the most active. IAA-aspartate showed only very low activity and higher concentrations showed only slight increases. These results show that IAA conjugates participate in the control of ethylene biosynthesis either directly or by the hydrolysis of the specific conjugates to yield IAA.

Methods for the analysis of endogenous indolic auxins play an important role in our studies of these compounds. We have used these methods to study a variety of different plant materials. In addition, we have applied these methods in order to measure the IAA levels, as well as ethylene evolution rates, in a fruticose lichen Ramalina duriaei collected from carob trees growing in northeastern Israel (8,9). IAA levels were estimated by GLC with electron capture detection of the pentafluorobenzyl (PFB) ester and also by ELISA following methylation. The identity of the isolated IAA was confirmed by GC-MS of both the PFB and methyl esters. IAA levels in lichens 1 year after transplanting to an air-polluted urban site were found to be lower than in the control thalli left at a non-polluted rural site. The material from the latter contained about 2.5 μ g/g F.W. free IAA and 0.5 μ g/g F.W. conjugated IAA, while the urban material contained 0.3 μ g/g F.W. each of free and conjugated IAA. Ethylene production rate was 1.0 nl/g F.W./hr in the material from the rural site and 1.5 nl/g F.W./hr in the material from the urban site. This is the first identification of IAA from a lichen and these data may suggest a possible interaction between the lichen and the tree on which it grows.

We next applied the basic concept of isotope tracking worked out so well in our studies of IAA metabolism in tobacco leaf disks, to look at IBA metabolism following treatment. We produced radioactive IBA for use in these studies both labeled with 14 C and, for the first time, we have produced (via contract) very high specific activity 3 H-IBA using specific tritium incorporation from the 5-halogenated indole. These radiolabeled compounds allowed us to study IBA metabolism in a number of plant systems. First, we studied IBA metabolism in mung beans treated with 3 H-IBA and followed this using DEAE-Sephadex chromatography followed by fractionation on PVPP TLC and HPLC (16,18).

Indole-3-butyric acid (IBA) was much more effective than indole-3-acetic acid (IAA) in inducing adventitious root formation in mung bean (Vigna radiata L.) cuttings. Prolonging the duration of treatment with both auxins from 24 to 96 h significantly increased the number of roots formed. Labeled IAA and IBA applied to the basal cut surface of the cuttings were transported acropetally. With both auxins, most radioactivity was detected in the hypocotyl, where roots were formed, but relatively more IBA was found in the upper sections of the cuttings. The rate of metabolism of IAA and IBA in these cuttings was similar.

Fig. 2. Time-course of the changes in the levels of labelled free IAA and IBA in section II of mung bean cuttings. Sections II of the cuttings were excised at different times after application of labelled auxins and the extracted radioactivity was analyzed by TLC. The radioactivity found in the zones corresponding to the free auxins is presented as percentage of total extractable radioactivity. Values are means of 3 replicates $\pm$ SE.

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Both auxins were metabolized very rapidly and 24 h after application only a small fraction of the radioactivity corresponded to the free auxins. Hydrolysis with 7 M NaOH indicates that conjugation is the major pathway of IAA and IBA metabolism in mung bean tissues. The major conjugate of IAA was identified tentatively as indole-3-acetylserpic acid, whereas IBA formed at least two major conjugates. The data indicate that the higher root-promoting activity of IBA was not due to a different transport pattern and/or a different rate of conjugation. It is suggested that the IBA conjugates may be a better source of free auxin than those of IAA and this may explain the higher activity of IBA.

These experiments in which mung bean cuttings were incubated in a labeled IBA conjugate showed that the conjugate is quickly hydrolyzed. After 6 h of incubation, most of the IBA taken up by the tissue was hydrolyzed. Most radioactivity was found at a retention time which corresponded to free IBA. These data demonstrate the capability of mung bean hypocotyls to hydrolyze IBA conjugates. The rate of hydrolysis of auxin conjugates may be an important factor in determining long-term effects of auxins on physiological processes such as rooting of cuttings. We also identified IAAsp as the major IAA conjugate in mung bean tissues. At least in bean tissues, IAAsp is hydrolyzed very slowly and therefore it is a poor source of free IAA (Bialek et al. 1983, S. Meir 1985. Thesis, Hebrew Univ. of Jerusalem, Israel). IBA, on the other hand, formed several conjugates which may be a better source of a free auxin. Support for this assumption comes from studies of ethylene biosynthesis in mung bean cuttings treated with IAA and IBA that showed that although IAA has a higher capability to stimulate ethylene biosynthesis than IBA, cuttings treated with IBA had significantly higher levels of 1-

aminocyclopropane-1-carboxylic acid (ACC), malonyl-ACC and ethylene during most of the rooting process than IAA-treated cuttings. Again, this higher auxin activity of IBA was probably derived from IBA conjugates because most of the added IBA had already been conjugated. It would seem that IBA conjugates are actually a better source of free auxin than IAA conjugates because they are more readily hydrolyzed by plants. This could explain why IBA is a better root-promoting compound than IAA.

Similar work with olive cuttings (15) showed no major difference in the rate of metabolism of IAA and IBA.

Testing of the rooting ability of softwood cuttings of various olive cultivars showed that some were difficult to root, some were easy to root, and some rooted only after treatment with IBA. In a study of the metabolism and transport of tritium-labeled IBA in 'Kalamata' (difficult-to-root) and 'Manzanillo' (easy-to-root), transport was found to be faster in the latter one. After 96 h of incubation, 40% of the radioactivity remained in the segment 1 (the first cm of the cutting), and 60% moved to segment 11 and 111 (2 and 4 cm from segment 1, respectively). In 'Kalamata' close to 70% of the label was found in segment 1 and the rest in segments 11 and 111. No major differences were found between IBA metabolites in the cuttings of the two cultivars. In both, IBA was the main compound after 96 h of incubation, and a new metabolite, probably IBA conjugate with a sugar, was formed.

Table 1. Effect of IBA (6000 mg/l) on rooting of olive cultivars. Oct. - Nov. 1986

Cultivar	Percent Rooting	
	No IBA	IBA
Cornezuelo	0	10±2.0
Giaraffa	21±6.0	25±5.5
Amigdalolea	0	30±7.0
Koreneiki	3±1.7	3±1.0
Picholine	3±1.0	45±6.0
Manzanillo	5±3.4	40±5.2
Kalamata	0	0
Suri	0	3±2.0
Leucokarpa	0	3±3.6
Kadesh K-12	25±3.6	41±5.5
Barnea K-13	38±7.9	80±4.5

Fig.1. Distribution of radioactivity (% of total radioactivity recovered) in cuttings of 'Manzanillo' and 'Kalamata' after incubation for various periods on H^5 -indole-3-butyric acid.

Fig.2. Chromatographic analysis of radioactive compounds from softwood olive cuttings, which were incubated for various periods on H^5 -indole-3-butyric acid. The chromatograms were run in chloroform:ethyl acetate:formic acid (4:5:1). The R_f of authentic IBA was 0.75.

One of the important findings was that applied IBA was metabolized rapidly to other compounds. We have shown that after 24 h of incubation only 5% of the applied IBA remains and we have isolated and purified several of the metabolic products produced during the first 24 h of incubation of mung bean hypocotyls (see also Ref. 20). A procedure was developed using selective solvent partitioning (butanol, ethyl acetate plus water phase). This was

followed by a PVPP step and then ion exchange chromatography on DEAE-Sephadex. Radioactive peaks were then analyzed by preparative HPLC and by GC-MS.

Treat for 24 hrs with ^3H -IBA

incubate

Grind in 70% acetone, filter, evaporate

pH 7.0

Fractionate on DEAE-Sephadex

Neutral
Fraction
(N-1)
38 ug

Acidic Fraction

(A-1)
22 ug

(A-2)
168 ug

HPLC

methyrate
4 major
peaks

(Ha, Hb, Hc, Hd, He, Hf)

I

II

HPLC

HPLC

III

IV

V

VI

VII

VIII

Compounds I-VIII were analyzed by mass spectrometry using both GC-MS techniques and direct probe. Compounds I-VII were shown to be amide conjugated based on base lability and presence of the n/z 217, 199, 186 ion trio:

m/z 189 = R CH₂ CH₂-CH₂-CO-

m/z 199 = R CH₂-CH₂ CH₂-CO-N-[-H]

m/z 217 = R CH₂-CH₂-CH₂-CO-NH-CH =

Compound VIII was shown to be an ester compound according to its base lability and due to the lack of the m/z 189, 199, 217 trio and the presence of m/z 203 ion:

m/z 203 - R CH₂-CH₂-CH₂-CO-O [=H]

The mass spectra for each of these are presented on the following pages.

IG90 EEE6.72 RT= 07:07 +E1 LRP 02/09/88 16:29 SUB
TIC= 224124 100% = 63331 E. E. #4 unknown, 3 μ d, .51s, m.c500, ppm1256, LRP-PROBE.

D550 EEB8.33 RT= 03:12 +EI LRP 02/10/88 12:40 SUB
TIC= 111236 100% 11782 E.E. #5 unknown, 35/d, .Sis, m/s 20, ppm 1406, LRP-PROBE.
100

1690 0060001.106 RT= 10:32 +CI LFP 02/09/86 12:25
TIC= 14679040 100% 44436 E. E. R8 unknown, 7ul, 3s/d... 51s, 100136S, LFP-PROBE
100

Table 1. IBA in various plants as determined by GLC-FID.

Plant	Organ	IBA
Avocado	Leaves	+
Corn	Dry kernels	++
Corn	Germinating kernels	++
Corn	Leaves	+
Cypress	Leaves	+
Mung bean	Shoots	+
Olive	Shoots	+
Olive	Leaves	+
Palm	Leaves	nd
Peach	Leaves	+
Sour lemon	Leaves	nd
Tobacco	Leaves	+
Yucca	Leaves	nd

nd = not detected

2) Isolation identification of IBA in plants: The auxin indole-3-butyric acid (IBA) is very effective in the promotion of rooting on a wide variety of plants and is being used commercially to root many plant species world-wide. It is well accepted that IBA is a synthetic auxin however there were some early reports of its natural occurrence by chromatography and/or bioassay of, for example, potato peelings and Nicotiana. We have found that IBA is present in a wide variety of plant species and have provided definitive data for the presence of both free and ester conjugated IBA in kernels and leaves of maize (Zea mays), an important monocotyledonous crop plant (12,13).

All of the initial isolations and identification were done at the Volcani Center Laboratory in Israel. Additional confirmation was provided by repeating the isolation and identification in the Plant Hormone Laboratory in Beltsville, U.S.A. Conditions for the analysis in both Israel and the U.S. were similar using primarily HPLC GC/MS for analysis. IBA was isolated and identified by GC-SIM-MS (ions m/z 130 and 217) and by full scan GC-MS in the Hazera 224 corn material in both locations; work on IBA in leaves was carried out in Israel. In addition to isolation of IBA from corn, we have found IBA as a natural constituent of cypress (11) as well as tobacco (12,13), avocado, mung bean, olive, and peach.

GC-MS identification of indole-3-butyric acid (as the methyl ester) isolated from maize (left spectra) and tobacco (right spectra).

IBA was identified in the kernels and leaves of Hazera 224 field corn by TLC, GLC and by GC-MS. We did not find detectable levels of IBA in Stowell's Evergreen corn kernels. TLC of extracts of dry corn kernels, and corn kernels and leaves seven days after sowing gave distinct blue spots at the R_f of IBA and IAA when viewed under UV light. Additional evidence was the characteristic blue color for IBA after dipping in Ehmann reagent which can detect nanograms of IBA. The intensity of the color shows that the tissue had more IBA after hydrolysis with 1N NaOH. This indicates that IBA was released from conjugates by the hydrolysis. The mass spectrum of IBA methyl ester obtained from IBA isolated from corn shows a peak at 25.5 min that yields the fragmentation pattern typical of 3- substituted indoles. The molecular ion ($m/z=217$) is quite stable and relatively little fragmentation occurs. The side chain cleavage with retention of the methylene carbon and ring expansion results in a base peak at $m/z=130$, the quinolinium ion.

We found that when cuttings of mung beans were incubated with IBA, more than 90% of the IBA was metabolized after 24 h. We also found (Epstein and Wiesman 12) that IBA-alanine increased the number of olive cuttings which rooted compared with IBA or IAA treatments. Epstein and Lavee (10) and Fawcett et al (13) demonstrated the conversion of IBA to IAA in plants. Taken together with our finding on IBA in plants the data demonstrates that plants are able to synthesize, catabolize and conjugate this hormone. Our failure to find IBA in the Stowell's Evergreen cultivar of maize and Chen's finding that IBA was not present in non-tumorous tobacco plants suggests that it is not continuously present in plant tissues or that its accumulation is under genetic and possibly also developmental control. Went and Thimann (17) proposed that some unknown compounds (which they termed rhizocalines) might exist in the plant and act in conjunction with IAA in the formation of adventitious roots. Based on the plethora of publications relating application of IBA to adventitious rooting and the results of the present studies, as well as our results indicating IBA is present in at least seven other species, we have formed as a working hypothesis the idea that IBA could be such a natural rhizocaline.

The natural occurrence of IBA in plants has important implications related to indole biochemistry in higher plants. It is clear that plants are able to make indolealkanoic acids with side chains longer than the three carbon present in tryptophan and are also able to reduce the chain length to form IAA. Knowledge of the biosynthesis of compounds such as IBA could provide new insight into the mechanism and regulation of auxin biosynthesis.

3) IBA and IBA conjugate effects on rooting: Of major interest has been our studies of conjugates of IBA for the induction of roots in a variety of species. We have found that the use of IBA-alanine was very effective for root induction in peach and difficult-to-root varieties of olive. In bogonvilia, however, we also have difficult-to-root, medium rooting and easily rooting cultivars. In this case, however, IBA-alanine provides only a modest increase in rooting over IBA treatment. We examined two

aspects of IBA conjugates use in root induction. In order to understand the species differences we see in the use of IBA conjugates we studied the transport and metabolism of both IBA and IBA-alanine. We found that transport was effected by the humidity of the air around the plants and by light. Under high humidity and applied IBA remains primarily in the bottom two sections of the mung bean hypocotyl while under low humidity conditions movement into the upper sections was much more rapid. In contrast, IBA-alanine moves rapidly to the leaves (within 6 hr) and is then transported back down into the hypocotyl over a 48 hr period where it accumulates in the lower sections. This quite different pattern of accumulation might explain why IBA-alanine is effective in root induction in some species and in some conditions. Both IBA and IBA-alanine are transported very slowly in the dark; thus, the transport of these compounds is regulated by either light or by processes occurring in the light. In dark conditions the major product of IBA is a polar metabolite with properties like those of a sugar conjugate while with IBA-alanine the major product is even more polar and may be a oxidation product.

Propagation of olive (*Olea europaea* L.) is done mainly by rooting of softwood cuttings with a small number of leaves. This method is very convenient since it provides a large number of plants of good quality from chosen cultivars in a short time. Treatments with IBA and use of mist during the rooting period improved greatly this method of propagation, but there are still many olive varieties, e.g. 'Kalamata' and 'Uovo de Piccione', which are difficult to root even when using these treatments and techniques. Auxins are found in vivo both in the form of the free acid and covalently attached to amino acids or sugars and this suggested that such conjugates might find utility for root initiation. Thus we determined the effect of application of amino acid conjugates of IAA and IBA on root induction of difficult-to-root softwood cuttings.

One-year-old olive shoots, about 50 cm long were taken from a 20-year-old olive orchard. Cuttings 10-15 cm. long, with four leaves, were prepared. After treatments the cuttings were planted in a bed of peat moss: perlite 1:1 with a basal temperature of 25°C and intermittent mist (10 sec every 10 min). Treatments were done by quick dip of the cuttings in the test solution. Experiments were done in July-August by a completely randomized design with four replicates of 20-40 cuttings in each. Rooting was assessed by counting the number of rooted cuttings (Table 1,2; Fig. 1).

Although synthetic auxins have been known and used for almost 50 years, there are still many plants which are difficult to root even after treatment with IBA or IAA. Recently it was found that auxin conjugates with amino acids or sugars could provide the auxin requirement of tissue culture or induce root formation. In our laboratory we synthesized IBA-alanine and showed its superiority over IBA both in easy- and difficult-to-root cultivars of olive (10). We assume that some species and cultivars do not respond to IBA treatments due to problems of permeability to IBA and slow transport. IBA conjugates, on the other hand, pass more easily through the membranes and are

subsequently hydrolyzed in the cells to the free acid, which in turn are metabolized to new conjugates and other compounds which eventually induce rooting.

Table 1. Influence of different auxin treatments on the rooting of 'Manzanillo' olive cuttings

Treatment	Number of Cuttings Treated	Number of Rooted Cuttings	Percent of Rooting
Experiment 1			
Control	40	13	32±4.4
IBA (0.3% powder)	40	10	25±3.5
IBA (1 g/l)	40	3	20±2.8
IBA-alanine (1 g/l)	40	20	55±9.0
Experiment 2			
Control	100	10	10±1.4
IBA (2 g/l)	200	34	17±2.2
IBA-alanine (2 g/l)	200	87	44±6.0
Ureido-IBA (2 g/l)	200	49	25±3.5

40 CUTTINGS PER TREATMENT
 CONT = WATER CONTROL
 IAGL = INDOLE-3-ACETYL-L-GLUTAMATE
 IAAS = INDOLE-3-ACETYL-L-ASPARTATE
 IAAL = INDOLE-3-ACETYL-L-ALANINE
 IBAL = INDOLE-3-BUTYRIC-L-ALANINE
 IBA = INDOLE-3-BUTYRIC ACID
 ALL COMPOUNDS AT 2 g/l CONCENT.

Fig. 1. Effect of IBA and different auxin conjugates on rooting of 'Uovo de Piccione' cuttings. Mean SE=3

Table 2. Influence of different auxin treatments on the rooting of 'Napoli' and 'Uovo de Piccione' olive cuttings

Treatment	Number of Cuttings		No. of Rooted Cuttings		Percent of Rooting	
	Napoli	Uovo Picc.	Napoli	Uovo Picc.	Napoli	Uovo Picc.
Control	500	100	95	3	20±3.6	2±0.3
IBA-Alanine (1 g/l)	500	125	220	20	44±6.5	16±2.3
IBA-Alanine-ethyl ester (1 g/l)	500	-	190	-	38±5.6	-
IBA-Alanine (Crystals)	250	125	90	34	36±5.3	27±4.0
IBA (8 g/l powder)	250	125	70	14	28±4.1	11±1.0
IBA (8 g/l)	250	125	70	12	28±4.1	10±1.5

Paclobutrazol treatment combined with IBA and urea-phosphate significantly enhanced the rooting of softwood peach (*Prunus persica* L. Batsch) 'Maravilha' cuttings (17,19). Application of paclobutrazol to the mother plants 8 months before removal of cuttings was more effective than application directly to the cuttings. The survival rates of rooted cuttings obtained from paclobutrazol-treated plants were significantly higher than those obtained from untreated plants.

Cuttings were prepared from peach and subjected to the following treatments prior to rooting: (a) dipping in 6 g liter⁻¹ IBA in talc powder; (b) dipping for 30 min in a suspension of 0.5 g. liter⁻¹ paclobutrazol in H₂O; (c) dipping for 30 min in 1 g. liter⁻¹ urea-phosphate in H₂O; (d) dipping in 6 g. liter⁻¹ IBA followed by 30 min dipping in 0.5 g. liter⁻¹ paclobutrazol; (e) dipping in a mixture of 6 g. liter⁻¹ IBA and urea-phosphate powder in a ratio of 9:1 (w/w), respectively; and (g) dipping in 6 g liter⁻¹ IBA plus urea-phosphate powder, g:1 followed by 30 min dipping in 0.5 g. liter⁻¹ paclobutrazol. Untreated cuttings served as control.

Cuttings from paclobutrazol-treated trees rooted similarly (ca. 20%) to cuttings from control trees when no additional treatment was applied to the cuttings (Table 1). IBA and particularly IBA plus urea-phosphate stimulated more rooting than IBA alone. This promotive effect was most evident in cuttings from paclobutrazol-treated trees. Ninety four percent of these cuttings rooted when treated with IBA plus urea-phosphate versus 46% for nonpaclobutrazol-treated trees.

Survival of rooted cuttings was also studied. Survival of untreated cuttings from control and paclobutrazol-treated trees was low, 16 and 17%, respectively (Table 1). IBA and IBA plus urea-phosphate significantly increased survival rates, particularly in cuttings from paclobutrazol-treated trees. Seventy two percent of the latter cuttings receiving IBA plus urea-phosphate survived after transplanting.

Paclobutrazol was also applied directly to the cuttings. Paclobutrazol or urea-phosphate alone were almost ineffective (Table 2). IBA stimulated rooting and again its promotive effect increased significantly when paclobutrazol or urea-phosphate were included. Cuttings treated with a combination of IBA, paclobutrazol, and urea-phosphate had the highest percentage of rooting (69%). This rooting percentage was considerably lower than that achieved with cuttings from paclobutrazol-treated trees which received IBA plus urea-phosphate (Table 1).

Our results demonstrate the beneficial effect of paclobutrazol particularly when applied as a pretreatment to the mother plants, in combination with IBA and/or urea-phosphate on rooting and survival of 'Maravilha' cuttings. Promotion of adventitious root formation by paclobutrazol has been attributed either to its anti-gibberellin activity or to its growth retarding effect which may increase the partitioning of assimilates and/or plant hormones to the base of the cuttings. Our data indicate that paclobutrazol is more effective when applied to the mother plants rather than to the cuttings. Cuttings from paclobutrazol-treated trees or cuttings treated with paclobutrazol had indeed more

roots per cutting and the roots were thicker and initially shorter than in control cuttings.

These results point to the possibility of using paclobutrazol for improving the rooting and survival of softwood peach cuttings of difficult to root cultivars. Propagation of scion cultivars as softwood cuttings in the spring may have an advantage over semihardwood cuttings, particularly for meadow orchards. Rooted softwood cuttings can be planted in the field at the beginning of the summer. Thus, they may attain considerable growth during the summer and bear the first crop the next year.

Table 1. Rooting and survival percentage of 'Maravilha' cuttings taken from plants treated with paclobutrazol when given basal treatment of IBA and urea-phosphate.

Treatment	Faclobutrazol			
	+		-	
Rooting (%)				
Control	x ²	20.0 c ^y	x	21.0 b
IBA	x	50.3 b	y	32.6 b
IBA + urea Phospate	x	93.6 a	y	46.0 a
Survival (%)				
Control	x	16.2 c	x	15.0 b
IBA	x	35.4 b	y	23.2 b
IBA + urea phosphate	x	72.7 a	y	38.5 a

z x,y indicate mean separation within rows by Duncan's multiple range test, P=5%.

y a,b indicate mean separation within columns by Duncan's multiple range test, F=5%.

Table 2. Rooting percentage of 'Maravilha' cuttings treated with IBA, urea-phosphate and paclobutrazol.

Treatment	Rooting (%)
Control	23.3 c ^y
IBA	36.3 c
Paclobutrazol	28.3 c
Urea-phosphate	26.3 c
Paclobutrazol + IBA	64.3 a
IBA + urea-phosphate	50.6 b
Paclobutrazol + IBA + urea-phosphate	69.3 a

^y Mean separation by Duncan's multiple range test, P=5%.

Enzymes catalyzing hydrolysis of IAA-amino acid conjugates

Due to the high instability of the Phaseolus enzyme that hydrolyzes IAA-conjugates, we decided to look at other species prior to beginning intensive efforts at purification and characterization. We have focused our research efforts on an IAA-amide hydrolyzing activity in crude cell-free extracts prepared from carrot cells grown in suspension culture. Carrot tissue was chosen as there is evidence that these cultures rapidly hydrolyzes IAA-amide conjugates in vivo. Enzyme stability studies showed that the most effective extraction buffer is 10 mM MES-NaOH containing 250 mM sucrose, 10 mM MnCl_2 , 0.05 mM dithiothreitol and 0.1% v/v Triton X-100, pH 6.0. Specific activities approaching 25 pmol L-alanine.hr⁻¹.mg protein can be assayed in crude extracts at 37°C, in which the pH has been adjusted to 9.00 at 0°C. Activity is relatively stable in extracts prepared by grinding in a mortar with pestle in the presence of an abrasive which improves recovery of protein. Either glass beads (diameter 66-160 μm ; 0.5 mg/g tissue) or acid washed sand (0.5 mg/g tissue) are suitable as abrasives and neither decrease the specific activity. Although a crude enzyme preparation is somewhat labile at 0°C, with a loss of about 75% of the activity in 66 hrs. the preparation can be stored frozen when supplemented with either 20% v/v glycerol or sucrose (to give a final sucrose concentration of 25% w/v). With glycerol or sucrose at -70°C or -16°C the enzyme can be stored for at least 3 days without measurable loss of activity. A 30% drop in specific activity was measured in such extracts thawed after storage in the absence of glycerol or excess sucrose. Inclusion of the serine protease inhibitor APMSF (p-amidinophenylmethylsulfonyl fluoride) or 0.5% w/v BSA in the extraction did not improve recovery of activity, nor were we able to prolong the longevity of the enzyme at 0°C by including APMSF in the extraction buffer. Addition of 5 mM EDTA to the extraction buffer lacking Mn^{2+} reduced the specific activity in the crude extract by at least 75%. Omitting EDTA and including 10 mM Mn^{2+} enhances this activity in excess of 10 fold.

After ultracentrifugation (100,000 x g. 30 min) of the crude enzyme preparation (extracted either in the presence or absence of 0.1% v/v Triton X-100) all the activity was recovered in the supernatant fraction. The enzyme is therefore likely to be either a soluble component of the cytoplasm or periplasmic space or very weakly associated with the cell membrane. About 85% of the activity in a crude extract was lost when fractionated with ammonium sulfate. The remaining activity was found in the fraction precipitated between 40-60% saturated ammonium sulfate.

Our use of mass spectral analysis to enhance our biochemical studies is a modest application of such an approach. In addition, as we begin to enhance such studies with plant lines developed from our genetic studies, we expect to ultimately accomplish a new level of understanding of the reactions involved in IAA regulation and how these reactions might be controlled.

Initial studies were begun (14) using carrot cell cultures to look at differentiation. Carrot cells in suspension grow well on MS medium supplemented with 1 mg/l of 2,4-D. When transferred to 2,4-D-free medium, the cell clusters usually differentiate and

eventually form somatic embryos. We determined that omitting 2,4-D from the medium increased production and excretion of IAA (Table). 2,4-D seems to also inhibit the formation of esterified IAA. The significance of these metabolic changes in relation to the process of embryogenesis remains to be clarified and will be an important aspect of our work under an approved bard which is scheduled to begin.

	Proliferating culture		Embryogenic culture	
	cells	medium	cells	medium
	ng/g	ug/l	ng/g	ug/l
Free IAA	17.0	2.7	28.9	6.8
Free + ester IAA	19.4	8.5	61.0	12.9
Total IAA	410.5	9.4	615.0	14.7

F: DESCRIPTION AND RESULTS OF COOPERATION

We have a good and long standing history of cooperation as was detailed in our grant request. This association was worked out very well over the period of this BARD project. We have been able to coordinate our research on metabolic analysis, with the group at Beltsville doing basic studies on the general approach to isolation using the tobacco system. The studies of the hydro-lase enzyme, initiation studies on developmental and mass spectral analysis of metabolites. The Israeli group has done the biological studies of rooting and much of the studies on IBA transport and metabolism. We established a computer link using Bitnet for day-to-day contact. This was a major help in really having a part in each others research work. We discuss almost every experiment prior to its initiation and suggested new approaches often. The metabolic studies required that the final steps in identification be done at the Beltsville laboratory. This was effective since it was possible for us to work together on samples during visits by Dr. Epstein to Beltsville. The work on lichens was done at both locations with simply the shipment of the samples and extensive Bitnet discussions. The cooperation with Dr. Riov worked well with a graduate student working on the BARD project with both Dr. Epstein and Dr. Riov. Dr. Roh initiated some studies on IBA-conjugates on rooting on diverse species.

G: EVALUATION OF THE RESEARCH ACHIEVEMENTS

Listed below are the six specific accomplishments of BARD project US-842-84. References listed following each accomplishment are the papers published concerning those areas. Each of the areas listed were fully completed, with the exception of the goals related to accomplishment 3, which was limited by the decrease in funding from the amount of our original request.

RECEIVED
1984-11-13

1) Uptake and transport of IBA and auxin conjugates in plant tissues (16,17,19).

By understanding the metabolic changes that occur following treatment of plants it is now possible to devise methods for propagation of difficult-to-root species in a way that is predictable that will and optimize the effectiveness of the growth regulator treatment.

2) Identification of IBA and auxin metabolites found in diverse plant species (1,2,3,4,19,21).

The identification of metabolites provided insight into the design and synthesis of potential new rooting agents and should provide direction for the continued biorational design of new rooting agents.

3) Enzyme studies of proteins catalyzing hydrolysis of auxin conjugates (7,15).

Conjugated forms of growth regulators appear to offer advantages for initiating rooting in selected species. Isolation and characterization of the enzyme(s) responsible for hydrolysis should ultimately increase our understanding of those processes and increase our ability to design and predict the effect of new rooting agents applied in conjugated form.

4) Isolation of IBA as a natural constituent of plant materials (12,13,14).

The finding of the natural occurrence of IBA opens up the possibility of explaining many agricultural problems thought to involve auxin but found to poorly correlate with IAA levels. In addition, new demands for evaluation and registration of IBA for continued use in agriculture will be simplified and less complex now that it is shown to be a natural product.

5) Testing of IBA conjugates for use in rooting (17,19,21).

IBA treatment results in the formation of IBA and IAA conjugates and those compounds were found to be resistant and effective on rooting agents. Knowledge of the specific metabolism of auxin conjugates will provide a powerful tool in developing techniques for rooting specific crop plants.

6) Treatment of mother plants to improve rooting (18,20).

Improved rooting of some plants can be obtained by prolonging the period of more juvenile growth and we found that chemical treatment with butrazol increases the utility of auxin induction of rooting.

PUBLICATIONS

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12. Epstein E, L Muszkat, JD Cohen 1988 Identification of indole-3-butyric acid (IBA) in leaves of cypress and corn by gas chromatography and gas chromatography-mass spectrometry (GS/MS) Alon Hanotea 42:917-919 (In Hebrew)
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17. Wiesman Z, J Riov, E Epstein 1988 Comparison of movement and metabolism of IBA and IAA in mung bean cuttings. The 13th International Conference on Plant Growth Substances, 282 Calgary, Alberta, Canada
18. Wiesman Z, J Riov, E Epstein 1988 Paclobutrazol and urea phosphate in the rooting of spring cuttings of peach cv Almog HaSadeh 68:2142-2143 (in Hebrew)
19. Wiesman Z, J Riov, E Epstein 1988 Comparison of movement and metabolism of indole-3-acetic acid and indole-3-butyric acid in mung bean cuttings Physiol. Plant. 74:556-560
20. Wiesman Z, J Riov, E Epstein Effect of paclobutrazol and urea phosphate on rooting and survival of peach "Maravilah" softwood cuttings. HortScience (In press)
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