

4



BARD

FINAL REPORT

PROJECT NO. I-908-85

Chemical Identification, Physiological Characterization and Horticultural Applications of a Rooting Promoter Extracted from Avocado Tissues

M. Raviv, K. Mudge, D. Becker, N. Bassuk

6

630.72

BAR/RAV

2nd copy

1990

33546



000000161817

Standard BARD Cover Page for Scientific Reports

Date: _____

BARD
P.O. Box 6
Bet Dagan, ISRAEL

BARD project No. I-908-85

Title

Chemical identification, physiological characterization and horticultural applications of a rooting promoter extracted from avocado tissues

Investigators' Names

Investigators' Institutions

M. Raviv
K.W. Mudge
D. Becker
N. Bassuk

A.R.O. Neve Ya'ar Research Station
Cornell University
Technion - Haifa
Cornell University

Project's starting date: August 10, 1986

Type of Report: 1st Annual ☐ 2nd Annual ☐ Final ☒

הספרייה המרכזית
למדעי החקלאות
בית-7

Michael Raviv
Signature
Principal Investigator

Yuval Eshdat
Signature
Dr. YUVAL ESHDAT
Head, Dept. for Research Projects and Budgets
Agricultural Research Organization
Institution's Authorizing Official

630.72 : 581.134.1 : 581.134.3 :
BAR/RAV
and copy 634.653

Outline

Page

Acknowledgments

i

Abstract

ii

1. General introduction

1

2. Detailed description of the objectives and results.

1

2.a. Chemical identification of ARP.

1

2.b. Organic synthesis of ARP.

13

2.c. Development of an immunoassay for ARP based on time resolved fluorescence.

19

2.d. Does ARP-like activity exist in plants other than avocado?

24

2.e. Can ARP stimulate rooting of cuttings of plant species besides avocado?

36

2.f. Can ARP improve propagation of plants via tissue culture?

46

2.g. Anatomical and histochemical study of ARP stimulated rooting.

59

2.h. ARP's translocation in plant tissues.

73

2.i. ARP's mode of action.

78

3. General conclusions.

87

4. Obtained vs. expected results and their contribution to agriculture in Israel and in the United States.

88

Acknowledgements

We thank Prof. E.E. Goldschmidt, Prof. Y. Riov, Dr. O. Reuveni and Dr. O. Sagee for fruitful discussions and help in laboratory techniques. Thanks are also due to Dr. B. Steinitz for some stimulating ideas, to Prof. W.P. Hackett for hosting one of us (M.R.) while writing this report and for Ms. H. Ward for skillfull typing work.

Abstract

A rooting promoter which was extracted from avocado leaves (ARP) was chemically identified, synthesized and its steric structure was determined. The native compound has two active optical centers. The molecule is fully active only when the two centers are in the R configuration. The racemic mixture, synthesized initially, has therefore, only half the activity of the native or the stereospecifically synthesized compound. A GC analysis method was developed and ARP existence was demonstrated in several species.

An immunoassay technique based on time resolve fluorescence was also developed.

An anatomical work accompanied a time course of ARP's vs. IBA's activity using mung bean hypocotyl cuttings. It was shown that following application ARP-induce primordia develop faster than IBA-induce primordia.

A preliminary work on the mode of action of ARP suggests that ARP increases the sensitivity of the target cells to auxin.

ARP affects positively adventitious root formation on some horticulturally-important crops. Recommendations for use cannot be given at this stage due to the preliminary nature of the results.

1. General Introduction

This report will follow the objectives of the project as outlines in the proposal. Each section will be preceded by citation of the relevant objective and concluded with our view of the obtained vs. sought for results.

Some additional subjects which emerged during the course of the research although not anticipated in advance will be presented in a separate section.

2. Detailed description of the objectives and results

2.a. Chemical identification of ARP

D. Becker, Y. Sahali, and M. Raviv

2.a.I. Objective

Our goal will be to isolate and identify the active compound(s) in ARP. In order to obtain good separation of the unpolar mixture, we have to apply liquid chromatography, high pressure chromatography and gas chromatography. The mixture has been obtained in quantities of 1 gram per 10 kg of avocado seeds. The structure of the isolated compounds will be determined by spectroscopic methods as Infra-Red, Nuclear Magnetic Resonance, Mass Spectroscopy and if necessary we can make a crystalline derivative and determine the structure by x-ray analyses. In case the structure of the active compound will be unknown, it might be important to carry out total synthesis. It is assumed that most of the separations and partially the structure determination could be accomplished within a year.

2.a.II. Results

The chemical identification of ARP was published after its completion in "Plant Growth Regulation" 4:371-374 and is reproduced here with minor omissions.

The chemical identification of root promoters extracted from avocado tissues

M. Raviv, D. Becker, and Y. Sahali

Abstract

From the Avocado Rooting Promoter (ARP) 4 compounds were isolated and identified as:

II - 1 acetoxy - 2,4 dihydroxy-n-heptadeca-16-en;

III - 1 acetoxy - 2,4 dihydroxy-n-heptadeca-16-yn;

2I - 1,2,4 trihydroxy-n-heptadeca-16-en;

2II - 1,2,4 trihydroxy-n-heptadeca-16-yn.

The rooting activity of the pure compounds was verified using the mung bean rooting bioassay. Compound 2II is the most active.

Introduction

Endogenous factors determining rooting ability of cuttings have been thoroughly investigated during the last sixty years.

The dedifferentiation of mature cells, enabling their division and primordium initiation, is considered as the most crucial step along the whole rooting process, yet the primary stimulus to the realization of this stage is still unknown [7]. IAA, the most studied rooting hormone enhances primordium development. It seems, however, that exogenous IAA has no positive effect on rooting if applied only at the time when cell dedifferentiation takes place [4, 8, 12, 13, 15, 16].

It was also found that the rise in endogenous levels of IAA occurs well after the completion of the dedifferentiation stage [3, 20].

Several other substances have been suggested as dedifferentiation inducers but no conclusive evidence was found as to their function and generality in the plant kingdom [11, 18]. The concept of various "Wound hormones" such as traumatic acid [5] or oligosaccharrines [1] cannot give an explanation to the numerous events of adventitious root formation on intact plants.

In a previous study [14] a substance(s) was found in methanolic extracts of avocado leaves which exhibited root promoting activity and its concentration in various clones correlated with their rooting

ability. The substance(s) was called Avocado Rooting Promoter (ARP). In a timecourse study, it was found that ARP is active in rooting of mung bean cuttings long before IAA could reveal its activity [15]. It also exhibited an inhibitory activity in two bioassays for auxin activity. It was postulated that ARP may activate cell dedifferentiation in mung bean and presumably in tissues of other species.

The aim of the present study was to chemically identify ARP and to verify the activity of the pure substance(s).

Materials and methods

Isolation and structure identification

ARP was extracted from avocado seeds as previously described. It was further subjected to flash chromatography using silica-gel G-60 (Merck). Up to 10 grams fresh weight can be chromatographed using hexane-ethyl acetate mixtures on 25 grams of silica-gel. The active fractions were eluted by a 2:1 hexane:ethyl acetate mixture to give two distinct fractions which were subsequently separated by AgNO_3 solution to olefins and acetylenes according to Kashman et al. [10]. This procedure yielded four fractions:

- II - 1 acetoxy - 2,4 dihydroxy-n-heptadeca-16-en;
- III - 1 acetoxy - 2,4 dihydroxy-n-heptadeca-16-yn;
- 2I - 1,2,4 trihydroxy-n-heptadeca-16-en;
- 2II - 1,2,4 trihydroxy-n-heptadeca-16-yn.

The compounds were identified using I.R., N.M.R. and M.S., Fractions II and III were compared with authentic samples.

Biological test

The pure substances were dissolved in ethanol. Water were added up to 1% ethanolic solutions. Mung bean rooting bioassay [9] served for the evaluation of the rooting activity of the various fractions. Preliminary checks and routine water controls showed that 1% ethanol in water has no effect on the test's results.

Only visible roots (> 1 mm) were counted. Each test were consisted of 20 cuttings per treatment. The tests were replicated 4 times and the results presented in table 1 are the average of the 4 tests.

Table 1. The effect of various ARP fractions on the rooting of mung bean cuttings as compared to control = 100%

Concentration	Fraction		Concentration	Fraction	
	II	III		2I	2II
$3 \cdot 10^{-7} \text{M}$	78	117	$3.5 \cdot 10^{-7} \text{M}$	100	123
$3 \cdot 10^{-6} \text{M}$	105	119	$3.5 \cdot 10^{-6} \text{M}$	111	166
$1.5 \cdot 10^{-5} \text{M}$	134	207	$1.75 \cdot 10^{-5} \text{M}$	134	280
$3 \cdot 10^{-5} \text{M}$	225	237	$3.5 \cdot 10^{-5} \text{M}$	142	*255
$1.5 \cdot 10^{-4} \text{M}$	*109	*83	$1.75 \cdot 10^{-4} \text{M}$	*248	*136

*Overdose symptoms

Results and discussion

The biological activity of tested fractions, as manifested by its rooting activity is presented in Table 1.

From the presented results it can be concluded that compounds containing an acetylenic moiety are more active than those containing an olefinic moiety.

Between the acetylenic compounds fraction 2II is more active, showing pronounced activity even at low concentrations. Fraction II, which is identical to the growth inhibitor extracted from avocado mesocarp by Gazit and Blumenfeld [6] and identified by Bittner et al. [2] is the least active in terms of rooting activity. Its occurrence in crude ARP probably explains the anti-auxinic activity which was observed in previous studies (14,15).

In higher concentrations, rooting activity was found in all the tested fractions. The possibility that this phenomenon may result from spontaneous transformations in the tested solutions of one compound to the others, occurring during the rooting period was tested by T.L.C. of the various solutions and may be ruled out (data not shown). Another possibility which has yet to be tested is that such transformations take place within the cutting's tissues.

The existence of closely related compounds which act as growth promoter in one form and as growth

inhibitor in the other is a reminiscent of the penol- > o-di-phenol system which profoundly affects auxin-induced growth (19).

The involvement of the above mentioned compounds in the control of such different functions as rooting and fruit growth (6) may indicate that this group of compounds has a certain role in the control of development processes which has yet to be studied.

References

1. Albersheim P and Darvill AG (1985) Oligosaccharins. *Sci Amer* 253 (3):44-50
2. Bittner S Gazit S and Blumenfeld A (1971) Isolation and identification of a plant growth inhibitor from avocado. *Phytochem* 10:1417-1421.
3. Blahova M (1969) Changes in the level of endogenous gibberellins and auxins preceding the formation of adventitious roots on isolated epicotyls of pea plants. *Flora* 160:493-499
4. Eliasson L and Arebald K (1984) Auxin effects on rooting of pea cuttings. *Physiol Plant* 61:293-297
5. English J Jr Bonner J and Haagen Smit AJ (1939) The wound hormones of plants. II. The isolation of a crystalline active substance. *Proc Nat Acad Sci Wash* 25:323-329
6. Gazit S and Blumenfeld A (1970) Cytokinin and inhibitor activities in avocado fruit mesocarp. *Plant Physiol* 46:334-336
7. Haissig BE (1974) Origins of adventitious roots. *N Z J For Sci* 4:299-310
8. Haissig BE (1974) Influence of auxins and auxin synergists on adventitious root primordium initiation and development. *N Z J For Sci.* 4:311-323
9. Hess CE (1965) Rooting co-factors, identification and functions. *Proc Intl Plant Prop Soc* 15:181-186
10. Kashman Y Ne'eman I and Lifshitz A (1969) New compounds from avocado pear. *Tetrahedron* 25:4617-4631
11. Mitsuhashi M Shibaoka H and Shimokoriyama M (1969) Portulal: A rooting promoting substance in *Portulaca* leaves. *Plant and Cell Physiol* 10:715-723
12. Mitsuhashi M Shibaoka H and Shimokoriyama M (1969) Morphological and physiological characterization of IAA-less-sensitive and IAA-sensitive phases in rooting of *Azuki* cuttings. *Plant and Cell Physiol* 10:867-874
13. Mohammed S and Ericksen EN (1974) Root formation in pea cuttings. IV. Further studies on the influence of IAA at different developmental stages. *Physiol plant* 32:94-96
14. Raviv M and Reuveni O (1984) Endogenous content of leaf substance(s) associated with rooting ability of avocado cuttings. *J Amer Soc Hort Sci* 109:284-287
15. Raviv M Reuveni O and Goldschmidt EE (1986) Evidence for the presence of a native, non-

- auxinic rooting promoter in avocado (*Persea americana* Mill.). *Pl Growth Regulation*. 4:95-102
16. Sircar PK and Chatterjee SK (1973) Physiological and biochemical control of meristemization and adventitious root formation in *Vigna* hypocotyl cuttings. *The Plant Propagator* 19:17-26
 17. Tognoni F and Lorenzi R (1972) Acidic root-promoting growth inhibitor(s) found in *Picea* and *Chamaecyparis*. *J Amer Soc Hort Sci* 97:574-578
 18. Tognoni F and Lorenzi R (1983) Identification of root promoting substances from *Picea glauca* var. *albertiana*. *HortSci* 18:893-894
 19. Tomaszewski M and Thimmann KV (1966) Interaction of phenolic acids, metallic ions and chelating agents on auxin-induced growth. *Plant physiol* 41:1443-1454
 20. Weigel U Horn W and Hock B (1984) Endogenous auxin levels in terminal stem cuttings of *chrysanthemum morifolium* during adventitious rooting. *Physiol Plant* 61:422-428

Upon completion of the chemical identification of ARP the next step was to determine the steric configuration of the active molecule and to test the stereospecificity of the four possible diastereoisomers. This subject investigated, prepared and accepted for publication by "Phytochemistry". It is reproduced here with some minor omissions.

The Effect of Absolute Configuration of the Avocado Rooting Promoter on Its Rooting Activity

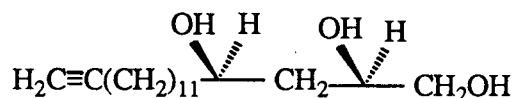
D. Becker, Y. Sahali, and M. Raviv

Abstract

All four diastereoisomers of the most active compound 2II of the Avocado Rooting Promoter (ARP) have been synthesized. Their root promoting activity was determined over the physiologically active concentration range. It was found that stereoisomer 2R, 4R exerts a rooting activity similar to that of the extracted and purified compound from avocado tissues. Stereoisomers 2R, 4S and 2S,4R had lower activity and stereoisomer 2S,4S had the lowest activity. It is concluded that the natural form, 2R,4R acts in the rooting process either in its original structure or after reaction which does not alter its chiral centers.

Introduction

In a previous paper, we described a native, non-auxinic rooting promoter extracted from various avocado tissues (1). It was later found that the partly purified fraction contains four closely related compounds which were identified and their root promoting activity was determined (2). It was found that compound 2II (scheme I) is the most active one. Ketones and furanes which may be formed by oxidation of I were also isolated from the avocado tissues (3). It was found that they have no rooting activity (unpublished data).

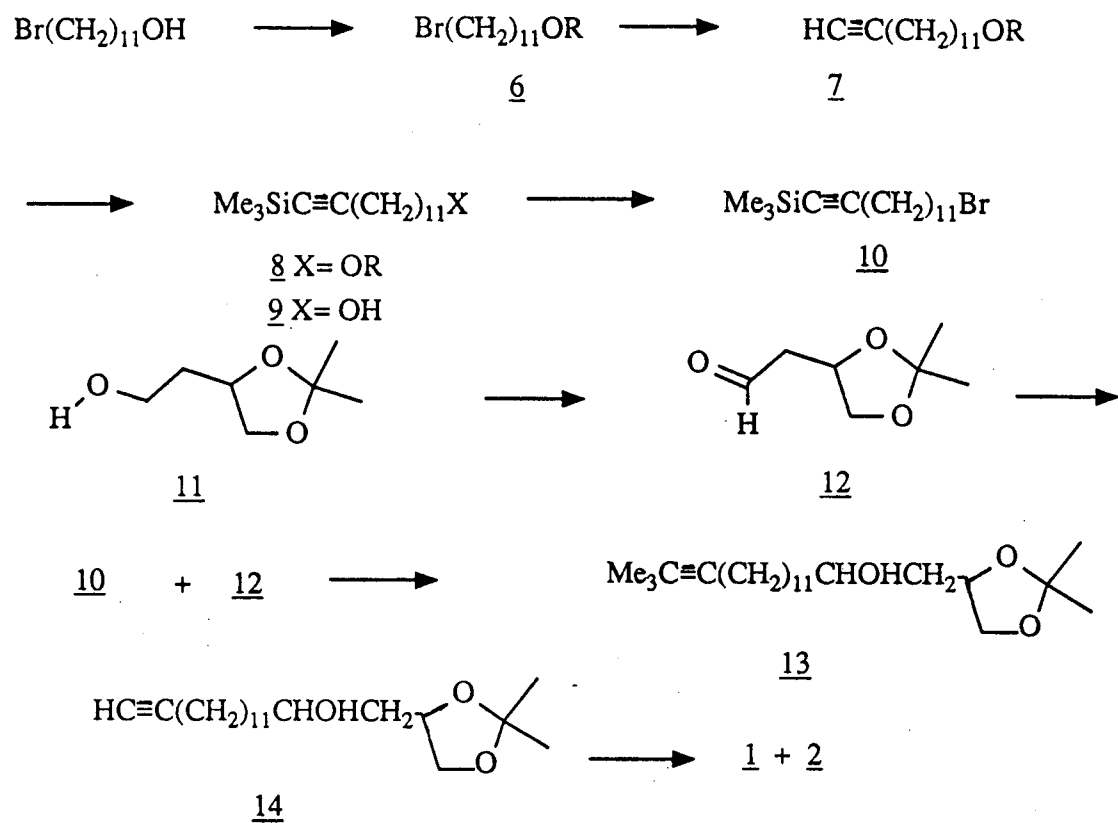


Scheme I

The purpose of the present study is to clarify the relation between the absolute configuration and the biological activity. All four possible stereoisomers of compound 2II were synthesized stereospecifically and their biological activity have been tested. The results may have theoretical and practical consequences.

Results and Discussion

The structure of compound 2I has been determined by Kashman (3). The chiral centers were determined by Sugiyama (4) to be 2R,4R-16-heptadecen-1,2,4-triol. We have modified the synthesis of 2I to the synthesis of 2II in the following way. A Grignard condensation between a four carbon chain carrying two protected alcohols to a thirteen carbons unit having protected terminal acetylenic group, led in one step to the skeleton of the target molecule in the proper oxidation state. The sequence of reactions employed for the preparation of the two units is described in scheme II. Having an (R) center in 11 enabled us to prepare by this route the two diastereomers 1 (2R,4R) and 2 (2R,4S) in a 43:57 ratio. The mixture was separated by chromatography to give two pure 1 and 2. The second pair of stereoisomers 3 (2S,4S) and 4 (2S,4R) were prepared by using (S) 11 as a starting material. The four new compounds were identified by spectroscopic methods and their purity was established by GC to be over 98%. The experimental part describes the sequence of reactions carried out on 11 (R), the same results were obtained for 11 (S).



Scheme II

The stereoselective synthesis of all four stereoisomers of 2II has been carried out successfully. The new compounds were purified and tested by known bioassay (5,6).

Table 2.* Root promoting activity of the 4 synthetic stereoisomers of compound 2II and the native compound (Average no. of roots/cutting of 3 replicates, percent of control = 100%).

Compound	Synthetic				Native
<u>Conc. (M)</u>	<u>(2R,4R)</u>	<u>(2R,4S)</u>	<u>(2S,4R)</u>	<u>(2S,4S)</u>	<u>(2R,4R)</u>
0	100	100	100	100	100
5.10-6	140	119	135	124	149
1.10-5	212	153	169	113	240
2.10-5	379	218	231	194	347

*The running numbers of the tables appearing in the report were arranged in a consecutive form.

It was found that the absolute configuration of the chiral centers at C-2 and C-4 does influence the activity of 2II as a rooting promoter. It can be concluded that the active compound that penetrates into the plant is chiral. The active site in the plant can tolerate better variation in 2II on carbon 2 than on carbon 4. The possibility that 2II is oxidized, to a ketone or furan, in the plant before it acts can be excluded as well. From a practical point of view, it seems that maximum efficiency will be obtained by using 2II either from natural sources or from a new cheap chiral synthesis which has yet to be developed.

Experimental

The instruments which were used: ¹H NMR, Varian T-60, Bruker WP-400; IR, Perkin-Elmer 257; GC Hewlett-Packard 5890, carrier gas helium, capillary column OV-101 25 m; HRMS, Varian MAT-711; Silica-gel Merck (250-400 mesh) keieselgel 60.

0.2 g of 1-Tetrahydropyrane-11-bromoundecane ether 6 : p-Toluenesulphonic acid and 10.1 g of 3,4-dihydropyrane (120 mM) were added to soln of 25.1 g (100 mM) 11- Bromo-1-undecanol (commercially available) in 100 ml of dry ether. The reaction was kept over night at room temp.

washed with 10% NaHCO_3 , H_2O , and dried (Na_2SO_4). The solvent was removed by reduced pressure to give, after chromatography over silica-gel eluting with hexane, 30.8 g of 6 in 90% yield.

1-Tetrahydropyrane-12-tridecyne ether 7: 1.5 equivalent of lithium acetylide was prepared according to Brandsma (7) in liquid ammonia. A soln of 10 g (30 mM) of 6 dissolved in 10.8 g of HMPA (2equ.) was added to the acetylide soln at -70°C and stirred for three hr, the cooling bath was removed and the reaction was stirred over night at room temp. Saturated soln of NH_4Cl (50 ml) were added and the organic material was extracted with hexane (4x30 ml), washed with H_2O and dried (Na_2SO_4). The solvent was removed by reduced pressure to give 6.6 g of 7 in 80% yield. IR 3320. ^1H NMR (60MHz, CCl_4) δ 4.4 (bs, 1H), 3.68-3.03 (m, 4H), 2.2 (m, 2H), 1.86 (1t, 1H).

13-Trimethylsilyl-1-Tetrahydropyrane-12-tridecyne ether 8: Soln of n-BuLi 10.8 ml (2.3M in cyclohexane) was added to soln of 6.5 g (23.6 mM) of 7 in 30 ml dry ether at 0°C and stirred at that temp. for 90 min. at room temp. The reaction was cooled to 0°C and 2.8 g (25 mM) of trimethylchlorosilane were added and stirred for one hr at 0°C and then for one hr at room temp. Water (50 ml) was added and the reaction was extracted with ether (3X30 ml), washed with brine (30 ml) and dried (Na_2SO_4). The solvent was removed by reduced pressure to give 7.6 g of 8 in 92% yield. IR 2178. ^1H NMR (60 MHz, CCl_4) δ 4.4 (bs, 1H), 3.85-3.05 (m, 4H), 2.1 (m, 2H), 0.15 (s, 9H).

13-Trimethylsilyl-12-tridecyn-1-ol 9 Ether 8 : 7.5 g (21.6 mM) was dissolved in 50 ml of MeOH, 0.1 g of p- toluenesulphonic acid were added and the soln was stirred over night at room temp. The acid was quenched by solid NaHCO_3 , the solids were removed by filtration and the solvent was removed by reduced pressure to give 5.1 g of 9 in 90% yield. IR 2189, 3640. ^1H NMR (60 MHz, CCl_4) δ 3.4 (t, 2H), 2.1 (m, 2H), 0.15 (s, 9H).

13-Trimethylsilyl-1-bromotridecyne 10: Methansulphonyl chloride 5.6 ml (24.3 mM) was added during 12 min to soln of 5 g (18.9 mM) of alcohol 9 and 3.37 ml (24.26 mM) of triethylamine in 25 ml of dry CH_2Cl_2 at 0°C . The reaction was stirred at 0°C for 30 min. and 2 hr at room temp., water (25 ml) was added and the organic layer was separated. The organic phase was washed with 1N HCl (20 ml), brine (20 ml), and dried (Na_2SO_4). The solvent was removed at reduced pressure to give crude mesylate in 95% yield. The crude mesylate was dissolved in 30 ml of dry THF and 2.9 g (33 mM) of dry lithium bromide were added at 0°C . The cooling bath was removed and the reaction was refluxed for two hr. Water (30 ml) was added, the solvents were removed by reduced pressure. The organic material was extracted by hexane (3X30 ml), washed with brine (20 ml) and dried (Na_2SO_4). The solvent was removed by reduced pressure to give 4.5 g of 10 in 73% yield. ^1H NMR (60 MHz, CCl_4) δ 3.2 (t, 2H), 2.08 (m, 2H), 1.9 (t, 1H), 0.15 (s, 9H). HRMS M^+ Cal. for $\text{C}_{16}\text{H}_{31}\text{BrSi}$: 330.1378, found : 330.1376.

(4R)-2,2-Dimethyl-1,3-dioxalane-4-ethanol 11: p-toluenesulphonic acid 0.2 g were added to soln of 27 g (0.26 mM) of (2R)-1,2,4-butanetriol (commercially available) in 350 ml of Me₂CO. The soln was kept at room temp. over night, the acid was quenched by NaHCO₃ (s) and the solids were removed by filtration. The solvent was removed by reduced pressure and the crude was distilled (BP 105-110° at 25 mmHg) to give 28 g of 10 at 74% yield. ¹H NMR (60 MHz, CCl₄) δ 4.3-3.23 (m, 5H), 1.73 (q, 2H), 1.34 (s, 3H), 1.3 (s, 3H).

(4R)-2,2-Dimethyl-1,3-dioxalane-4-ethanal 12 : Oxally chloride 1.53 g (21 mM) was dissolved in 25 ml of dry CH₂Cl₂ and the soln was transferred to three necked flask equipped with thermometer and two dropping funnels. The soln in the three necked flask was cooled to -60°C, soln of 1.9 g DMSO (10.1 mM) in 5 ml CH₂Cl₂ was added during five min and stirred for 10 min at -60°C. The second soln, 1.5 g (10.1 mM) of alcohol 11 in CH₂Cl₂, was added keeping the temp. below -50°C, the mixture was stirred for 15 min and 1.1 g (55 mM) ethyl diisopropyl amine was added and the cooling bath was removed. Water (30 ml) was added at room temp. and the organic material was extracted by CH₂Cl₂ (3X10 ml). The organic phase was washed with cold 10% HCl, H₂O and dried over Na₂SO₄. The solvent was removed at reduced pressure to give 1.3 g of crude 12 which was purified over silica-gel column using hexane-CH₂Cl₂ 8:1 as eluant to give 1.2 g of 12 in 76% yield. IR 1735. ¹H NMR (60 MHz, CCl₄) δ 4.43-3.86 (m, 2H), 3.4 (dd, 1H), 2.6 (m, 2H), 1.33 (s, 3H), 1.3 (s, 3H).

(2R,4RS) 1,2-Acetonide-17-trimethylsilyl-16-heptadecyn-1,2,4-triol 13 : Grignard reagent was prepared as usual from 1 g of Bromide 10 (3.06 mM) and 90 mg of magnesium in 5 ml dry Et₂O. The reagent was cooled to 0°C and soln of 445 mg of 12 (3.09 mM) in 2 ml of Et₂O was added drop wise. The reaction was stirred over night at room temp., 5% NH₄Cl (20 ml) were added. The organic material was extracted by Et₂O (3X10 ml), washed with H₂O (15 ml) and dried over Na₂SO₄. The solvent was removed and the crude was purified over Florisil column using hexane-CH₂Cl₂ (2:1) as eluant to give 780 mg of 13 in 65% yield. IR 3520, 2180, 1380. ¹H NMR (400 MHz, CDC13) δ 4.08 (dd, 1H), 3.81 (m, 1H), 3.55 (dd, 2H), 2.18 (t, 2H), 1.66 (m, 2H), 1.42 (s, 3H), 1.36 (s, 3H), 1.26 (wm, 18H), 0.15 (s, 9H). HRMS molecular weight M+ Cal. for C₂₃H₄₄O₃Si 396.3123, found: 396.3120.

(2R,4RS) 1,2-Acetonide-16-heptadecyn-1,2,4-triol 14: To soln of 700 mg of 13 (1.78 mM) in 3 ml of dry THF 2.2 ml of tetrabutylammonium fluoride (1M in THF) were added at room temp. and stirred for 10 min. Water (10 ml) were added and the product was extracted by Et₂O (5X10 ml), washed with brine and dried over Na₂SO₄. The solvent was removed by reduced pressure to give 510 mg of oily compound 14 in 90% yield. IR 3520, 1320. ¹H NMR (400 MHz, CDC13) δ 4.08 (dd, 1H), 3.81 (m, 1H), 3.55 (dd, 2H), 2.16 (dt, 2H), 1.93 (t, 1H), 1.66 (m, 2H), 1.42 (s, 3H), 1.36 (s, 3H),

1.26 (wm, 18H).

(2R,4RS) 16-heptadecyn-1,2,4-triol 1+2: A soln of 500 mg of 14 (1.56 mM) and 50 mg of p-toluenesulphonic acid in 5 ml of MeOH was kept for 10 hr at room temp. The acid was quenched with NaHCO_3 (s) and the solids were filtered. The solvent was removed by reduced pressure to give 375 mg of solid mixture of 1+2 in 43 : 57 ratio respectively. The isomers ratio was determined by GC. The alcohols were converted to the corresponding silyl derivatives (by O,N-bis (trimethylsilyl)-trifluoroacetamide) before the analysis. The synthetic 1 was found to be identical to 1 from natural sources by GC IR and NMR. Diastereomers 1 and 2 were separated over silica-gel column, as eluant was used hexane- Me_2CO in (2 : 1) ratio, 1 is less polar than 2. For activity test the samples were over 98% pure according to GC analysis.

Biological Test

Rooting of mung bean cuttings as described by Hess (5) and modified by Raviv et al. (6) used in order to evaluate the rooting activity of the tested compounds. concentrations were in the range of $1 \times 10^{-6} \text{M}$ - $5 \times 10^{-5} \text{M}$. The lowest concentration is close to the actual concentration in the plant tissue (Raviv et al., unpublished) and has no activity when applied exogenously. The highest concentration causes overdose symptoms. The presented concentration range is therefore $5 \times 10^{-6} \text{M}$ - $2 \times 10^{-5} \text{M}$. The bioassay was replicated 3 times with 20 cuttings per treatment in each replicate. The presented results are the average of the three experiments expressed as percent of control = 100%. Actual number of roots in the water control were 5-6 roots/cutting with SE lower than 0.35 in all cases.

References

1. Raviv M Reuveni O and Goldschmidt EE (1986) Plant Growth Reg. 4:95-102.
2. Raviv M Becker D and Sahali Y (1986) Plant Growth Reg. 4:371-374.
3. Kashman Y Ne'eman I and Lifshitz A (1969) Tetrahedron 25:4617-4631.
4. Sugiyama T Sato A and Yamashita K (1982) Agric. Biol. Chem. 46:481.
5. Hess CE (1965) Proc. Intl. Plant Prop. Soc. 15:181-186.
6. Raviv M and Reuveni O (1984) J. Amer. Soc. Hort. Sci. 198:284-287.
7. Brandsma L Preperative Acetylenic Chemistry, Amsterdam, New-York, Elsevier Pub. (1971)

2.a.III. Conclusions

It is our understanding that the chemical identification of ARP was completed. During the course of this project, it was found that other compounds extracted from various plant species, and behave

chromatographically similar to ARP exist. It can be therefore concluded that ARP is an example for a new class of plant growth regulators which has yet to be identified and investigated. This task, however, was beyond the scope of this project.

2.b. Organic synthesis of ARP

D.Becker and Y. Sahali

2.b.I. Objectives

For preliminary biological tests, the fastest and cheapest way to obtain the active known compounds will be to separate them from Avocado seeds. Seeds are available during the season in kilogram quantities. However, for further applications, it is important to develop a total synthesis from readily available starting materials.

One possibility which will be tested is to use the nonactive olefins as precursors for the preparation of the active acetylene.

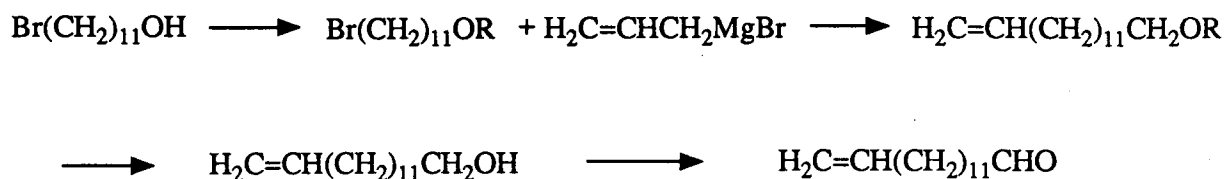
2.b.II. Results

Our first assignment was to supply active material for the biological tests. The source for the active compounds was Avocado seeds. The seeds were vacuum dried and grounded to fine powder in kg. scale.

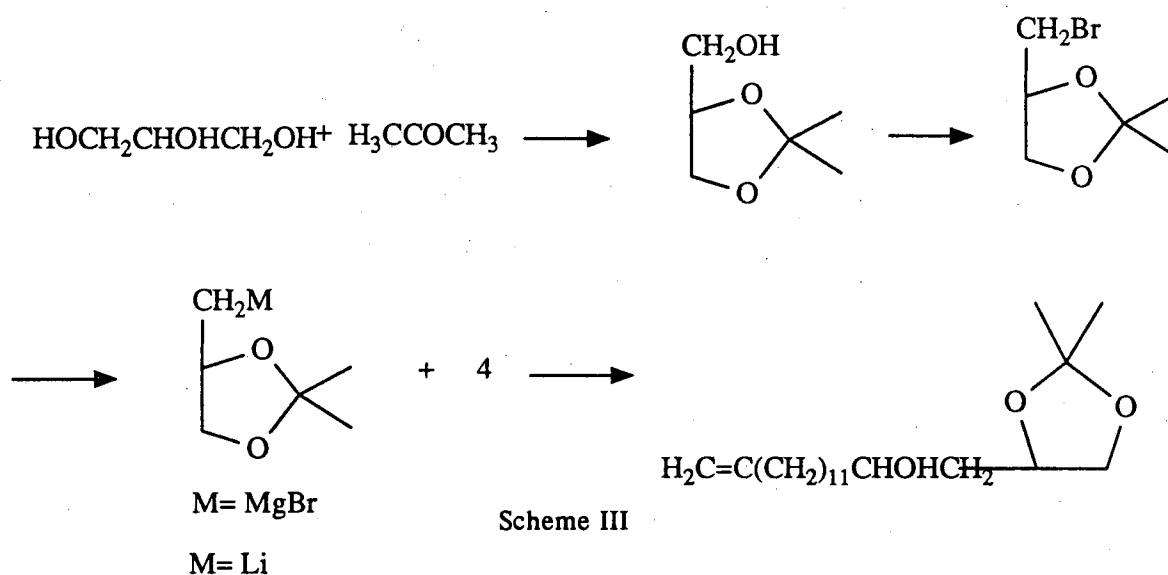
In order to produce the large quantities needed for the physiological and horticultural parts of this study, we tried to increase the yields of the extraction procedure. A continuous extraction with numerous solvents was carried out but none was superior to boiling the plant material in hexane followed by crystalizing the first crop of the active compounds. A yield increase was achieved, however, by hydrolyzing the mixture of the four active compounds (1I, 1II, 2I, and 2II) to give a mixture of 2I and 2II. The separation of the most active compound (2II) was done using its silver salt.

Total organic synthesis of ARP took about 2 years to be accomplished, after experimenting several unsuccessful routes. Part of the way will be described below.

The first approach is described in scheme III.



4

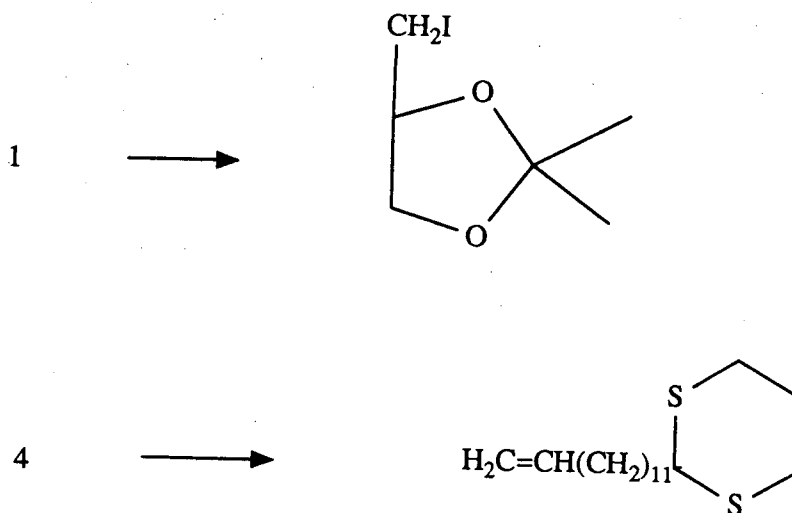


Scheme III

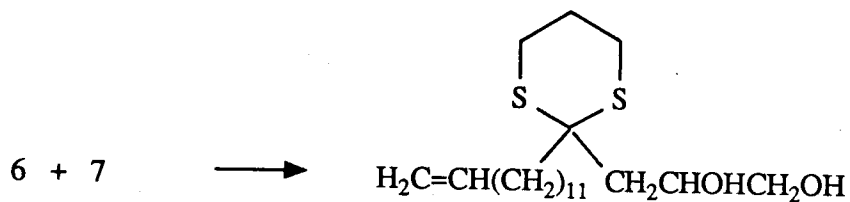
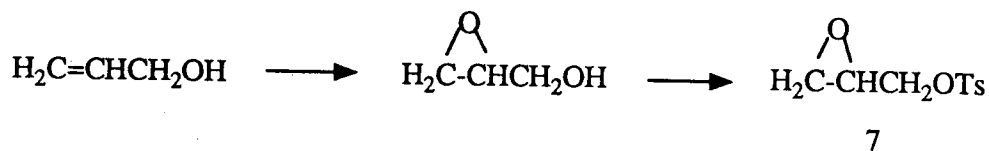
The fact that chiral ketals of glycerol are commercially available makes this route particularly attractive. The methodology was tested naturally on racemic mixture which was prepared from glycerol and acetone in the usual way. The ketal alcohol 1 was converted via the mesylate to the corresponding bromide 2 in good yield and the organometal reagent 3 was prepared by known methods. The aldehyde 4 was made from bromohydrine C₁₁H₂₂ as described in scheme III in good yield. To our disappointment, we could not isolate the expected product from the reaction mixture by reacting the aldehyde 4 and the organometallic reagent 3. It is known in the literature that having an oxygen in the vicinity of Grignard reagent might interfere with its efficiency. In some cases, keeping the temperature below 25°C and working with excess of magnesium should solve the problem. In our system, this was not the case.

In scheme IV, we describe an alternative approach based on umpolung the activity of the aldehyde by converting the carbonyl to thioacetal. A condensation between the carbanion with the corresponding iodide made from ketal of glycerol will form the expected compound. Thioacetal 6 was made in good yield from the aldehyde 4 and iodide 5 were condensed in the usual way, but we could not identify the expected product in the reaction mixture. It is known that thioacetal reacts slow and some time in very low yield. We have tried to change conditions such as temperature and solvents but nothing changed the negative results.

Another approach is described in scheme V. The ketal iodide 5 was replaced by tosyl epoxide 7 that is expected to be more reactive toward nucleophilic substitution. Another advantage to work with epoxides is a result of modern techniques that were developed to prepare chiral epoxyalcohols from the corresponding olefin. We have made the epoxide and it seems that we got new products in the alkylation. Unfortunately, none of these products was identical to ARP.

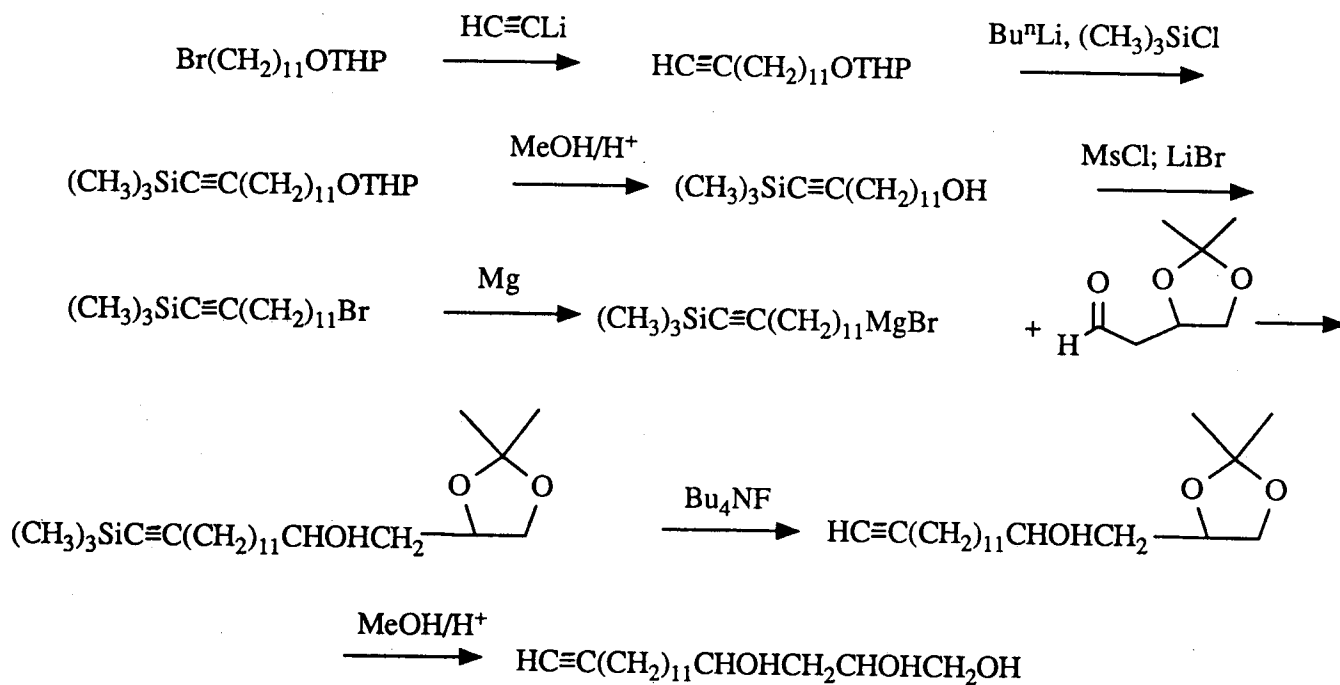


Scheme IV



Scheme V

The synthesis which finally yielded a mixture of the four optical isomers of compound 2II is described in scheme VI.



Scheme VI

The above described organic synthesis of compound 2II is long and expensive.

The optical mixture of compound 2I can be obtained by reducing 2II with Lindlar catalyzator. The rooting activity of the synthetic racemic mixture of compound 2II was tested against the native compound and the results appear in Table 3.

Table 3. Average number of roots/mung bean cutting as affected by extracted vs. synthetic compound 2II.

Source	Synthetic	Extracted
Concentration		
(M)		
0	6.0 d*	6.0 d
5.10-7	6.1 d	6.7 d
5.10-6	6.3 d	8.5 d
1.10-5	6.9 d	10.5 d
2.10-5	10.8 d	17.4 c
5.10-5	19.3 bc	30.8 a**
1.10-4	24.4 b**	24.5 b**

*Figures following with the same letter are not statistically different at the 5% level of confidence.

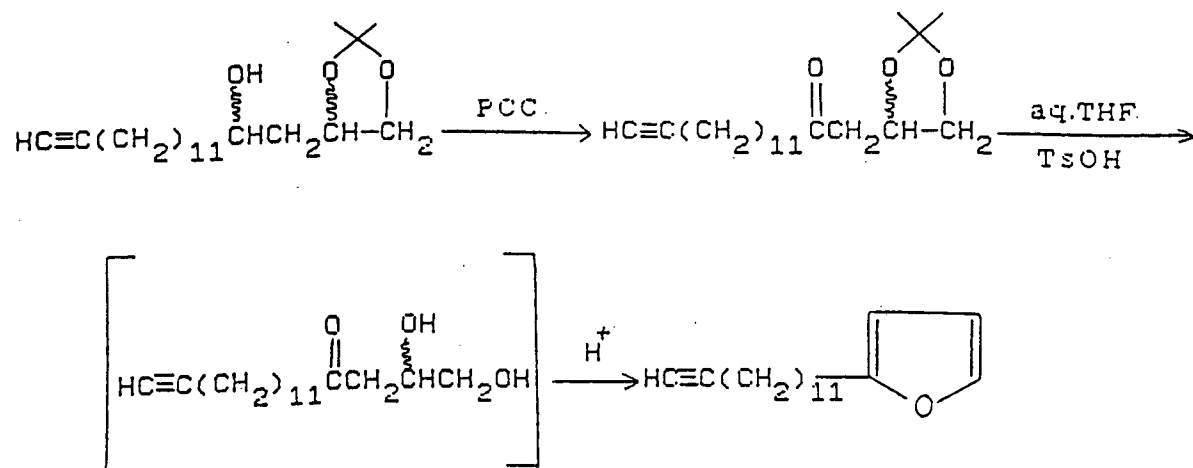
**Over dose symptoms.

These results indicate a clear stereospecificity of the rooting process therefore the four diastereomers of compound 2II were prepared and tested for biological activity. The results appear in section 2.a.II.

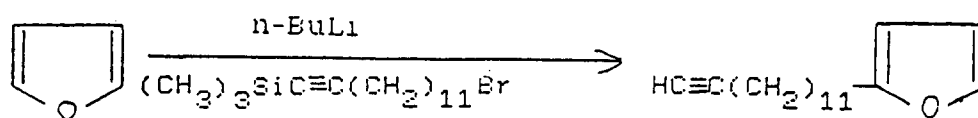
The importance of both optical centers (on carbons 2 and 4) is evident suggesting that the root promoting activity resulted from compound 2II *per se* and not from any of its putative metabolic products.

Another approach to test this conclusion used one of its putative metabolites - the furan.

The furan was prepared according to Schemes VII and VIII.



Scheme VII



Scheme VIII

The resulted compound was found as not active in the rooting bioassay. We also tested the possibility that this absence of activity is due to compartmentation and that exogenously applied furan cannot enter the target cells although it can be produced there from compound 2II.

Mung bean cuttings were tested for furan existence with or without ARP feeding. The furan was not found in the tissue, nor in the feeding solution. Altogether, these findings do not rule out completely the possibility that ARP serves as a precursor for as yet unknown another active compound. An enzymatic system which may take part in such a process should be characterized before testing further this hypothesis but this is beyond the scope of the present research.

2.b.III. Conclusions

Although being long and expensive, a laboratory method was developed to enable the organic synthesis of ARP. As a by-product of the process we gathered two lines of evidence suggesting that the steric structure of ARP is essential for its activity thus suggesting a role for ARP *per se* and not as a precursor in the rooting process.

This finding is of significance taking into account the special structure and the low water solubility of ARP which intuitively led us to the assumption that ARP should be converted to other, more mobile molecule in order to have a biological activity.

2.c. Development of an immunoassay for the avocado rooting promoter based on time resolved fluorescence.

F. Kohen, Dept. of Hormone Research, The Weizmann Institute of Science, Rehovot.

2.c.I. Objective

The original objective of this section of our research was to carry out radioisotope labeling of ARP so as to enable study of ARP translocation in plant tissues. Due to various technical and financial difficulties, it was not possible to complete this part of our proposal. Instead, we approached Dr. Fortune Kohen of the Dept of Hormone Research, Weizmann Institute of Science, and she kindly accepted to develop an immunoassay for ARP based on time resolved fluorescence. Enclosed herewith is her full report.

Rationale:

The purpose of this study was to develop a sensitive non-isotopic immunoassay for the Avocado Rooting Promoter (ARP). At present, four types of labels have emerged as major competitors to radiolabelling. These include particles (e.g. gold sols), enzymes, fluorophores (e.g. Fluorescein, umbelliferone, rare-earth chelates) and chemiluminophores (e.g. isoluminol, acridinium ester, etc.). Of these labels, the lanthanide chelates (e.g. Europium) are capable of yielding higher specific activities than commonly used radioisotopes and have different fluorescence emission wavelengths which can be discriminated from each other using appropriate filters (1). In addition, the fluorescence half-life of lanthanide chelates is up to 6 magnitudes longer than conventional fluorophores, and by using the principles of time-resolution, the fluorescence of these chelates can be distinguished from the inherent fluorescence of biological materials which has a very short half-life (nanoseconds). Consequently, the use of lanthanide chelates as labels in immunoassay procedures permitted the development of "in house" (i) sensitive competitive type immunoassays for the direct measurement of haptens (e.g. steroids, drugs, etc.) and two-site immunoassays for macromolecular molecules (e.g. polypeptide hormones, growth factors) based on biotin-avidin interaction (2); (ii) and non-separation immunoassays for urinary steroid metabolites (e.g. estrone-3-glucuronide) (3) for the delineation of the fertile period in women and for use in *in vitro* fertilization programs.

Accordingly, in pilot studies, we examined the feasibility of developing a non-isotopic immunoassay for ARP based on time-resolved fluorescence. For this purpose, ARP was rendered antigenic by coupling it to bovine serum albumin (BSA). ARP-BSA conjugate served as an immunogen to immunize rabbits. After three booster injections, the serum of the immunized rabbits were tested for antibody production. The preliminary results that we obtained are described in the following sections.

2.c.II. Results

Preparation of Avocado-Rooting-Promoter Carrier Conjugates

1,2,4-trihydroxy-n-heptadeca-16-yn, the most active compound of the ARP complex (4) was rendered antigenic by coupling the hydroxy groups to a macromolecular carrier (e.g. bovine serum albumin, egg albumin, etc.) via a hemisuccinate linking group using methods developed in our laboratory (5).

Generation of Antibodies of ARP-Carrier Conjugate

Two rabbits (#1 and #2) were immunized with the ARP-carrier conjugates using procedures developed in our laboratory (5). The rabbits were bled after the third booster injection, and the sera

were characterized for antibody titer using the procedure shown in flow sheet 1. Briefly, microtiter plates were coated with ARP-ovalbumin conjugate. The plates were then incubated with various dilutions of the bleedings obtained from the immunized rabbits. After washings, the plates were further incubated with donkey anti-rabbit-europium conjugate. The fluorescence of europium was then measured using the ARCUS time-resolved fluorometer. The serum of rabbit #1 showed titers ranging from 1:1000 to 1:4000, and was chosen for further studies.

Flow sheet 1. Flow sheet for check antibody titer for ARP by time-resolved fluorescence.

- 1) Coat microtiter plates with ARP-ovalbumin conjugate.
- 2) Incubate for 2 hrs with varying dilutions of sera of rabbits immunized with ARP-BSA.
- 3) Incubate for 2 hrs with donkey anti-rabbit-europium conjugate.
- 4) Add enhancement Solution
- 5) Measure fluorescence using an ARCUS time-revolved fluorometer.

Development of a competitive immunoassay for ARP based on time resolved fluorescence.

The serum of immunized rabbit #1 was chosen for this study. For this purpose, ARP-ovalbumin was absorbed to microtiter wells at two different concentrations, 52 ng and 125 ng/well respectively. Competitive binding reactions were then carried out using rabbit antibody to ARP at two different dilutions (1:2000 and 1:4000 respectively) and varying doses of unaltered ARP. After the immunological reaction, the plates were washed, and incubated for 2 hrs with donkey anti-rabbit-Europium conjugate. The plates were then washed and processed for time-resolved fluorescence. The results are shown in Fig. 1.

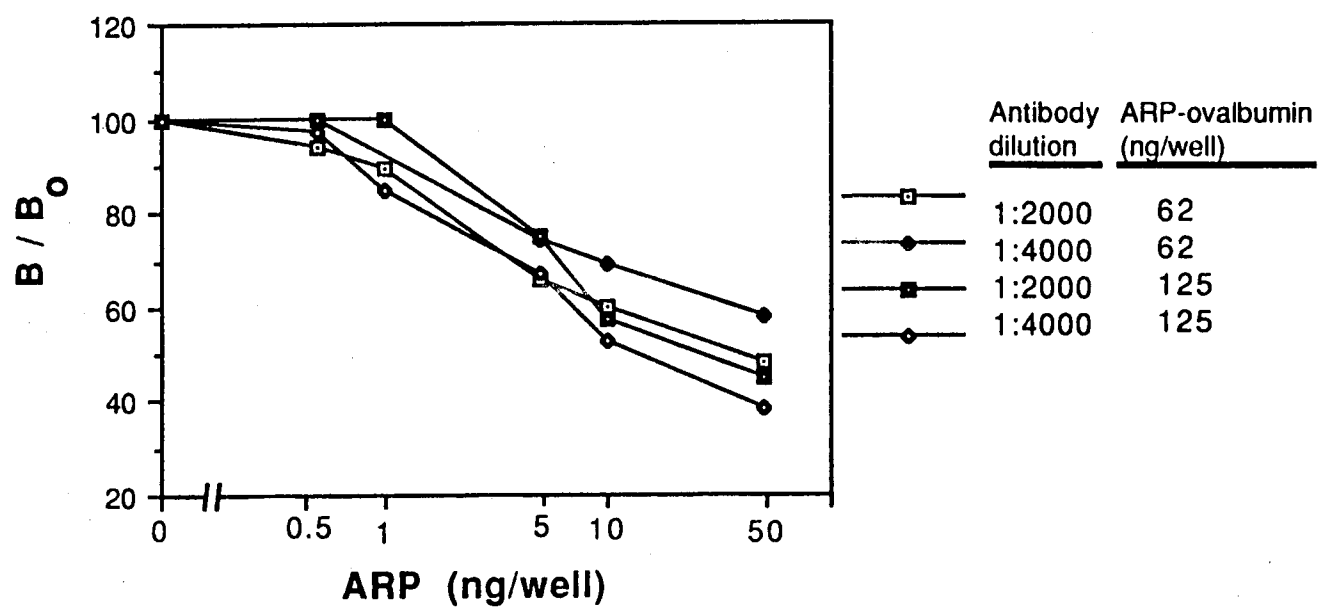


Fig. 1. Dose-response curve for ARP 2II

Remarks

The results of this preliminary study indicated that it is feasible to generate antibodies to ARP (see Fig. 1). The sensitivity of the assay at this stage is rather poor; however, we feel that the sensitivity can be improved by trying other immunoassay formats (6,7).

References

1. Barnard G (1988) The development of fluorescence immunoassays. In: Non-Radiometric Assays: Technology and Application in Polypeptide and Steroid Hormone Detection, Alan R. Liss, Inc., pp. 15-37.
2. Strasburger C Barnard G Toldo L Zarmi B Zadik Z Kowarski A and Kohen F (1989) Somatropin as measured by a two-site time-resolved immunofluorimetric assay. Clin. Chem., 35,913-917.
3. Barnard G Lichter S Kohen F Mikola H and Lovgren T (1989) The measurement of estrone-3-glucuronide in urine by a rapid non-separation time-resolved fluoroimmunoassay. Clin. Chem. 35, 555-559.
4. Raviv M Becker D and Sahali Y (1986) The chemical identification of root promoters extracted from avocado tissues. Plant Growth Regulation, 4, 371-374.
5. Kohen F Bauminger S and Lindner HR (1975) Steroid immunoassay. In: Proc. Fifth Tenovus Workshop, Cardiff, E.H.D. Cameron, S.G. Hillier and K. Griffiths, eds. Alpha Omega Publishing Ltd., Cardiff, Wales, pp. 11-32.
6. Kohen F Amir-Zaltsman T Strasburger CJ Bayer EA and Wilchek M (1987) The avidin-biotin reaction in immunoassay. In: Complementary Immunoassays. W.P. Collins, ed., John Wiley, New York, pp. 57-69.
7. Strasburger L Amir-Zaltsman Y and Kohen F (1988) The avidin-biotin reaction as a universal amplification system in immunoassays. In: Non-Radiometric Assays, B. Albertson and F. Hazeltine, eds., Alan Liss Inc., N.Y., pp. 79-100.

2.c.III. Conclusions

We are aware to the difference between radioisotopic labeling and immunological methods. Our feeling is that by this change in direction, we opened possibilities either greater or otherwise non-existent with radiolabeling. Possible applications include easy and accurate determination of ARP in plant tissues, *in situ* localization of ARP and tracing ARP translocation in tissues lacking the native compound.

2.d. Does ARP-like activity exists in plants other than avocado?

M. Raviv, Sh. Medina, D. Becker, Y. Sahali, A. Cose, K.W. Mudge, N.J. Glassbrook

2.d.I. Objective

We intend to test the hypothesis that ARP or similar activity may be generally present in a wide range of easy-to-root plants. In order to do this we will use the improved extraction and separation techniques developed for Avocado during the feasibility study. Other plant species will be tested for ARP-like activity (assayed using the mung bean rooting bioassay). A range of plants will be surveyed including other plants in the same family as Avocado (Lauraceae) as well as number of well-documented systems which have easy- and difficult-to-root members.

2.d.II. Materials and methods

ARP extraction

Unless otherwise stated ARP extracted according to previously described procedure (Raviv et al., 1986 a, b) The crude extract served for two purposes: For GC analysis an additional purification was conducted (see below). For rooting bioassay the crude extract was paper chromatographed (Raviv and Reuveni, 1984) and the last section (R_f 0.9-1.0) served for the bioassay. In all cases we tested several concentrations but for the sake of clarity presented only the optimal one.

Mung bean Rooting bioassay

Playing a central role in this project the bioassay itself was a subject for further modification. The bioassay was developed by Hess (1965). It was later modified by Fernqvist (1966) , Blazich and Heuser (1978) and Jarvis *et al.* (1984).

Initially we used the procedure as describe by Hess with some modifications (Raviv and Reuveni, 1984). During the course of the present research we tested some variables and introduced more modifications.

1. Throughout the project we used one mung bean cutting per vial thus avoiding possible interactions among the cuttings themselves. This also implies that concentration can be

expressed on a per cutting rather than on a per vial basis. The solution volume was daily adjusted to 3.5 ml.

2. Characterization of the age-dependent responsiveness of the mung bean rooting bioassay to ARP and IBA.

In a previous study, (Bassuk and Howard, 1981) it was shown that the rooting response of mung bean cuttings to IBA is age-dependent. When using the standard procedure of cotyldeon removal, the cuttings revealed a gradual increase in responsiveness to IBA (conc. $10^{-5}M$). Since it was not certain that the optimally sensitive seedling age for responsiveness to ARP would be the same as those for IBA it was necessary to determine this empirically. Two parameters served as guidelines: a) the extent of the response as compared to the control and b) the extent of variability ($SE \cdot t_{0.05}$ as a percentage of the average). The results are shown in Table 4.

Based on these and on the previous results (Bassuk and Howard, 1981) it seems that the standard procedure of taking 7-day-old cuttings may lead to higher variability problems without adequate compensation in terms of responsiveness. As a result of these experiments all the subsequent trials were done with either 5- or 6-day-old cuttings.

At a later stage of this research we decided to select a mung bean line having higher extent of response to root promoters.

In order to select a line of general value, IBA rather than ARP served as the effector.

Seeds of the resulted line are currently in use in our laboratory (A.R.O.). It was necessary to determine the age-related responsiveness of this line, too. The results of this experiment are shown in table 5.

As a conclusion of these experiments, all the subsequent trials conducted with the selected line were done with 5-day-old cuttings.

3. The involvement of certain nutrients in the quality and responsiveness of mung bean cuttings.

Following the findings of Jarvis and co-workers (1984) we tested the effects of Calcium and Boron in our system. We were not able to corroborate their conclusions. However, it was found that adding $Ca(NO_3)_2$ at 140 ppm and H_3BO_3 at 1 ppm to the irrigation water of the mung bean seedlings resulted with shorter but much healthier and uniform plants. As a result, this became the routine irrigation solution for our mung bean seedlings.

Table 4. Effect of mung bean seedling age on cutting responsiveness to IBA and ARP.

Age (days)	Treatment parameter	Water control	IBA 10^{-6} M	IBA 10^{-5} M	ARP 0.1 gr/vial
4	R/C ¹	5.1	5.6	6.6	9.9
	R/C as % of control	100	110	129	194
	SE•t _{0.05} ²	0.86	1.52	1.36	3.89
	SE•t _{0.05} as % of R/C	17	27	21	39
5	R/C	5.2	7.0	13.0	16.0
	R/C as % of control	100	135	250	308
	SE•t _{0.05}	1.06	1.47	2.08	3.69
	SE•t _{0.05} as % of R/C	20	21	16	23
6	R/C	7.8	12.0	20.0	24.1
	R/C as % of control	100	154	226	309
	SE•t _{0.05}	1.31	2.35	7.44	9.03
	SE•t _{0.05} as % of R/C	17	20	37	37
7	R/C	10.3	28.4	18.7	21.8
	R/C as % of control	100	258	182	212
	SE•t _{0.05}	2.62	9.16	8.48	11.38
	SE•t _{0.05} as % of R/C	25	32	45	52

¹R/C = roots per cutting

²SE•t_{0.05} = Standard Error x students t at the 0.05 level of confidence.

Table 5. Effect of mung bean seedling age on cutting's responsiveness to IBA.

Age (days)	Treatment parameters	Water Control	IBA (M)			
			$1 \cdot 10^{-6}$	$5 \cdot 10^{-6}$	$1 \cdot 10^{-5}$	$5 \cdot 10^{-5}$
5	R/C ¹	6.5	14.8	23.1	35.3	50.8
	R/C as % of control	100	229	358	545	786
	SE·t _{0.05}	0.60	2.72	2.02	3.78	4.96
	SE·t _{0.05} as % of control	8.8	18.4	8.7	10.7	9.8
6	R/C	7.0	18.5	25.1	33.5	49.9
	R/C as % of control	100	264	359	479	712
	SE·t _{0.05}	0.83	2.26	2.48	4.17	4.85
	SE·t _{0.05} as % of control	11.9	12.3	9.9	12.4	9.7

¹R/C = roots per cutting

²SE·t_{0.05} = Standard error x student's t at the 0.05 level of confidence.

4. Light intensity during mung bean growth and rooting

Using various growth chambers during the course of this project forced us to determine the optimal photosynthetic Photon Flux Density (PPFD) for both the growth and rooting stages of the bioassay.

Several trials were conducted in the range of $50\text{--}300 \mu\text{M}\cdot\text{sec}^{-1}\cdot\text{m}^{-2}$. Interactions among PPFD's kept during the two stages were tested, too. It was found that the low ($50 \mu\text{M}\cdot\text{sec}^{-1}\cdot\text{m}^{-2}$) light level at the stage of seedling growth causes the development of very long and weak cuttings. Although this does not affect rooting results in a negative manner, it creates logistic as well as uniformity problems and hence is not desirable. The highest light level at this stage resulted with very short plants and poor rooting response. Optimal light level at time of seedling growth is $100\text{--}150 \mu\text{M}\cdot\text{sec}^{-1}\cdot\text{m}^{-2}$. Light intensity while rooting has somewhat lower effect. Low light intensity causes some decrease in root numbers while high light intensity leads to leaf yellowing and eventual abscission. As for the growing stage optimal light intensity for rooting is $100\text{--}150 \mu\text{M}\cdot\text{sec}^{-1}\cdot\text{m}^{-2}$.

GC analysis of ARP

Crude methanolic extracts of various samples of plant material were further purified according to the following procedure:

- Evaporation of solvent until dryness.
- Dissolving in minimal amount of methanol, filtration.
- Hydrolyze in 2% KOH W/V in 20 ml methanol, 4h. room temp.
- Add 10 ml water, evaporate methanol.
- Extract x3 times with CH_2Cl_2
- Evaporate to dryness - dissolve in minimal amount of CH_2Cl_2 , Filter.
- Running of Florisyl column (Merck 50-100 mesh, 15 cm height, 1 cm internal diameter) with the following solvents:

Hexane: CH_2Cl_2	1:1	40 ml
CH_2Cl_2 :		40 ml
CH_2Cl_2 : EtAc 4:1	60 ml	
CH_2Cl_2 : EtAc	2:1	40 ml

ARP will appear in the last fraction.

After evaporation till dryness the sample is dissolved in 200 ml of dry CH_2Cl_2 to which 200 ml of Bis(trimethylsilyl) trifluoroacetamide (BSTFA, Aldrich) is added. The mixture is shaken and stayed for 20 minutes at room temp. Typically equivalent of 10 grams FW were passed through this procedure and finally dissolved in 0.4 ml of the silylizing agent. Samples of 1 ml were used for GC injection.

The analysis conditions were:

SE-54 capillary column, 60 meters length, 0.25 mm internal diameter.

Splitless injection, H_2 flame detector

Column headpressure: 25 psi

Chromosorb precolumn inlet

Make-up gas (He) 30 ml/min

Carrier gas (He) 1.4 ml/min

Air 400 ml/min

H_2 40 ml/min

Purge 3 ml/min

Purge time 3 min

Initial temp 50°C

Initial time	3 min.
Temp. increase	35°C/min
Final temp.	200°C
Final time	20 min

Currently, this method allows us the qualitative detection of compounds 2I and 2II of the ARP complex. It will be possible, however to calibrate the procedure in the future so as to enable direct quantitative analysis. Compounds 1I and 1II are being oxidized during the preparation thus cannot be analyzed.

2.d.III. Results

During the first stages of this work, only ARP-like activity was tested in purified extracts using the mung bean rooting bioassay. At a later stage a new analysis method was developed for ARP. The method was described above and results of direct analysis, verified with biological assay will be presented.

Is ARP Similar to Water Extractable Rooting Stimulator of Willow?

Kawase (1964) reported that centrifugation of willow stems results in a water extract ("willow water") which stimulated rooting of mung bean cuttings. Although previous work suggested that only slight ARP activity was present in extracts of avocado wood and cortex, the possibility was tested that a water extract of avocado might contain root promoting activity similar to that found in "willow water" and, in fact, ARP might be the same or similar to the (unidentified) active material in willow. Furthermore, if ARP activity is extractable into water, it might be more easily purified than that obtained by methanol extracts. ARP activity in a water extract would also provide evidence for its mobility within avocado.

The technique developed by Kawase (1964) was used for both willow and avocado. Willow stems (*Salix alba* var. *tristis*) were separated to current and previous year's growth. Approximately 7.5 g.f.wt. were immersed in 20 ml water before centrifugation.

Avocado stems are much thicker and heavier so that a sample of ca. 110 g. was used. Avocado stem taken from 5-month-old seedlings (seed harvested from the variety Topa-Topa) grown in a greenhouse were cut into 10 cm segments and centrifuged as above with 20 ml water either apical end up or basal end up. The exudates were kept in a freezer until the beginning of the rooting assay. Aliquots of 10, 1.0 and 0.1 gram fresh weight equivalent for avocado and of 0.5, 0.1, and 0.05 g fresh weight equivalent for willow were used for the bioassay.

Unusually long roots appeared on cuttings in this bioassay. This is quantified below.

Table 6. Rooting activity of aqueous exudates of willow and avocado stems.

Treatment	Gram F.W. Equivalent	Roots/ cutting	SD	% roots		
				< 1cm	1-3 cm	>3 cm
Willow, current growth	0.05	6.4	1.95	100	0	0
Willow, current growth	0.10	7.4	2.22	100	0	0
Willow, current growth	0.50	10.2	3.62	79	21	0
Willow, previous season's growth	0.05	7.7	1.72	90	10	0
Willow, previous season's growth	0.10	7.7	1.63	88	12	0
Willow, previous season's growth	0.50	8.4	2.42	48	52	0
Avocado, apex up	0.10	7.4	1.31	92	8	0
Avocado, apex up	1.00	9.2	4.93	68	32	0
Avocado, apex up	10.00	8.8	2.58	0	0	100
Avocado, base up	0.10	7.1	1.90	100	0	0
Avocado, base up	1.00	7.8	3.05	14	67	19
Avocado, base up	10.00	7.8	2.94	0	6	94
Water control	---	7.3	3.10	100	0	0

No considerable rooting activity was detected in stems exudates of willow or avocado. The unusual long roots which appeared in some cases may be attributed to the nutritional effect of photosynthates exuded from them into the bathing solutions.

ARP-like activity of several woody species

ARP was extracted and partially purified as described above from several woody species. The rooting activity of the supposedly-active fractions was tested using the mung bean rooting bioassay and a summary of the results are shown in table 7.

Table 7. Rooting activity of ARP-like extracts of several woody species.

Plant material	Typical rooting ability	Extracted tissue	Concentration in gr. eq. FW/cutting	Roots/cutting (percent of control = 100%)
Apple "Robusta"	D ¹	leaf	0.1	158
Apple "Novole"	E ²	leaf	0.1	184
<i>Hedera helix</i> , Mature	D	young leaf	0.2	261
<i>Hedera helix</i> , Juvenile	E	young leaf	0.2	296
<i>Ficus benjamina</i>	E	young leaf	0.5	157

¹D - difficult-to-root ²E - easy-to-root

Although there appears to be only a weak correlation between rooting ability and the activity of the extracts the results were encouraging enough to pursue this line of work particularly when the possibility of accompanying the bioassay with a direct analysis realized. Understandably the first plant material to be tested were avocado leaves.

Leaves were sampled from a mature tree of difficult-to-root cultivar (Fuerte) and at time of the year (March) during which avocado rootability is low (Raviv and Reuveni, 1979). Consequently, only moderate rooting activity was detected. (11.3 root/cutting vs. 6.6 r/c for the control. Significant difference at $p < 0.01$). Compound 2II was found in this case.

Other members of the *Lauraceae* family

1. *Laurus nobilis* L.

Bay laurel (*Laurus nobilis* L.) is a tree, native to Israel and to other Mediterranean countries. It belongs to the family *Lauraceae* to which the avocado genus belongs too. In many respects (morphology, growth habit, being dioecious) it is very different from avocado. However, rooting capability of its cuttings is very low as is the case for many avocado cultivars. In a previous work (Raviv et al., 1983) we selected and propagated wild clones of bay laurel which differed greatly in their rooting ability.

Leaves of two of these clones: an easy- and difficult-to-root were extracted and tested for ARP - like activity. Root promoting activity of the ARP fraction was tested using the mung bean bioassay and the results are presented in Table 8.

Table 8. Average number of roots/mung bean cutting as affected by two concentrations of ARP extracted from leaves of an easy- and difficult-to-root bay laurel clones.

Concentration gr.eq. FW	Clone			
	Easy-to-root		Difficult-to-root	
	R/C	SE	R/C	SE
0	7.4	± 1.9	7.4	± 1.9
0.01	22.0	± 3.3	17.3	± 2.8
0.05	23.7*	± 8.3	11.8*	± 3.1

*Overdose effects

There is an apparent association between the clone's rooting capability and the rooting activity of the ARP fraction purified from the leaves. The GC analysis revealed a similar association: both extracts contains only compound 2I but its concentration in the easy-to-root is about ten-fold its

concentration in the difficult-to-root one. The similar trends revealed in both rooting activity and actual ARP content offer at least partial explanation for the difference observed in rooting capability of the clones and suggest a role for ARP in their rooting process.

American members of the *Lauraceae*

Plant material of some American members of the *Lauraceae* family were collected, lyophilized and shipped to Israel where they were extracted and analyzed. Bioassay and GC analysis results are shown in Table 9.

Table 9. ARP-like activity (Av. no. of roots/cutting) and ARP identification in extracts of some American *Lauraceae*. Each mung bean cutting was treated with 0.05 gr. eq. D.W.

Plant material	Av. no. of roots/cutting \pm SE	ARP identified
Water control	5.5 \pm 0.45	
<i>Lindera benzoin</i> (shoots)	29.5 \pm 3.03	2II
<i>Litsea aestivalis</i> (shoots)	13.8 \pm 2.28	NC*
<i>Persea borbonia</i> (shoots)	18.9 \pm 2.83	NT**
<i>Persea borbonia</i> (fruits)	14.6 \pm 1.84	None
<i>Sassafras albidum</i> (fruits)	18.4 \pm 2.40	2II, 2I

*NC - Analysis results are not conclusive **NT - Not tested

The results shown in table 9 demonstrate the existence of 2I and/or 2II in some of the tested genera. Only in one case - fruit of *Persea borbonia* ARP was not found. The amount of *Persea borbonia* shoots was too small to enable analysis.

For *Litsea aestivalis* there is a need for different extraction and purification procedure due to heavy masking. *Lindera benzoin* shoots and fruits of *Sassafras albidum* contain detectable amounts of 2II. The last genus contains also 2I.

Families other than *Lauraceae*

Mangifera indica

Mango, which belongs to the *Anacardiaceae* family, produces seeds which, in many cases, yield apomictic plants. These plants resulting from purely maternal tissue are identical to the mother tree thus enabling vegetative propagation of these plants. Unfortunately, some other mango cultivars

doesn't possess this property and if needed as rootstock must be propagated using stem cuttings. Dr. O. Reuveni, of the Institute of Horticulture, ARO selected some easy- and difficult-to-root clones and kindly supplied us with leaves of 2 of these clones. Extracts of these leaves prepared as described previously and were subjected to rooting bioassay and to GC analysis. The results are shown in Table 10.

Table 10. ARP-like activity (Av. no. of roots/cutting) and ARP identification in extracts of easy- and difficult-to-root Mango clones

Clone	Av. no. of roots/cutting	ARP identified
Water control	6.8 \pm 0.66	
131/1 (Easy-to-root) 0.5 gr eq. F.W.	20.6 \pm 2.94	2I
Ein 2 (Diff.-to-root) 0.5 gr. eq. F.W.	21.0 \pm 4.55	None

These results suggest the occurrence of ARP-like compounds, close in chemical structure to the already known ARP's compounds, in mango. The content of these compounds however is probably not the limiting factor to rooting of the tested difficult-to-root mango clone.

Rosa indica major

Rosa indica cuttings serve as the sole rootstock for rose bushes in Israel and in many other parts of the world where mild winters prevail. Vegetatively propagated clones of *R. indica* differ greatly in their rooting ability (M. Raviv, unpublished). Stock plants were grown in controlled conditions and their leaves were sampled for ARP analysis bioassay. At the same time a rooting experiment of these clones was conducted. None of the members of the ARP complex was found in the GC analysis. The results of the rooting and root promoting activity experiments are shown in Table 11.

Table 11. Rooting of two clones of *R. indica* cuttings and root promoting activity of the ARP fraction extracted from their leaves.

Parameter clone	Roots/rose cutting	Roots/mung bean cutting (at optimal concentration of ARP solution)*
Easy-to-root	6.3 \pm 0.8	11.3 \pm 1.5
Difficult-to-root	0.15 \pm 0.12	13.7 \pm 1.9

*Rooting of control cuttings - 5.2 \pm 0.3 roots/cutting.

As in the GC analysis, no marked difference in root promoting activity was found in the ARP fraction extracted from both easy- and difficult-to-root rose leaves and it may be concluded that endogenous ARP has no endogenous role in the process of rooting of rose cuttings.

Rutaceae

Two species of the *rutaceae* family *Citrus reticulata* "Rangpur", an easy-to-root and *Citrus grandis* "Okoblanco", a difficult-to-root one were tested while rooting their cutting. Both leaves and the cortex taken from the cutting's base were tested for rooting activity at excision time and 19 days later. Only leaves sampled at these two dates served for ARP identification.

Table 12. ARP-like activity (Av. no. of root/cutting) and ARP identification in extracts of Rangpur and Okoblanco. Each mung bean cutting was treated with 0.05 gr. eq. D.W.

Plant material		Av. no. of roots/cutting \pm SE	ARP identified
Rangpur	Leaves O ¹	10.6 \pm 1.47	2I, 2II
Rangpur	Leaves E ²	10.7 \pm 1.48	2I, 2II
Okoblanco	Leaves O	8.5 \pm 1.51	2I, 2II
Okoblanco	Leaves E	8.3 \pm 1.42	2I, 2II
Rangpur	Cortex O	8.7 \pm 1.35	NT ³
Rangpur	Cortex E	12.6 \pm 2.80	NT ³
Okoblanco	Cortex O	8.1 \pm 1.41	NT ³
Okoblanco	Cortex E	7.6 \pm 1.02	NT ³
Water Control		6.1 \pm .065	---

¹O = start, ²E = end of rooting period, ³NT = not tested

Although at the present stage it is not possible to do accurate quantitative analysis, it is safe enough to state that quantities of both 2I and 2II were higher in Rangpur as compared to Okoblanco. This exemplifies again the possible association between the levels of components of the ARP complex and the species rootability.

The rooting and tissue sampling was done by Dr. Oded Sagee, Department of Citriculture, A.R.O. Bet Dagan.

Conclusions

Compounds 2I and/or 2II of the ARP complex were positively identified in GC analysis of purified extracts of leaves of several species of the *Lauraceae* family as well as in one easy-to-root clone of *Magnifera indica* (*Anacardiaceae*) and two species of the *Rutaceae*. In some of these cases, ARP-like activity is related to the rooting ability of the tested plant material (tables 8 and 12). At present it is still difficult to quantify ARP concentration from the GC result. However, on a comparative basis, it is safe to state that ARP concentrations have the same trend as ease-of-rooting for three different cases: easy- versus difficult-to-root clones of bay laurel, mango and two *Rutaceae* species.

No ARP-like activity was found in exudates of avocado and willow stems as well as in *Rosa indica* leaves. Some ARP-like activity was found in apple, ivy *Ficus benjamina*, *Persea borbonia* and *Litsea aestivalis*. GC analyses were not conducted for the first three and are not conclusive for *Litsea*. No ARP was found in *Persea borbonia* fruits.

Only in a few cases (rose, *Persea borbonia* fruit, leaves of difficult-to-root mango clone) we found no ARP (according to the current sensitivity of the method). We thus conclude that ARP has a relatively wide-spread distribution within the plant kingdom.

The preparation of plant material for analysis is a labor-consuming process. Taking into account the short period which elapsed between the time the method became available and the termination of the project, we believe that a great number of plant samples had been processed and analyzed.

References

- Bassuk NL and BH Howard (1981) Factors affecting the use of mung bean (*Vigna radiata* L. Wilczek) cuttings as a bioassay for root promoting substances. Jour. Hort. Sci. 56:295-300.
- Blazich FA and CW Heuser (1978) The mung bean rooting bioassay: a re-examination. Jour. Amer. Soc. Hort. Sci 104:117-120.
- Fernqvist I (1966) Studies on factors in adventitious root formation. Ann. Agric. Coll. Sweden 32:109-244.
- Hess CE (1965) Rooting co-factors, identification and functions. Proc. Intl. Plant. Prop. Soc. 15:181-186

- Jarvis BC *et al.* (1984) The interaction between auxin and boron in adventitious root development. *The New Physiologist* 97:197-204
- Kawase M (1964) Centrifugation, rhizocaline and rooting in *Salix alba* L. *Physiol. Plant.* 17:855-865.
- Raviv M and O Reuveni (1979) Seasonal effects upon the rooting of avocado cuttings. *Alon Hanotea* 33:449-452 (in Hebrew).
- Raviv M *et al.* (1983). Native bay laurel (*Laurus nobilis* L.) as an ornamental plant. *Acta Hort.* 132:35-42.
- Raviv M and Reuveni O (1984) Endogenous content of leaf substance(s) associated with rooting ability of avocado cuttings. *J. Amer. Soc. Hort. Sci.* 109:284-287
- Raviv M Reuveni O and Goldschmidt EE (1986) Evidence for the presence of a native, non-auxinic rooting promoter in avocado. *Pl. Growth Reg.* 4:95-102
- Raviv M D Becker and Y Sahali (1986) The chemical identification of root promoters extracted from avocado tissues *Pl. Growth Reg.* 4:371-374

2.e. Can ARP stimulate rooting of cuttings of plant species besides avocado?

2.e.I. Objective

During the course of the feasibility study, we have investigated the effect of ARP on the rooting of several plants species not previously tested including carnation, oak, and linden. Of these, carnation rooting was stimulated by ARP. Thus, the number of plant species in which rooting is stimulated by ARP is five out of the seven tested. Furthermore, in the experiments with carnation, oak and linden, only a single concentration of ARP was tested. We propose to test the effect of ARP at a range of concentrations and formulation (aqueous soak, alcohol quick dip, powder dip) on an expanded range of plants species including temperate as well as subtropical plants.

2.e.II. Materials and methods

Since in this section we will describe situations which are very different from each other, materials and methods will be described for each case separately.

- a. *Lindera benzoin* Shoot cutting assay
K.W. Mudge and N.J. Glassbrook.

Materials and methods

Terminal shoot cuttings were collected during the first week of July from a native stand of young spicebush trees, approximately three- to five-years-old. Cuttings were held overnight in a cooler at approximately 4°C. Cutting bases were freshly cut and dipped for 3 seconds in 50% ethanol solutions including 3 levels of ARP 2 II (0, 10^{-3} M, 10^{-2} M) and IBA (0, 2500 or 5000 ppm) arranged in a complete factorial design. The stem cuttings were approximately four inches long with two terminal leaves remaining. These terminal leaves were trimmed to approximately half their original length to reduce transpiration. After the ethanol evaporated the cuttings were arranged in wooden flats containing moist perlite then placed in the greenhouse under intermittent mist. There was one replicate row of 5 cuttings of each of the 9 treatments arranged randomly in each of 8 replicate flats of perlite. Cuttings were checked periodically for root formation and after eight weeks they were harvested and evaluated for percent rooting and the number of roots per cutting.

Results

IBA, either alone or in the presence of ARP 2II, significantly increased the percentage of *Lindera* cuttings that rooted ($p=.0001$, $p=.0035$, $p=.0001$ for 0, 1, and 10 μ M ARP respectively, by linear regression), Figure 2, and significantly increased the number of roots per cutting ($p=.0018$, $p=.0015$, $p=.0062$ for 0, 1, and 10 μ M ARP respectively), Figure 3. The application of ARP 2II, alone or in combination with IBA, had no significant effect on percentage rooting or number of roots per cutting.

ARP 2II did not produce the expected rooting enhancement when applied as a quick dip in the *Lindera* assay. Furthermore, because the quick dip method also used relatively large quantities of ARP 2II which was available only in limited amounts, further rooting studies with ARP 2II were confined to the dilute soak method of application.

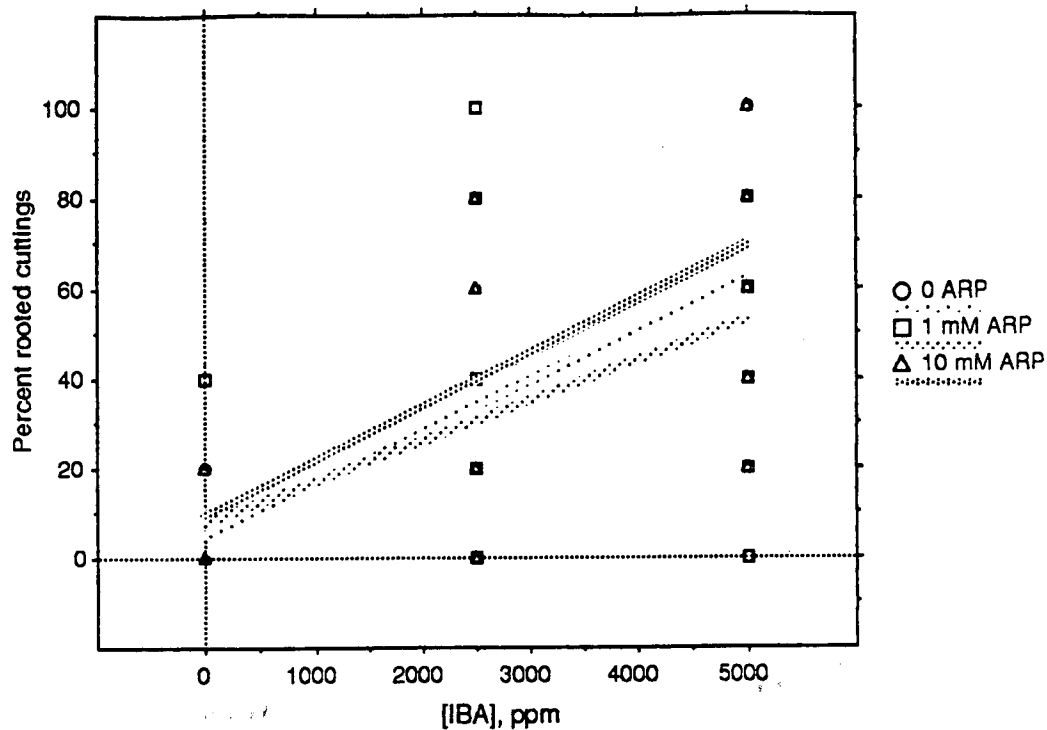


Figure 2. Rooting response of Lindera shoot cuttings to quick dip application of ARP 2II and IBA - percent rooted. Regression lines are shown for [IBA] vs % rooting at three ARP 2II levels.

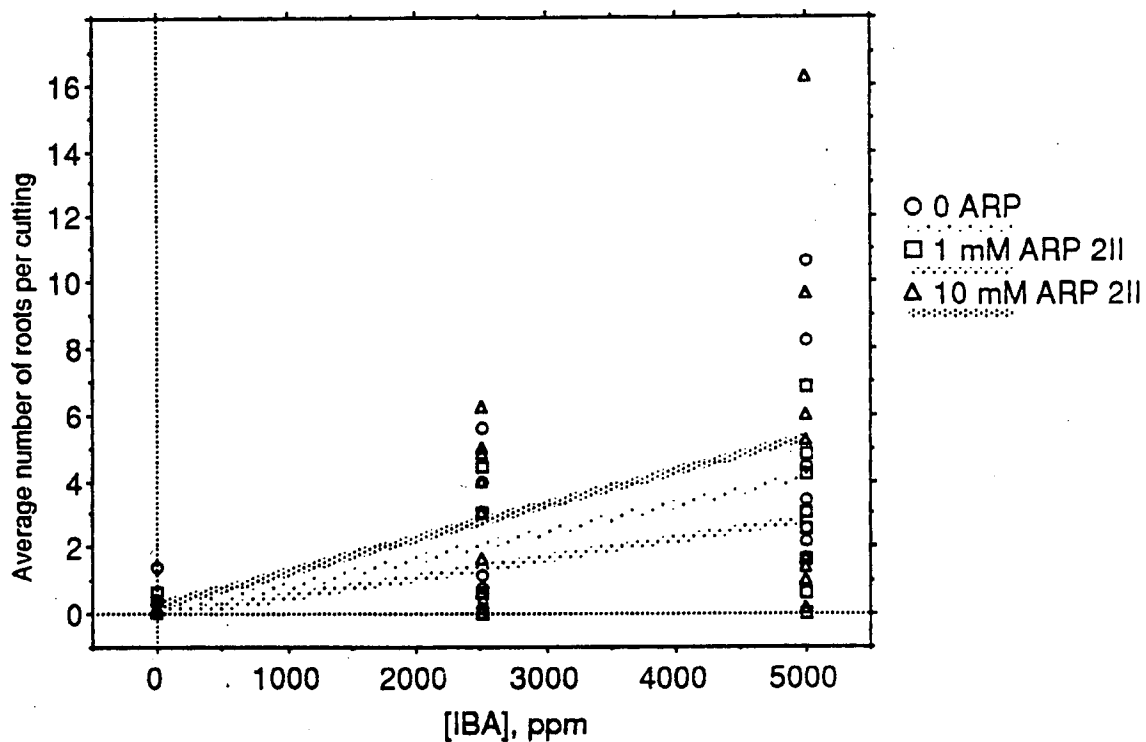


Figure 3. Rooting response of Lindera shoot cuttings to quick dip application of ARP 2II and IBA - roots per cutting. Regression lines are shown for [IBA] vs no. roots per cuttings at three ARP 2II levels.

b. Chrysanthemum shoot cuttings assay.

K.W. Mudge and N.J. Glassbrook

Materials and methods

Cultivars of *Chrysanthemum morifolium* that vary in ease of rooting are commercially available. Commercially prepared cuttings of Chrysanthemum 'Bright Golden Anne', a moderately easy to root cultivar, was obtained from Yoder Bros. (Barberton, Ohio). Cuttings were trimmed to 0.5 cm internode length with one to two fully expanded leaves remaining. Leaves were trimmed or removed as necessary to leave approximately equal leaf area on each cutting. Treatment solutions consisted of 0, 10, 20, and 40 μ M ARP 2II. Cuttings were then placed into 4 dram vials containing 15 ml of treatment solution. There were five rows (reps) of each treatment with ten cuttings per row for a total of fifty cuttings per treatment. The cuttings were harvested after two weeks and evaluated for the number of roots per cutting.

Results

The application of ARP at 10 to 20 μ M significantly increased the number of roots per mum cutting ($p=0.0226$, by linear regression analysis of the 0, 10 μ M, and 20 μ M data). The number of roots on cuttings treated with 40 μ M ARP 2II was not significantly different from the control, Figure 4. The observed increase in number of roots was statistically different but small (12 roots on treated vs 10 for the control) and was not evident by qualitative observation.

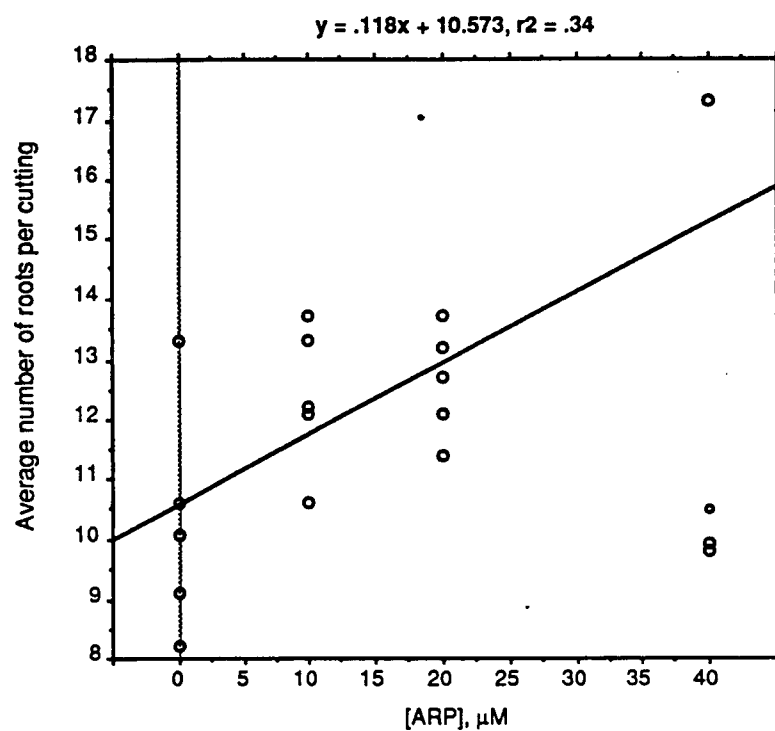


Figure 4. Rooting response of Chrysanthemum shoot cuttings to ARP 2II number of roots per cutting. Regression line shown is for [ARP 2II] (excluding 40 μM level) vs number of roots per cutting.

c. Bench-grafted rose cuttings

M. Raviv, Sh. Medina and Sh. Yadid

Materials and Methods

The effect of ARP with or without IBA on rooting and graft healing of bench grafted rose cuttings was tested in 2 experiments, using 2 *Rosa indica* clones as rootstocks and 2 commercial cultivars as scions. All the plant material which was used for these experiments is virus-free, grown under controlled conditions in soilless culture.

Experiment a.: Scions of the CV. "Mercedes" were side-grafted onto *R. indica* clone 1B and planted in 2" speedling trays, filled with peat-lite medium. Rooting was done under intermittent mist (5 sec. each 5 min., 08:00-17:00). Bottom heat - 20°C. Minimal air temperature - 17°C. Light intensity - 45% of the natural sunlight.

Treatments to the cuttings' bases consisted of 5% ethanolic solutions of $1 \cdot 10^{-4}$ M IBA and/or ARP. Cuttings were dipped for 15 min., grafted immediately afterwards and inserted under the mist. Rooting period: 10.1.89 - 31.1.89

Experiment b.: Scions of the CV. "Golden Times" were side-grafted onto *R. indica* clone 1T and planted in the same conditions as described above. In this experiment, the scions were also treated with the same solutions as in exp. a. except that ethanol concentration was in all cases 1% and dipping time for scions was 1 min. Rooting period: 23.2.89 - 14.3.89

Results - Exp. a

The effect of growth regulators, applied to the rootstock of this specific shy-rooting clone was significant (table 13).

Both IBA and ARP promoted rooting alone as well as in combination. The combined effect was especially beneficial in terms of root growth which has a considerable significance from practical viewpoint. Some stimulation of the scion's bud growth was also observed although the effect in this case is probably secondary and hence, less dramatic.

Table 13. The effect of IBA, ARP and their combination on rooting and scions' bud growth of bench-grafted rose cuttings.

Treatment	Rooting (%)	Roots/cutting \pm SE	Root length/rooted cutting (mm.)	Bud swelling (%)	Length of new growth (mm) \pm SE
Control, 5% Ethanol	0	--	---	44	1.44 \pm 0.18
IBA	56	3.67 \pm 1.58	97.2	67	2.22 \pm 0.36
ARP	56	3.44 \pm 1.54	61.2	56	1.89 \pm 0.35
IBA & ARP	78	6.67 \pm 2.58	235.3	78	1.89 \pm 0.20

Exp. b.

The purpose of the second experiment except for verifying the results of the first one was dual:

1. To test the effect of the growth regulators on degree of the graft union healing.
2. To test the effect of the growth regulators vs. the known inhibitory effect of the CV. "Golden Times" on rooting and graft-healing of bench-grafted rose cuttings. This experiment suffered from a severe *Botrytis* attack.

Table 14. The effect of IBA, ARP and their combination on rooting, graft union healing and scion's bud growth of bench-grafted rose cuttings.

Treatment	Rooting (%)	Roots/cutting	Root length/rooted cutting	Complete healing (%)	Bud swelling (%)
Rootstock treated with 1% EtOH	15	2.4	375	59	62
Rootstock treated with IBA + ARP	29	3.2	134	86	71
Scion treated with 1% EtOH	10	0.3	15	46	0
Scion treated with IBA	40	3.8	183	48	0
Scion treated with ARP	55	7.5	412	100	55
Scion treated with IBA + ARP	38	2.6	128	96	23
Scion and rootstock treated with IBA and ARP	90	11.4	254	67	50

Although clone 1T usually roots better than 1B (Raviv, unpublished) the rooting in this experiment was not significantly better than in the previous one, probably due to an extensive leaf drop caused by the botrytis attack. This may be the reason for some puzzling results such as the reduced root elongation of cuttings treated with IBA and ARP to their rootstock as compared to the control.

The main findings of this experiment are:

1. Both IBA and ARP enhanced rooting when given not only to the rootstock but also to the scion. It was especially apparent when both rootstock and scion were treated.
2. ARP had a striking effect on the graft union healing when applied to the scion alone. IBA revealed no effect although in some other cases IBA showed beneficial effect on graft union healing of roses (Raviv and Gilad, unpublished). Application of IBA and ARP to the rootstock caused enhanced healing, too, which, from unknown reasons was somewhat reduced when the scion was treated, too.
3. All scion-applied treatments inhibited the growth of its bud, probably as a response to ethanol which was only partially counteracted by the growth regulators.

Taken together these results call for more horticultural work aimed at fine calibration of concentrations, solvents, duration and site of application for this promising combination of growth regulators. Also the carry-over effect of these treatments on subsequent sapling's growth should be studied.

d. Rooting of tomato cuttings

M. Raviv, Sh. Medina and Sh. Yadid

In a previous study (Raviv et al. 1986) a positive effect was found for ARP on rooting of cuttings of tomato of an Israeli cultivar. Recently mutants of the CV. VFN8 were selected and described by Zobel (1973, 1986). These mutants are a recessive mutant that does not develop lateral roots (*dgt* = *Diageotropica*) and another recessive mutant which does not develop adventitious root (*ro* = *rosette*).

The effect of ARP on adventitious root formation by cuttings taken from the 3 lines was tested.

Material and methods

Tomato seedlings of the 3 lines were grown in a greenhouse (minimal night temp. 17°C) in a peat-lite mixture until the first pair of true leaves was fully expanded. At this time, cuttings were

harvested, consisting of these leaves, an apical bud and a 3-4 cm. stem segment. The cuttings were planted 1 cm. deep in containers filled with aerated solutions. Rooting solutions consisted of 1/2 Hoagland + 0, 1, 5, 20 μ Mol ARP. Rooting was done in a growth chamber under similar conditions as the mung bean tests (see section 2.d.II for details). Rooting period was 5 days, after which roots were counted.

The experiment was replicated three time with similar results and the results of one of these experiments are presented, calculated on the basis of control = 100%.

Table 15. Average relative response of tomato cuttings of 3 lines to various ARP concentrations (control = 100%).

Concentration Tomato line	0	1	5	20
VFN 8	100	151	150	114
<i>dgt</i>	100	114	173	150
<i>ro</i>	100	144	260	0

The actual number of roots varied greatly among the lines - from ca. 5 per *dgt* and ca. 10- per *ro* control cuttings to ca. 48 roots per VFN8 control cutting.

The easy-to-root line (VFN8) showed relatively low response to ARP which was satisfied by low concentration. *dgt* and especially *ro* responded in a more vigorous way although even at higher concentrations of ARP produced relatively low number of root per cutting as compared to VFN8.

The highest ARP concentration prevented rooting of *ro* cuttings during the time frame of the experiment. No toxicity signs were apparent but the cutting's base was splitted.

It may be therefore assumed that although not being the primary cause, ARP may contribute to rooting of these difficult-to-root mutants. Two typical properties of *dgt* are its low auxin sensitivity (Kelly and Bradford, 1986, Hicks *et al.* 1989) and its low ethylene production rate (Zobel, 1973). Such contributions may be therefore by increasing its sensitivity to auxin (see section 2.i) or by the ability of ARP to enhance ethylene production rate (Mudge and Raviv, unpublished) and thus to evoke an ethylene-induced root formation (Mudge, 1988).

Conclusions

Rooting, as we perceive it, is the culmination of a complex and long sequence of events. Each of these events has its own set of requirements and one should not expect any sole treatment (be a chemical, environmental condition, type of plant material chosen, etc.) to compensate for any other unfulfilled requirement. It was beyond the scope of the present research to analyze in depth cases of low regeneration abilities so as to find these for which ARP is the presumed missing prerequisite. Without performing such an analysis, it is not possible to predict in advance whether and to which extent ARP will stimulate regeneration processes. During the course of the 60 years passed since the first observation of auxin-stimulated rooting numerous cases of lack of response to auxin were documented. It is not surprising, therefore that so is the case for ARP.

While first detected using an easy-to-root test plant (mung bean), ARP was associated with genetic- and age-related events in which a very difficult-to-root species - avocado (*Persea americana*) exhibited improved rooting potential (Raviv and Renveni, 1984, Raviv *et al.* 1987). Its application to cuttings of this species, apparently deficient in ARP, promoted its rooting. It was also active when given to some easy-to-root species. During the course of the current research ARP showed potential for rooting enhancement of another easy-to-root species (*Chrysanthemum*) and of a species having medium rooting capacity (*Rosa indica*). It was also tested on two types of difficult-to-root types of plant material. No root promoting was achieved in the case of *Lindera benzoin*. Since ARP was found in this species (Table 9) it may be assumed that level of ARP is not the limiting factor to rooting of cuttings of this species.

Two difficult-to-root mutants of tomato (*Lycopersicum esculantum*) responded to ARP application by a significant increase in the number of roots. Since normally preformed root primordia occur in tomato stems we believe that the effect in this case is of different nature than in the other cases. However, it may be the outcome of a common basic mechanism such as by rendering the treated tissue higher sensitivity to auxin. For further discussion of this subject, see section 2.i.

References

- Hicks GR DL Rayle and TL Lomax (1989) The *dgt* mutant of tomato lacks high specific activity auxin binding sites. *Science* 245:52-54.
- Kelly MO and KJ Bradford (1986) Insensitivity of the *dgt* tomato mutant to auxin. *Plant Phys.* 82:713-717.

Mudge KW (1988) Effect of ethylene on rooting (pp. 157-161) In: Davis TD BE Haissig and N Sankhla (eds.): Adventitious root formation in cuttings. Dioscorides Press Portland.

Raviv M and O Reuveni (1984) Endogenous content of a rooting promoter extracted from leaves of difficult- and easy-to-root avocado cuttings. Journ. Amer. Soc. Hort. Sci. 109:284-287.

Raviv M O Reuveni and EE Goldschmidt (1986) Evidence for the presence of a native non-auxinic rooting promoter in avocado. Pl. Growth Reg. 4:95-102.

Raviv M O Reuveni and EE Goldschmidt (1987) The physiological basis for loss of rootability with age of avocado seedlings. Tree Physiology 3:115-122.

Zobel RW (1973) Some physiological characteristics of the ethylene-requiring tomato mutant *diageotropica*. Plant Physiol. 52:385-389.

Zobel RW (1986) Rhizogenetics (root genetics) of vegetable crops. HortScience 21:956-959.

2.f. Can ARP improve propagation of plants via tissue culture?

K.W. Mudge and N.J. Glassbrook

2.f.I. Objectives

The demonstration during the feasibility study that ARP can stimulate rooting of raspberry explants *in vitro* was encouraging. The effect of ARP will be investigated *in vitro* on both induction of adventitious roots as well as tissue dedifferentiation leading to callus formation and adventitious bud induction. The two micropropagation systems currently being investigated in the lab of one of the investigators (KWM) are mugo pine and red raspberry. These will be used to test the effects of ARP. Mugo pine is especially appropriate since it is a system in which root induction in the micropropagated shoots from young mugo pine explants has proved to be very difficult and ARP will be investigated for its usefulness here as well.

2.f.II. Results and discussion

ARP's heat stability

As part of our initial evaluation of ARP 2II we tested it's heat stability. This was particularly applicable to ARP 2II's use in tissue culture systems where media are routinely autoclaved before use.

Mung bean cuttings were rooted in previously autoclaved (121°C for 18 min) or unautoclaved treatment solutions containing either 0 or 40 μ M ARP 2II. From the data shown in Figure 5, it is apparent that ARP activity was not diminished by autoclaving. In fact cuttings in the autoclaved 40 μ M ARP solutions had higher root numbers than in non-autoclaved solutions. This enhancement of rooting may be due to reduced microbial contamination in the solutions, and hence less xylem plugging during the rooting period.

Similar experiment was conducted in Newe-Ya'ar. ARP concentration was 20 μ M in this case. From the results shown in table 15, it can be concluded that ARP is indeed heat resistant. The main difference between the two systems is the use of fresh glass distilled water vs. nutrient solution as controls in Newe-Ya'ar and in Cornell, respectively. Apparently, xylem plugging is more likely to occur in cuttings treated with unsterilized nutrient solution than in freshly-prepared glass distilled water.

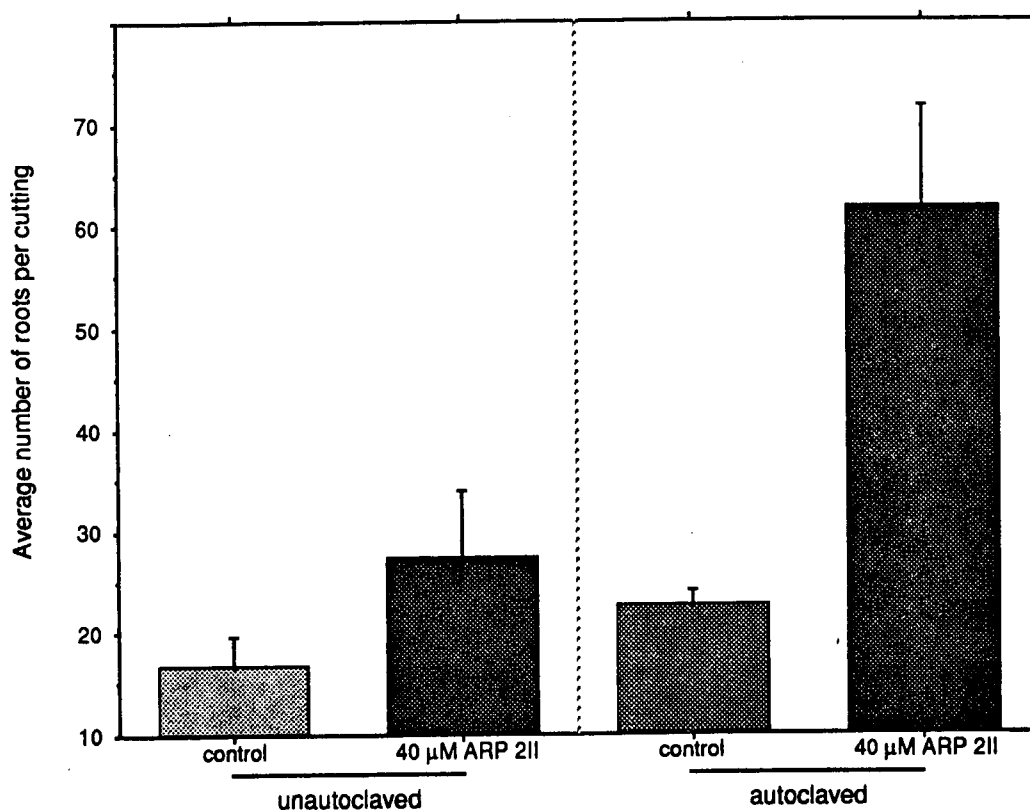


Figure 5. Rooting response of hypocotyl cuttings from eight-day-old mung bean seedlings to autoclaved and non-autoclaved control and 40 μ M ARP 2II solutions.

Table 15. Rooting response of mung bean cuttings to autoclaved and non-autoclaved control (glass distilled) and 20 μ M ARP 2II solutions.

Treatment	Roots/cutting
Water, not sterilized	5.5 a*
Water, sterilized	6.1 a
ARP, not sterilized	11.0 b
ARP, sterilized	12.6 b

*Numbers followed by different letters are statistically different at the 5% level of confidence.

Effect of ARP on In Vitro Rooting of Tissue Cultured Red Raspberry

Tissue culture propagation of a number of plant species is limited not by their ability to establish (Stage I) or proliferate shoots (Stage II) in culture, but rather by their ability to root (Stage III) in culture (Klein, 1982; Sriskandarajal and Mullins, 1981). This experiment was designed to test the hypothesis that ARP can improve *in vitro* (Stage III) rooting of red raspberry (*Rubus ideaus*) shoot culture. The variety tested was '883' shoot explants which had been previously proliferated using standardized tissue culture procedures for this crop on Anderson's shoot proliferation media (Anderson, 1975). Shoots were aseptically transferred to Anderson's rooting media (same as above except minus adenine sulfate and Benzyl amino purine) containing compounds to be tested. Three levels of ARP (0, 10 and 20 g.fr.wt. equivalent) and three levels of the auxin IBA (0, 0.5, and 1.0 mg/L) were tested in a complete factorial arrangement of treatments. Nine shoots were transferred to each vessel (Magenta GA 7 boxes) containing 50 ml of agar solidified test media. Each treatment was replicated with 4 boxes. After 10 weeks in the light at 24°C, rooting of explants was evaluated. Results are presented in Table 16.

The results indicate that ARP does indeed increase the percentage of Red Raspberry shoots which rooted *in vitro* especially at a concentration of 10 g.fr.wt. equivalent. As in the case of ARP stimulated rooting of mung bean cuttings, ARP was effective either in the presence or absence of added auxin.

Table 16. Effect of ARP and auxin on in vitro rooting of tissue cultured red raspberry.

A. Percentage of Explants Rooted

IBA concentration mg/L	ARP concentration, g. fresh wt. equivalent		
	0	10	20
0	6.0 ±3.5	19.5±13.2	26.0 ±12.0
0.5	5.5 ±3.2	20.0± 9.4	8.3 ± 5.3
1.0	13.7 ±5.3	36.0± 7.0	16.5 ± 3.2

B. Mean Number of Roots per Rooted Explant

IBA concentration mg/L	ARP concentration, g. fresh wt. equivalent		
	0	10	20
0	1.5 ±0.5	1.7±0.6	1.8 ±0.5
0.5	1.0 ±0.0	1.8±0.3	2.3 ±1.3
1.0	2.4 ±1.1	2.3±0.4	1.2 ±0.5

When pure ARP 2II became available, 2 follow-up experiments were conducted. One-month-old Stage II shoot cultures of red raspberry, *Rubus ideaus* cv. 'Heritage', grown on agar-solidified Anderson's medium [Anderson, 1975] were subdivided, and large healthy shoots were placed in sterile GA-7 (Magenta) containers with 40 ml of fresh media amended with various levels of ARP 2II and/or auxin. In the first experiment, the culture medium contained 0, 1, 10, or 100 μM ARP 2II, plus or minus IBA at 2.45 or 4.5 μM . There were five shoots per GA-7 container and five containers per treatment. The microcuttings were allowed to root under cool white florescent lights (PAR 70-100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at ca 25°C. After six weeks the cuttings were harvested and the number of roots counted. In the second evaluation, the culture medium contained 0, 10, 50, or 100 μM ARP 2II, plus or minus IAA at 0.5, 1.0, or 5.0 ppm. There were four shoots per GA-7 container and five containers per treatment. After six weeks the cuttings were harvested and evaluated for the percentage of rooted cuttings. In both raspberry rooting trials, crystals of ARP (precipitation) could be seen in the culture medium that contained 100 μM ARP 2II, so the 100 μM concentration should be considered nominal.

As reported in the first annual report, the addition of ARP 2II to the culture medium in the first experiment significantly increased the mean number of roots per explant at the highest concentration of ARP 2II (100 μM), but not at the lower concentrations. Addition of IBA increased the number

of roots on the microcuttings at both levels used regardless of ARP concentration, Figure 6. A high proportion of the cuttings rooted and there was little difference in rooting percentage between treatments.

In the second experiment, the addition of ARP 2II alone to the medium had no effect on the percentage of microcuttings that rooted, but significantly decreased the percentage of cuttings that rooted in the presence of IAA ($p=0.0003$ for 0.5 ppm IAA, $P=0.0001$ for 1.0 and 5.0 ppm IAA), Figure 7. Percentage of rooted cuttings was selected as the parameter for this second experiment because most of the rooted cuttings had only one or two roots.

The observed effect of ARP on rooting was different in this two experiments. In the first, ARP enhanced rooting at the highest concentration in the presence or absence of auxin, whereas in the second experiment, ARP reduced the rooting, but only in the presence of auxin, particularly at the highest concentration. In neither experiment did ARP 2II compare favorably with auxin with regard to the concentration necessary to produce horticulturally acceptable rooting.

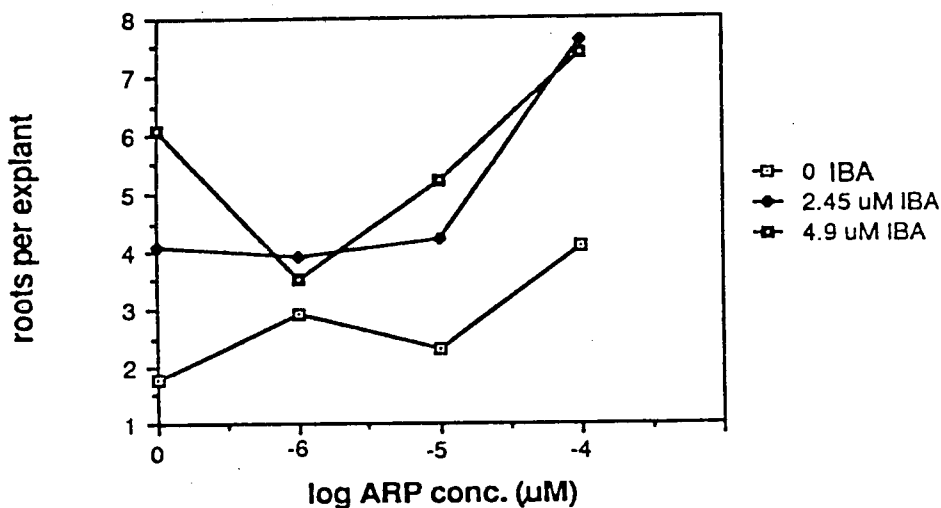


Figure 6. Rooting response of red raspberry microcuttings to ARP 2II and IBA - experiment #1.

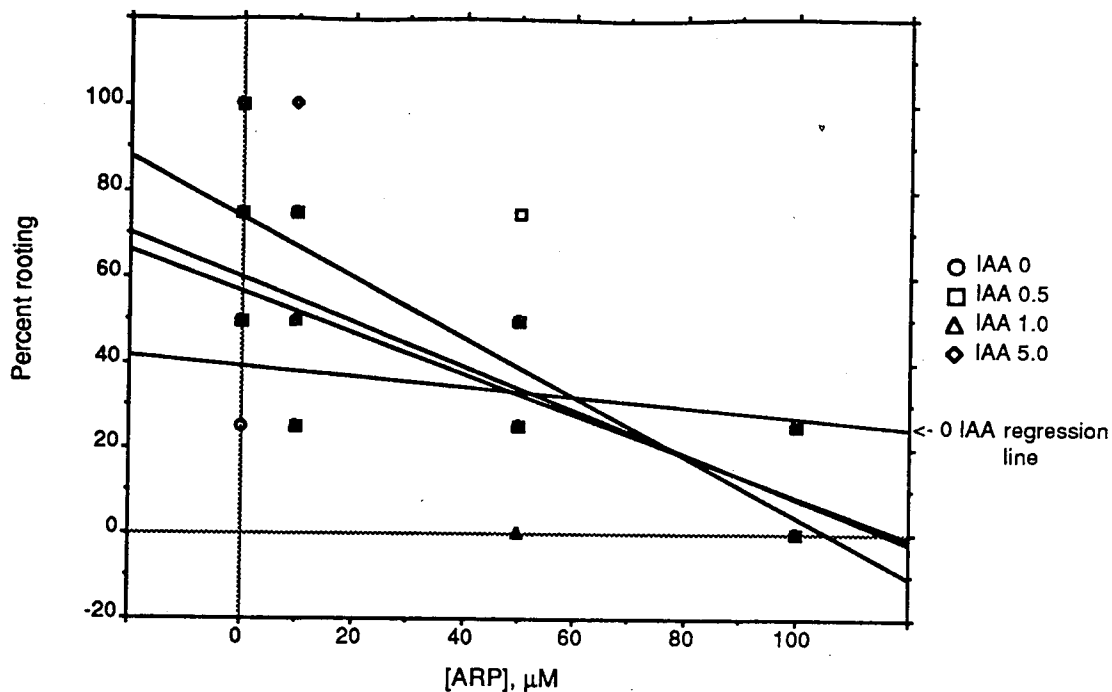


Figure 7. Rooting response of red raspberry microcuttings to ARP 2II and IAA - experiment #2.

Chestnut microcutting rooting assay. Chinese chestnut (*Castanea mollissima* L.), clone #131, Stage II shoot cultures that had been grown on Woody Plant Medium (WPM) [Lloyd and McCown, 1980] for six weeks were subcultured and placed on fresh WPM amended with 0, 1, 10, or 100 μM ARP 2II, plus or minus IBA at 4.9 μM , in a complete factorial arrangement of treatments. Four large healthy shoots were transferred into each GA-7 container, which contained 40 ml of medium. At six weeks and periodically until 10 weeks, cultures were examined for rooting.

In this experiment, only one plantlet rooted. That plantlet was on medium containing both 1 μM ARP 2II and 4.9 μM IBA. Hence ARP 2II was not effective for in vitro rooting of this particular difficult to root woody species.

Carnation microcutting rooting assay.

Nodal microcuttings from Stage II shoot cultures of *Dianthus* 'China Doll' grown on Propagation Medium [Glassbrook, unpublished] were placed on 25 ml of fresh media in baby food jars and sealed with Magenta closures. The media was amended with 0, 10, 20, or 40 μM ARP 2II plus or minus 0.5 ppm IBA. There were three microcuttings per container and nine containers of each treatment. The microcuttings were allowed to root in the lighted culture room for approximately 6 weeks in a culture room at ca 25°C and PAR of 100 $\mu\text{mol m}^{-2}\text{s}^{-1}$ before they were evaluated for the number of roots per cutting. The results are presented as the number of roots per cutting in each of the uncontaminated vessels.

IBA, by itself, increased the number of roots per cuttings, but the observed increase was not statistically significant. The addition of ARP, without IBA significantly increased the number of roots formed on the cuttings. The addition of ARP 2II with IBA had no significant effect on rooting. These results are summarized in Figure 8. Note that the curve fit to the 0 IBA data points on the graph is a second order polynomial ($p=0.005$) and not a linear regression line.

Effects of ARP on Adventitious Bud Formation

As stated in the original proposal, it was our objective to determine if ARP activity is limited to root promotion, or if it has a promotive effect on adventitious bud formation as well. The observation described in section 2.g that the optimum period for ARP activity in the mung bean hypocotyl rooting bioassay precedes that of auxin by at least 24 hours suggests that it may be acting on a earlier stage of development prior to the "commitment" of a primordium to become a root, and hence under some conditions, particularly in the presence of cytokinin, it might promote adventitious bud rather than root formation. This hypothesis was tested in several assays described below.

Petunia leaf disk assay

The in vitro petunia leaf disk assay is a relatively well characterized tissue culture system in which roots or shoots can be induced to form on leaf disk explants in response to hormones present in the culture media. ARP 2II was tested in this system to determine if ARP 2II would influence root or shoot formation either alone or in the presence of low levels of cytokinin or auxin.

Petunia leaves of intermediate age from 12-week-old growth chamber-grown 'Blue Picotee' petunia plants were disinfested with a solution of 0.5 % sodium hypochlorite plus 2 drops of Tween 20 per 100 ml of solution. After 3 rinses in sterile distilled water, disks approximately one cm in diameter were cut from the leaves with a sterile #4 cork borer. Three disks were placed adaxial side down on each plate of Murashige and Skoog minimal organics medium (Sigma, St. Louis, MO) with 3% sucrose and solidified with 0.8% Bactoagar. Nine plates (reps) were prepared for each treatment. The medium was amended 0, 10, 20, or 40 μM ARP 2II with or without 0.1 mg/l BAP, or 0.1 mg/l NAA (hence a 4 x 3 complete factorial with a total of 12 treatments). The leaf disks were allowed to develop in the culture room under cool white florescent tubes (PAR 70-100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at ca 25°C. The disks were checked periodically for four to eight weeks and then were harvested and evaluated for root or shoot formation.

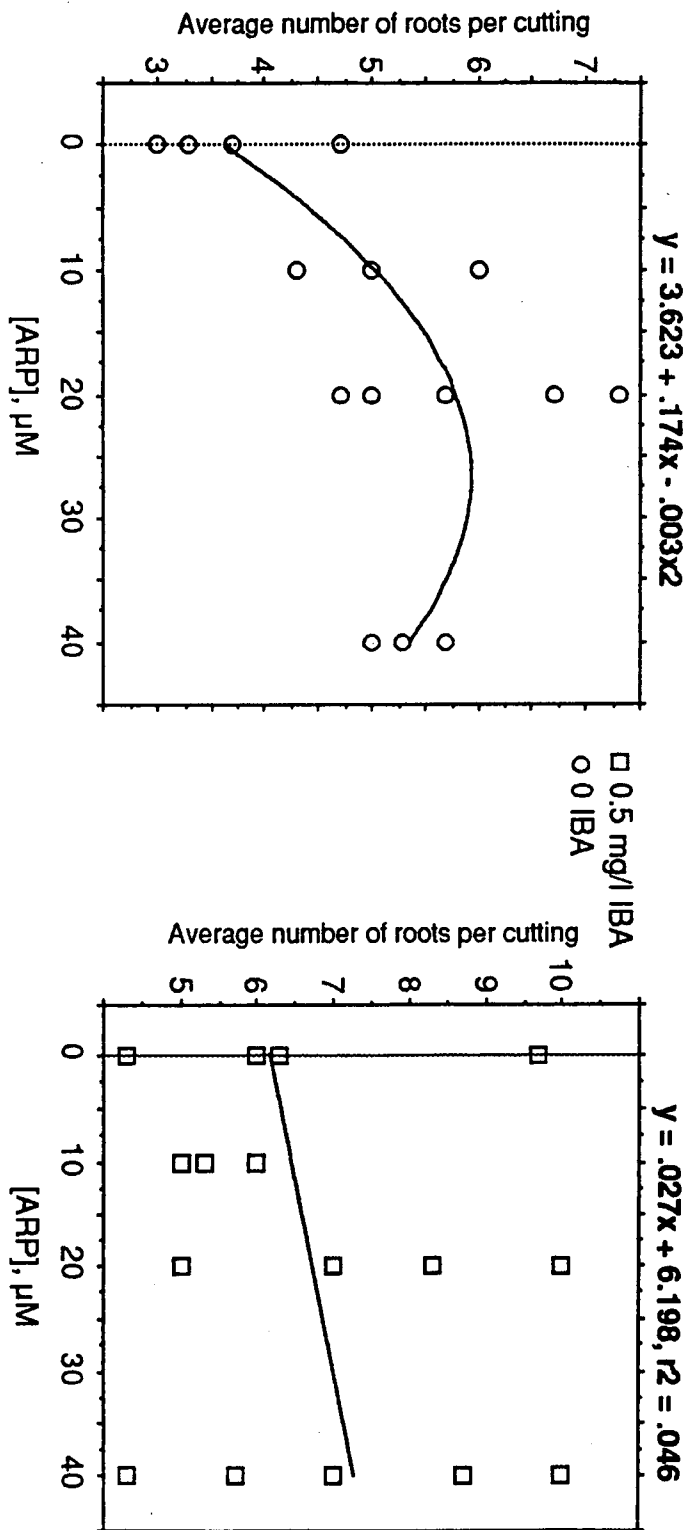


Figure 8. Rooting response of Carnation microcuttings to ARP 2II and IBA.

No roots or shoots formed on the hormone free medium or on media that contained ARP 2II alone. Shoots did form on BAP containing media, but ARP 2II inhibited the formation of BAP induced shoots in a dose dependent manner ($p=0.001$) (Figure 9). Although callus did form on NAA treated disks, no roots formed on any of these disks regardless of ARP level. Hence in this system, ARP 2II did not induce adventitious bud formation. On the contrary, ARP 2II inhibited shoot formation, which would be expected for a substance with auxin-like activity. However, in contrast to the root promoting effect of ARP 2II in other systems, it did not stimulate rooting by itself or in combination with a low concentration of NAA in the petunia leaf disc system. An interaction of ARP 2II with auxin might be more evident in a repeat of the petunia assay using higher levels of NAA or using different auxins such as IBA or IAA.

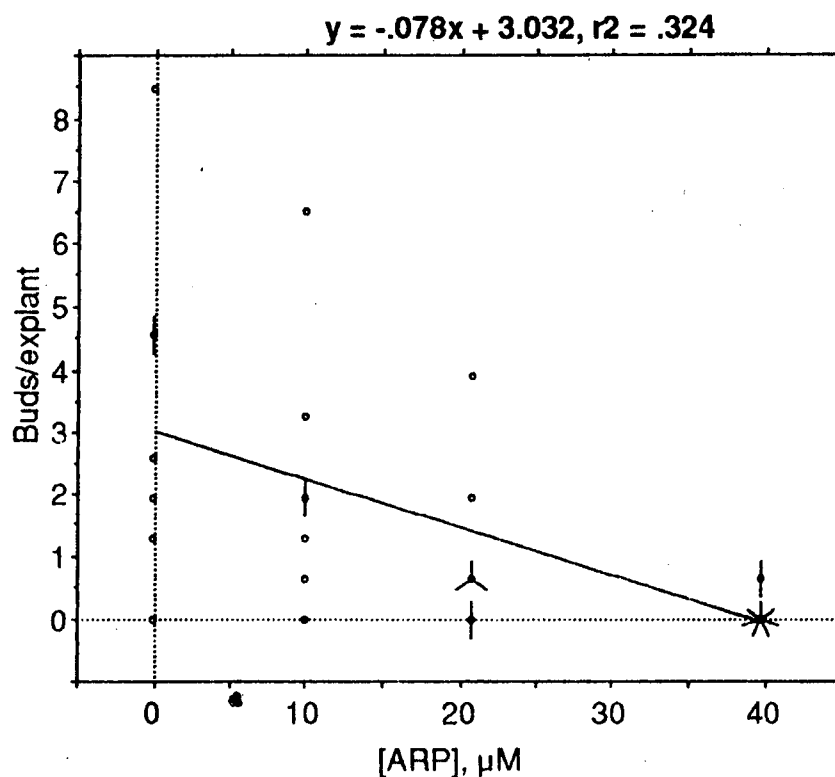


Figure 9. Number of adventitious buds formed on petunia leaf discs as affected by ARP 2II, NAA and BAP.

Pine cotyledon assay

Pine embryo culture is another relatively well characterized tissue culture system in which adventitious buds can be induced to form on explants in response to hormones present in the culture medium. In this system, the presence of a small amount of auxin (NAA) enhances adventitious bud formation on cotyledons treated with high levels of cytokinin (Mudge, 1986; Mott and Amerson, 1981).

From preliminary experiments it became evident that microbiological contaminants were difficult to eliminate from *Pinus radiata* seeds. Hence the following 3 stage decontamination procedure was adopted. Seeds were first washed in 0.5% sodium hypochlorite amended with two drops of Tween 20 per 100 ml of solution. The seeds were imbibed overnight in an antibiotic solution containing 100 mg/l thiabendazole, 200 mg/l piperacillin, 50 mg/l rifampicin, and 20 mg/l trimethoprim with 1 drop of Triton X-100 per 100 ml of solution. Finally, just prior to excising the embryos, seeds were disinfected with 30% H₂O₂. Intact embryos were excised with a sterile scalpel. Firm healthy embryos were placed four per plate on Gresshoff and Doy medium (cited in Mudge, 1986) containing 10 mg/l BAP, 20 g/l sucrose, and 8 g/l Bactoagar. The medium was amended with 0, 10, 20, or 40 μ M ARP 2II \pm 0.01 mg/l NAA. There were seven plates (reps) of each of the eight treatments. The plates were allowed to develop for twelve weeks in the culture room under cool white florescent tubes (PAR 70-100 μ mol m⁻² s⁻¹) at ca 25°C. The explants were evaluated for the number and type of buds (adventitious or axillary) on each explant.

ARP significantly reduced the number of buds formed on embryos grown on media containing both BAP and a low concentration of NAA, but it did not reduce the number of BAP-induced buds to level below that formed on embryos grown without NAA. ARP 2II did not affect the number of buds formed on media without NAA, (Figure 10). These results indicate that ARP 2II can inhibit the auxin-stimulated component of bud induction but not that due to BAP alone.

Lily bulb scale assay

Lily bulb scales are commonly propagated by technique referred to as "scaling" in which scales are remove from bulbs and placed in a moist medium where both adventitious bud (bulblet) and root formation occurs at the base of the scale. The application of a small amount of auxin (NAA) often enhances bulblet formation.

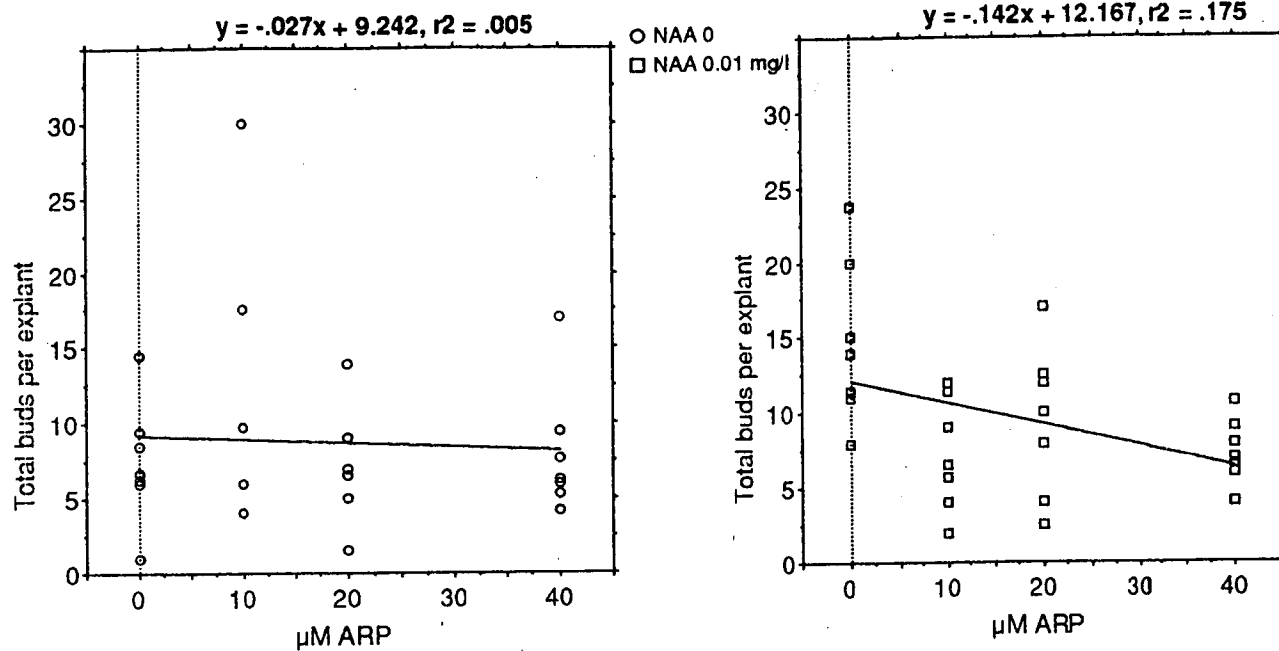


Figure 10. Bud induction response of pine embryos to ARP in the presence of BAP ± NAA.

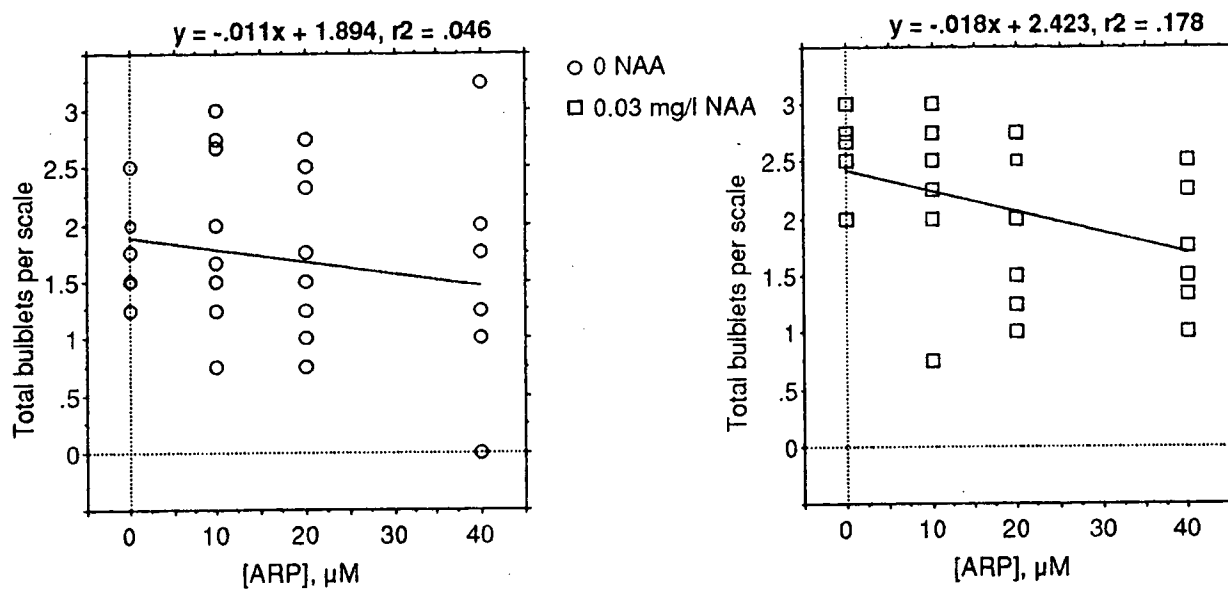


Figure 11. Effect of ARP 2II on bulblet formation on lily bulb scales.

To determine if ARP 2II would affect bulblet formation (i.e. adventitious bud formation), ARP 2II was evaluated in an *ex vitro* lily bulb scale assay. Moderate sized scales with no obvious damage from soil contact were selected from the interior of *Lilium* 'Angelica' bulbs (purchased from B&D Lilies, Port Townsend, WA). Scales were disinfested with 0.5 % sodium hypochlorite for 30 minutes. A shallow lateral cut was made 1 cm above the base of the scale and the end of a 2 x 5 cm strip of seed germination paper was inserted into the cut. The other end of this paper wick was inserted into an inverted 25 mm Bellco Kap-ut containing ca 3 ml of treatment solution. The base of the scale was supported by the paper wick and the edge of the cap. Four scales prepared in this manner were placed in a closed GA-7 culture vessel (rep=vessel) to reduce water loss. There were eight reps of each treatment. The treatments consisted of 0, 10, 20, or 40 μ M ARP 2II \pm 0.03 mg/l NAA in nutrient solution. The culture vessels were placed in the dark at ca 25°C for incubation. The scales were harvested at 10 weeks and the number of bulblets formed were counted. ARP 2II significantly decreased the number of bulblets formed ($p=.0161$) on lily bulb scales, (Figure 11).

In this system, like the pine embryo system described above, ARP 2II did not promote adventitious bud formation, and in fact inhibited an auxin induced response.

2.f.III. Conclusions

As for crude ARP extracts (Raviv, 1981) pure ARP 2II is autoclave-stable which facilitates its study in sterile cultures.

ARP showed some root-promoting activity in two of the four studied systems. The results, however, were not fully reproducible in the case of red raspberry.

The presence of ARP 2II in media containing high levels of cytokinin and low levels of auxin, significantly reduced the production of adventitious buds on pine cotyledons. ARP in the absence of auxin did not stimulate adventitious bud formation, nor did it inhibit bud induction below the level achieved by the controls which contained no auxin.

ARP 2II significantly reduced the number of bulblets produced on lily bulb scales in the presence of exogenously applied auxin, but not on the control bulb scales.

These results suggest that the inhibition of bud or bulblet induction is not simply an inhibition of growth, but may be a modification of tissue response to auxin. With hind sight, weight analysis of the leaf explants or bulblets would give a more definitive answer on the question of growth inhibition.

ARP 2II's inhibitory properties were not limited to auxin induced responses. The compound reduced the number of buds formed on petunia leaf disc explants in the presence of a small amount of cytokinin.

Although the rooting enhancement observed in some assays was statistically significant, the enhancement of rooting was only obtained at concentrations that are relatively high for a hormone-like effect. This suggests that ARP 2II either has a relatively low activity or that it penetrates tissue poorly. Also, the amount of rooting enhancement obtained with ARP 2II was small compared to that obtainable with low levels of auxin. ARP 2II inhibited a number of horticulturally-desirable, phytohormone-induced, organogenic responses.

Given the high levels of ARP needed to enhance rooting and its seemingly contradictory behavior in different systems, ARP 2II in its current form has limited potential for practical use in *in vitro* organogenesis.

In order for ARP 2II or a similar compound to become horticulturally practical, there would need to be further studies with ARP and ARP analogues to determine in more detail which portions of the molecule are important to activity and perhaps obtain a compound that is active at lower concentrations.

References

- Anderson WC (1975) Propagation of rhododendrons by tissue culture: Part 1. Development of a culture medium for multiplication of shoots. *Proc. Intl. Plant Prop. Soc.* 25:129-35.
- Klein CM (1982) Tissue culture propagation of raspberry *Rubus idaeus*, *Rubus occidentalis* and *Rubus neglectus*. M.S. thesis, Cornell University, Ithaca, NY.
- Lloyd G BM McCown (1976) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. *Proc. Intl. Plant Prop. Soc.* 30:421-427.
- Mott RL HV Amerson (1981) Tissue culture plantlets produced from *Pinus monticola* embryonic materials. *Forest Science*, 27:299-304.
- Mudge KW (1986) Micropropagation of mugo pine from embryonic and seedling explants. *HortScience* 21:298.

Sriskandarajal C MG Mullins (1981) Micropropagation of Granny Smith apple: Factors affecting root formation *in vitro*. Jour. Hort. Sci. 56:71-76.

2.g. Timecourse and Anatomy of ARP and IBA induced rooting of Mung Bean Hypocotyl Cuttings.

K.W. Mudge, N.J. Glassbrook, J.P. Lardner, N.L. Bassuk, and B.K. Maynard

2.g.I. Objective

The preliminary finding that ARP may act to stimulate cell dedifferentiation will be investigated by studying the response of mung bean hypocotyls to ARP over time. Paraffin microtechnique will be used to study events at the cellular and tissue level. A similar approach has been used to study the time course of rooting in a number of other systems including *Vigna*, *Pinus*, and *Phaseolus*.

2.g.II. Introduction

As described in earlier annual reports, an experiment was initiated to evaluate the time course of the response of mung bean cuttings to ARP and auxin. These growth substances were pulsed for a 24 hour treatment period on either the 1st, 2nd, 3rd or 4th day after cutting. At the end of each pulse period, and at the end of each subsequent 24 hour period, up to 4 days after cutting, a subset of cuttings from each treatment was fixed for anatomical evaluation, while the remaining cuttings were allowed to root for the normal 8 day period. The goal of this work was to envisage the earliest events associated with rooting, to determine which cell stages are sensitive to ARP and auxin, and how these growth regulators are affecting the timing and/or extent of these early events.

2.g.III. Materials and Methods

In the first replicate of this experiment, during 1987, cuttings were pulsed for 24 h either with 4 mg/L IBA or 10^{-4} M ARP. IBA and ARP solutions contained 1% EtOH and 0.01% TWEEN-20 (to maximize solubility of ARP). During the second repeat of the experiment in 1989, a lower ARP concentration was used (4×10^{-5} M, without Tween), because persistent problems with ARP solubility (even in the presence of Tween 20). The second experiment also differed from the first in that growth regulator and control treatment solutions contained dilute inorganic nutrients. In both experiments, times of application (pulses) were 0-24, 24-48, 48-72, and 72-96 hours. Pulses were preceded and followed by tap water.

For each experiment, 2 cuttings were harvested for anatomical evaluation at the end of the pulse period, and at the end of each additional 24 h period up to 96 hours after the start of the experiment (time 0 is the time at which cuttings were taken). Harvesting for anatomical observation involved cutting a 3 cm section from the base of the hypocotyl cutting and fixing it in FAA. After fixation for a minimum of several weeks, each of the 3 cm sections was cut in half and dehydrated in an ethyl alcohol/tert-butyl alcohol series, infiltrated and imbedded in paraffin, sectioned at 20 μm with a rotary microtome, and stained with safranin and fast green (Johanssen, 1940). Four groups of 5 serial 20 μm thick sections from the basal 1.5 cm long hypocotyl segment were mounted on glass microscope slide for microscopic evaluation. The first group of five sections (totaling 100 μm thick) began at 900 μm from the base of the cutting, and each successive group was separated from the previous one by 200 μm . Since no root primordia were found in the apical 1.5 cm segment in the first experiment, it was not evaluated in the second experiment, and all results discussed below are from the basal segment of each hypocotyl cutting.

Root primordia were most commonly located in the phloem parenchyma exterior to and between each of the 4 paired bundles of xylem elements, Plate 1. Occasionally primordia developed at an atypical position intermediate between paired xylem bundles, exterior to the single xylem bundle that occurs at this location, Plate 1. The occurrence of root primordia in these atypical locations was random, and unassociated with any particular growth regulator or pulse time treatments. For a given series of five 20 μm sections each of the 4 ("A" through "D") potential primordium locations (exterior to a pair of xylem bundles) was scored for the maximum stage of primordium development (as defined below) reached in that series. The "maximum developmental stage" referred to in Figure 13 was the highest rating recorded for all 4 series for a given treatment (pulse time x growth substance x harvest time combination). The "average developmental stage" referred to in Figure 14 was an average of the maximum stage of development recorded for each of the 4 series for a given treatment.

Stages of development are defined as follows:

1. nucleus small, elongated, weakly stained and found close to the cell wall, Plate 2.
2. nuclei enlarged, occupying a central position in the cell; nucleoli enlarged, and prominent, Plate 3.
3. cytoplasmic staining density increased compared to stages 1 and 2; phloem parenchyma cells beginning to divide, Plate 4, and Plate 5.

4. abundant (rapid) divisions of phloem parenchyma cells, yielding many small densely cytoplasmic cells. Mitotically active cells include phloem, starch layer (a more or less distinct ring of relatively small cells just interior to the cortex), and occasionally, the inner cortex, Plate 6.

5. well developed root primordia enlarged by division of initials, daughter and surrounding cells; adjacent cells contribute to primordia by forming root cap-like covering over advancing primordia. The most advanced primordia have disrupted the cortex, and nearly emerged, Plate 7.

For each treatment, in addition to the cuttings harvested as described above for anatomical evaluation, 6 replicates of 3 cuttings each were left in distilled water for a full 8 days, after which time they were harvested and emerged roots were counted.

The optimal 24 hour treatment period (pulse) for ARP differed from that for IBA, as is apparent from Figure 12. In experiment 1 the most effective ARP pulse was day 2 (24-48 h), while for IBA there were 2 peaks of maximum sensitivity - day 1 and a slightly greater peak at day 3. In experiment 2 there was a similar unimodal sensitivity to ARP, and a bimodal sensitivity to IBA, but the maximum peak for each growth substance was one day later than in experiment 1, ie. peak ARP sensitivity was at day 3, while the maximum (later) peak of IBA sensitivity was at the day 4 pulse. In both experiments the period of maximum sensitivity to ARP preceded that for IBA by 24 hours. This is consistent with the finding of Raviv, Reuveni, and Goldschmidt (1986) who reported peak sensitivity to ARP at day 2, followed by peak sensitivity to the auxin IAA at day 3. They did not report the day 1 peak for IAA as we observed for IBA in this experiment, but Raviv has noted a similar bimodal response for IBA (unpublished).

In general the stages of development for primordia in ARP treated cuttings was identical to those for IBA treated cuttings (Plates 2 - 7). Figure 13 and Figure 14 show the effect of IBA or ARP pulse time on maximum or average primordium development at 4 days after the start of the experiment. The trends apparent at harvest day 4 are similar to those for earlier harvest times. Primordium development data from both experiments were pooled since response was similar in both.

In interpreting these data it is helpful to keep in mind that there are essentially two groups of primordia developing in any cutting treated with either IBA or ARP. Those primordia induced by wounding (the act of making the cutting at time zero), which presumably begin developing from time zero. The second group of primordia are those which are induced to begin developing in

response to growth substance application, which, depending on pulse time and rate of development may begin developing after wound induced primordia. One would expect that if growth substance induced primordia began to develop at or shortly after the growth substance pulse the average stage of primordia development (both groups) in growth substance treated cuttings would be less than in control cuttings since the average will include not only the more developed (older) wound induced primordia but also the less developed growth substance induced primordia. Surprisingly, this lowered average stage of development is observed for ARP treated cuttings (compared to the control) to a greater extent than for IBA treated cuttings, Figure 14. This result suggests that primordia induced in response to APR treatment begin development more rapidly than those induced by IBA, i.e., fewer early stage growth substance induced primordia were present in IBA than in ARP treated cuttings, so that the average of primordia initiated early by wounding plus those initiated later by growth substance was decreased relatively less by IBA treatment than by ARP treatment. In other words, at day 4 more early stage ARP induced primordia were present than IBA induced primordia, resulting in a lower average stage of development. The delayed pulse time optima for IBA (day 3 or 4) compared to ARP (day 2 or 3) shown in Figure 12 suggests that (some of) the roots initiated in response to IBA regardless of the timing of application, begin to develop later (day 3 or later) than those developing in response to ARP (day 2 or 3).

The bimodal sensitivity to IBA, though not particularly relevant to this discussion of ARP physiology, is none the less interesting. It suggests that IBA may promote rooting by 2 separate routes - an early acting phase (day 1) in which IBA synergizes the effect of wounding on primordium initiation, and a later phase which is independent of wound induced rooting (3 to 4 days post wounding). The fact that IBA is more active than IAA in stimulating both ethylene production and rooting of mung bean hypocotyl cuttings, and the fact that only IBA but not IAA exhibits the early stage of sensitivity suggests that the early stage of sensitivity to IBA may be related to IBA induced ethylene production and not to the usual auxin-induce root formation as affected by the native auxin - IAA.

References

Johansen DA (1940) Plant Microtechnique. McGraw Hill, New York.

Raviv M O Reuveni and EE Goldschmidt (1986) Evidence for the presence of a native, non-auxinic rooting promoter in avocado. Pl. Growth Reg. 4:95-102.

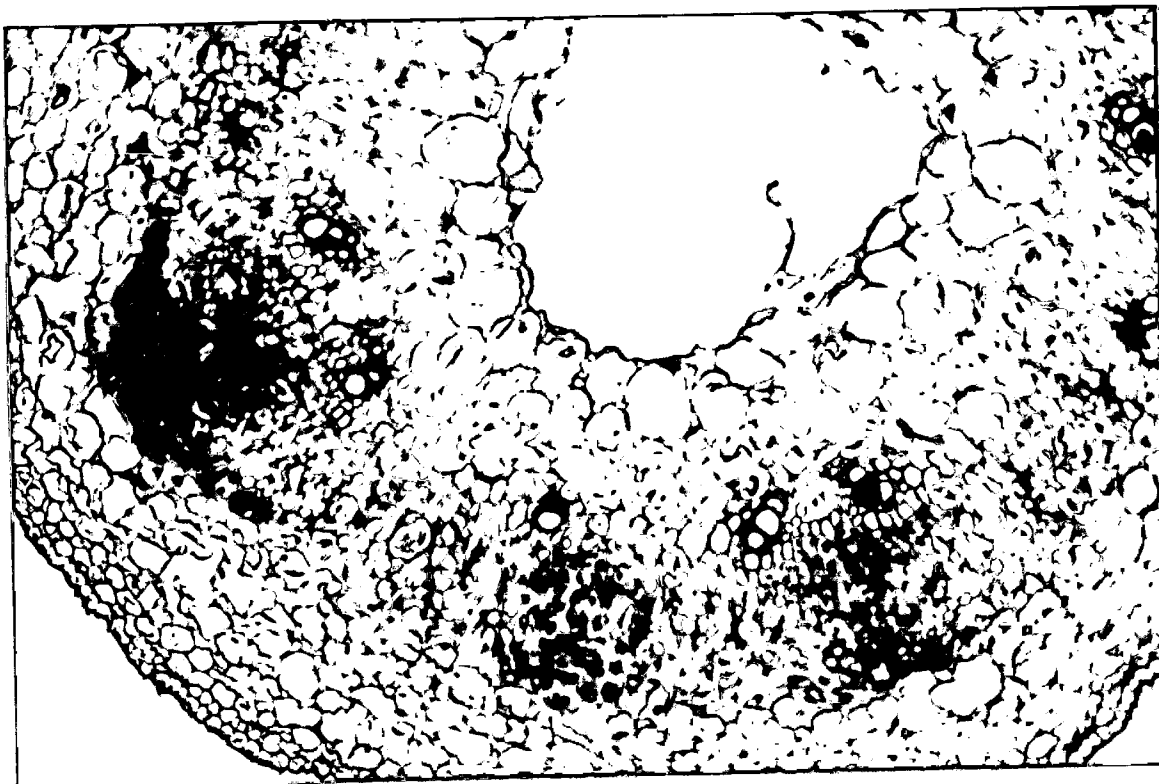


Plate 1. Cross section of a mung bean hypocotyl cutting showing typical and atypical locations for root primordium development. Primordia typically develop exterior to paired xylem bundles, and atypically occur between xylem bundle pairs, exterior to the single (unpaired) xylem bundle that may occur at this location.

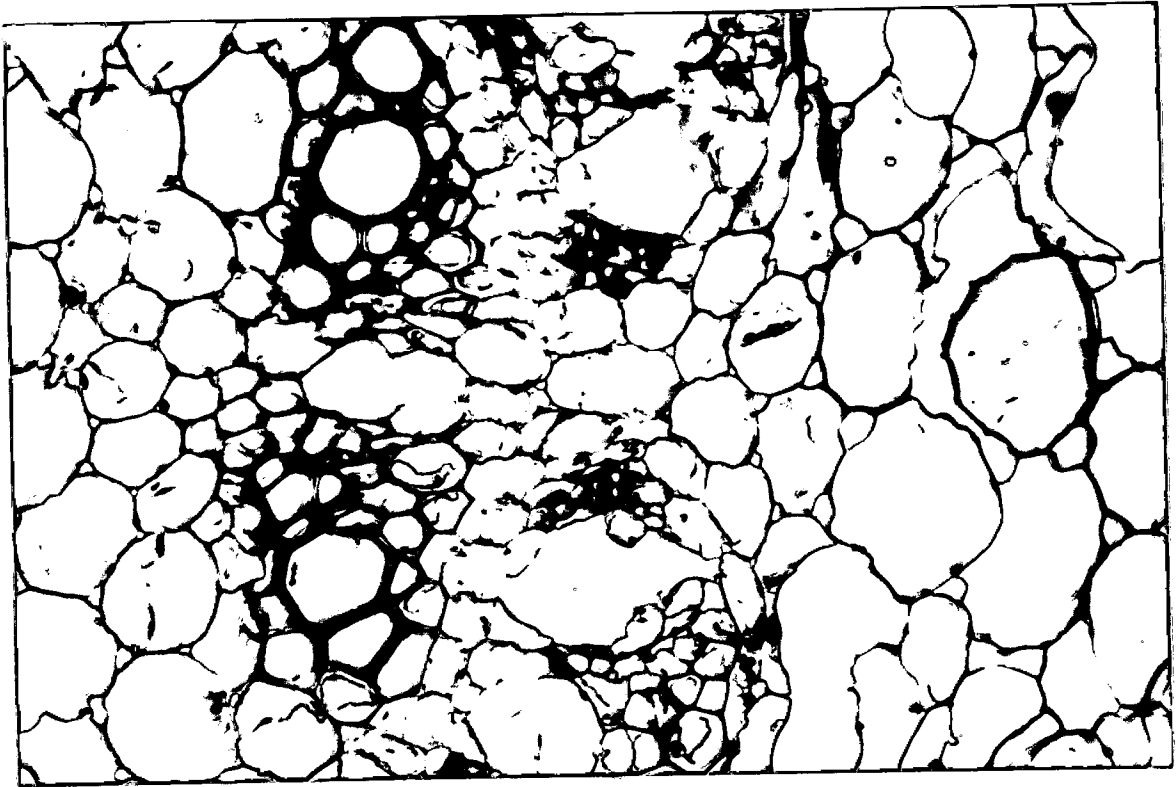


Plate 2. Cross section of a mung bean hypocotyl cutting showing a typical stage 1 root primordium.

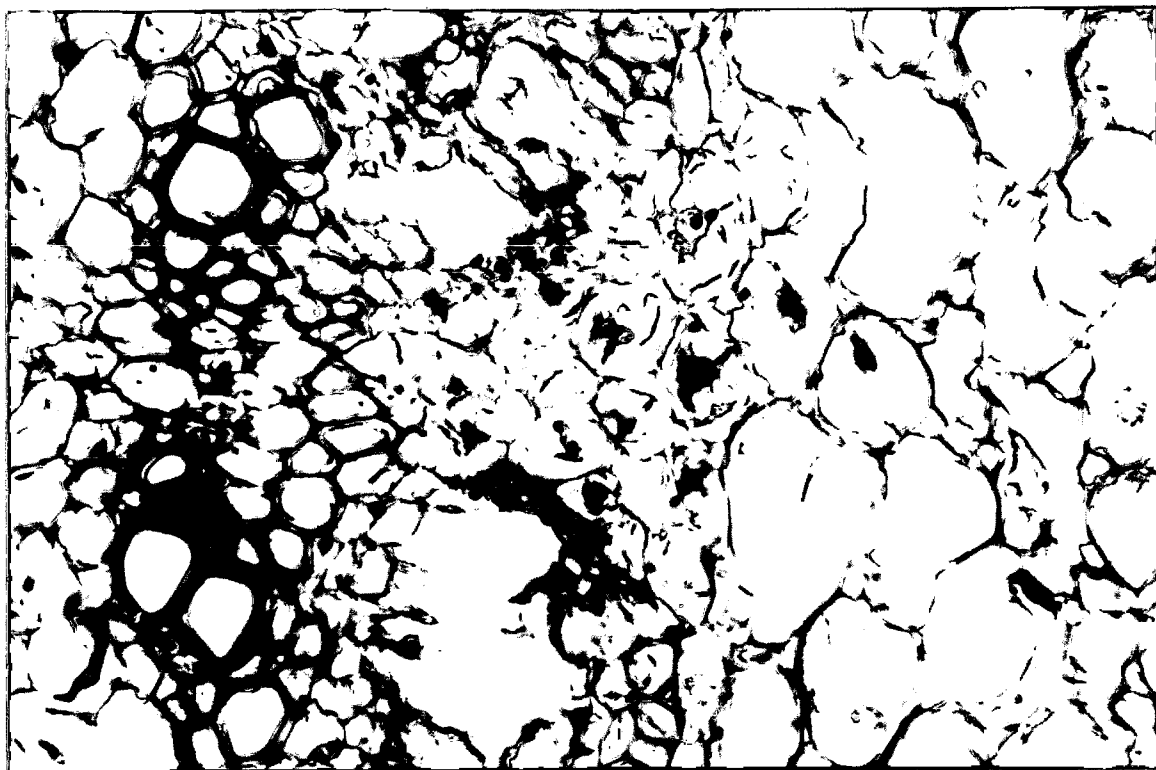


Plate 3. Cross section of a mung bean hypocotyl cutting showing a typical stage 2 root primordium.

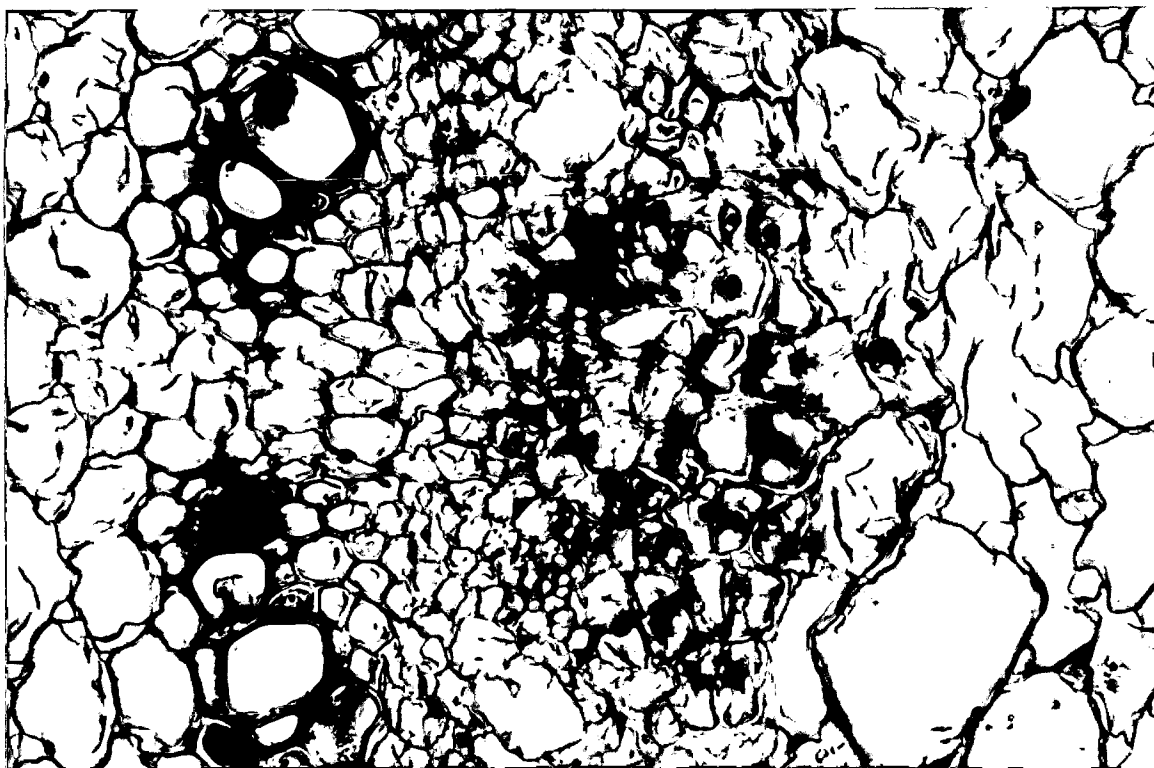


Plate 4. Cross section of a mung bean hypocotyl cutting showing a typical stage 3 root primordium.

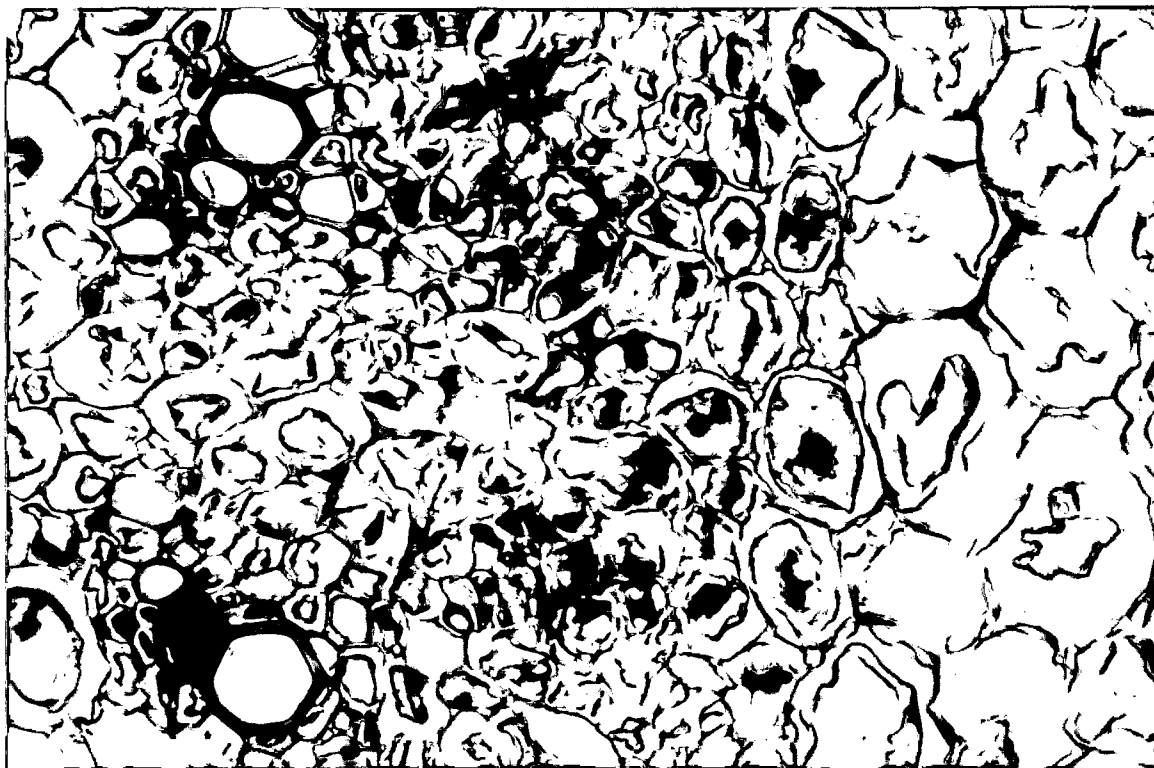


Plate 5. Cross section of a mung bean hypocotyl cutting showing a typical stage 3 root primordium. Note mitotic activity (cell division) in upper right region of the primordium just interior to the cortex

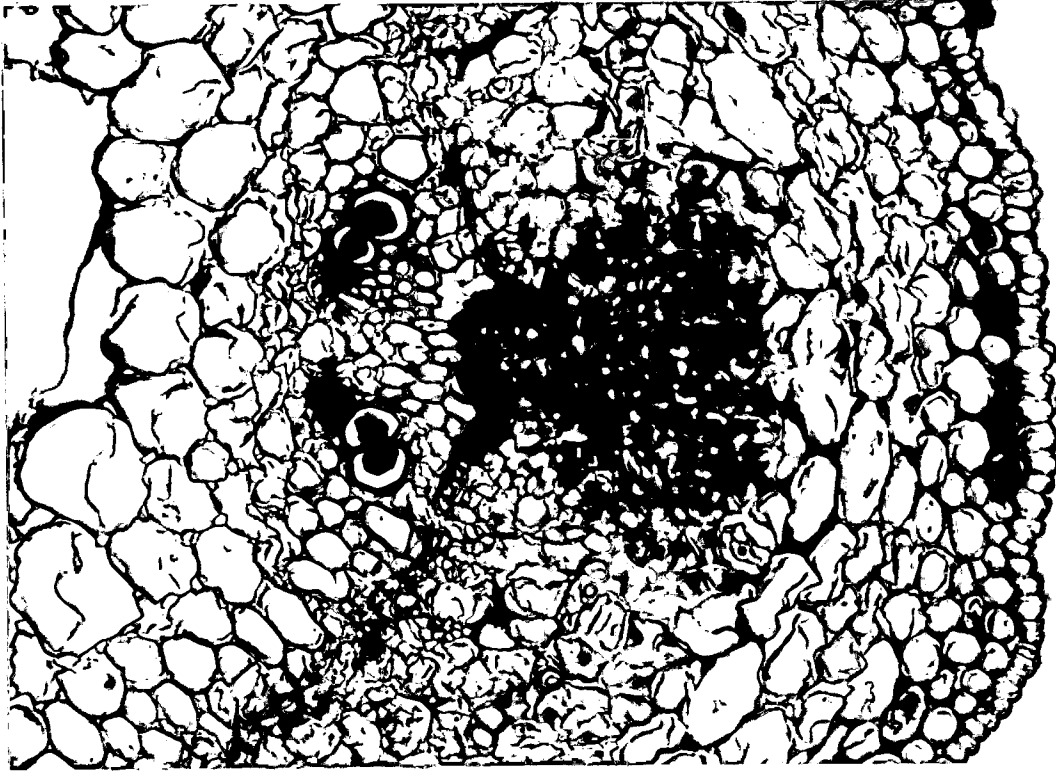


Plate 6. Cross section of a mung bean hypocotyl cutting showing a typical stage 4 root primordium.

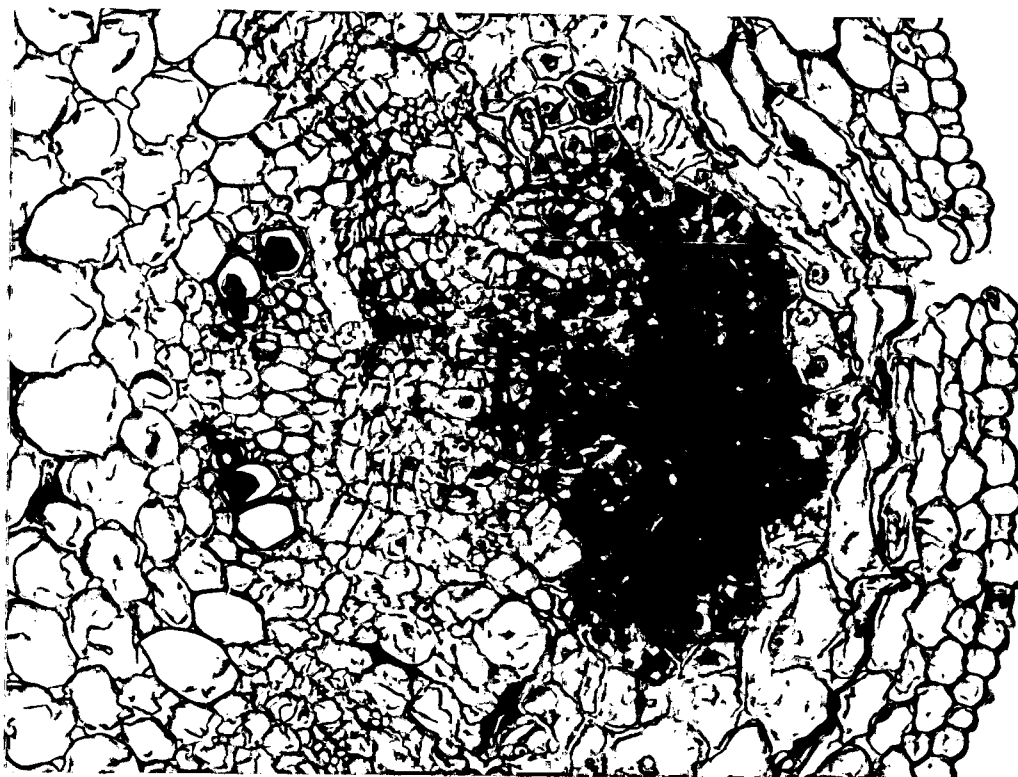


Plate 7. Cross section of a mung bean hypocotyl cutting showing a typical stage 5 root primordium.

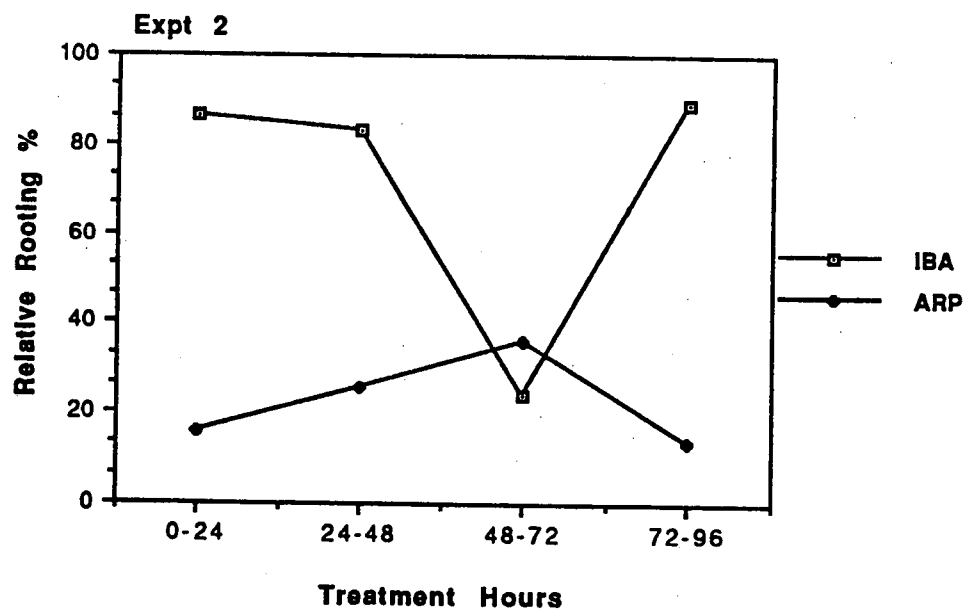
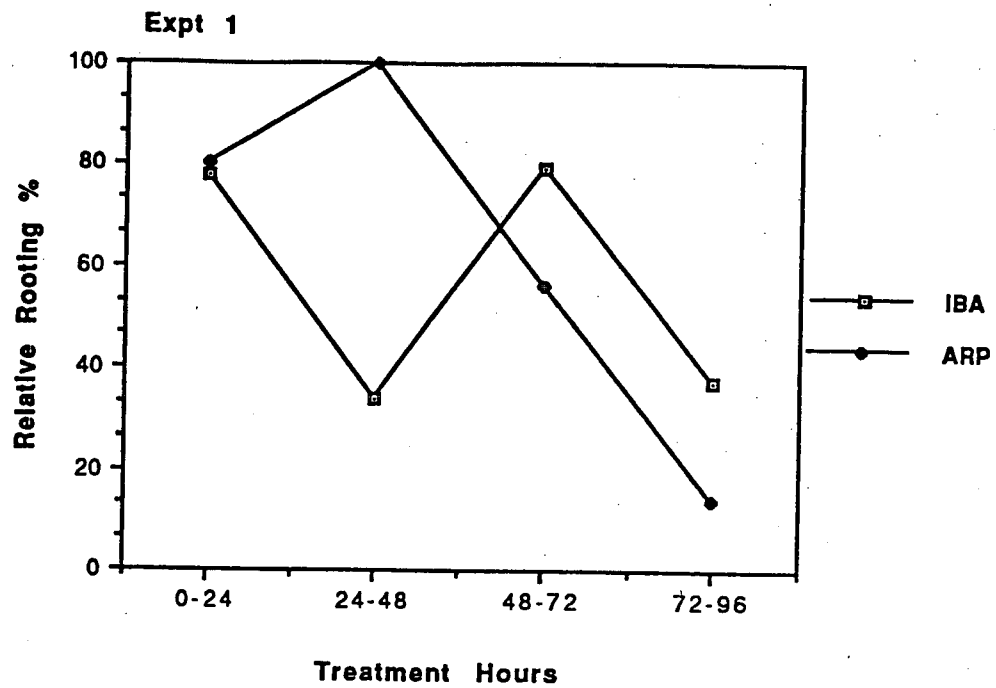


Figure 12. Effect of IBA or ARP pulse (treatment hours) on rooting of mung bean hypocotyl cuttings eight days after cutting.

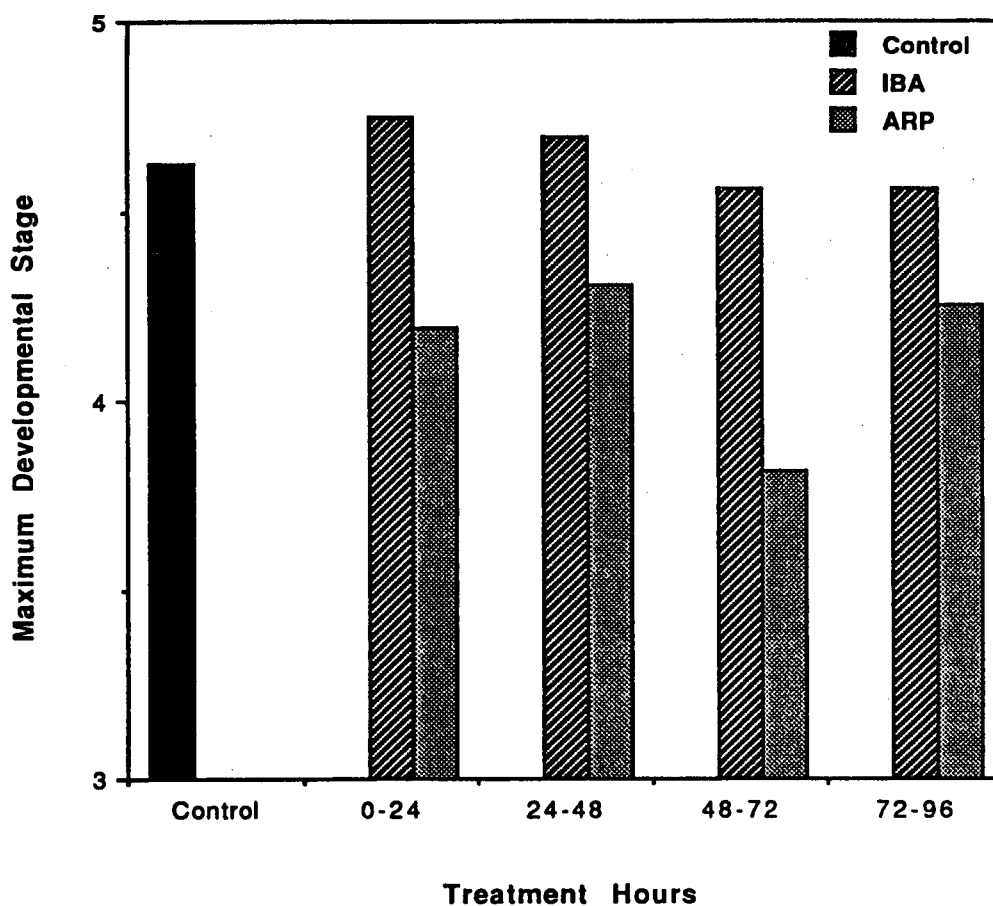


Figure 13. Effect of IBA or ARP pulse (treatment hours) on maximum developmental stage of root primordia in mung bean hypocotyls harvested 96 hours after cutting.

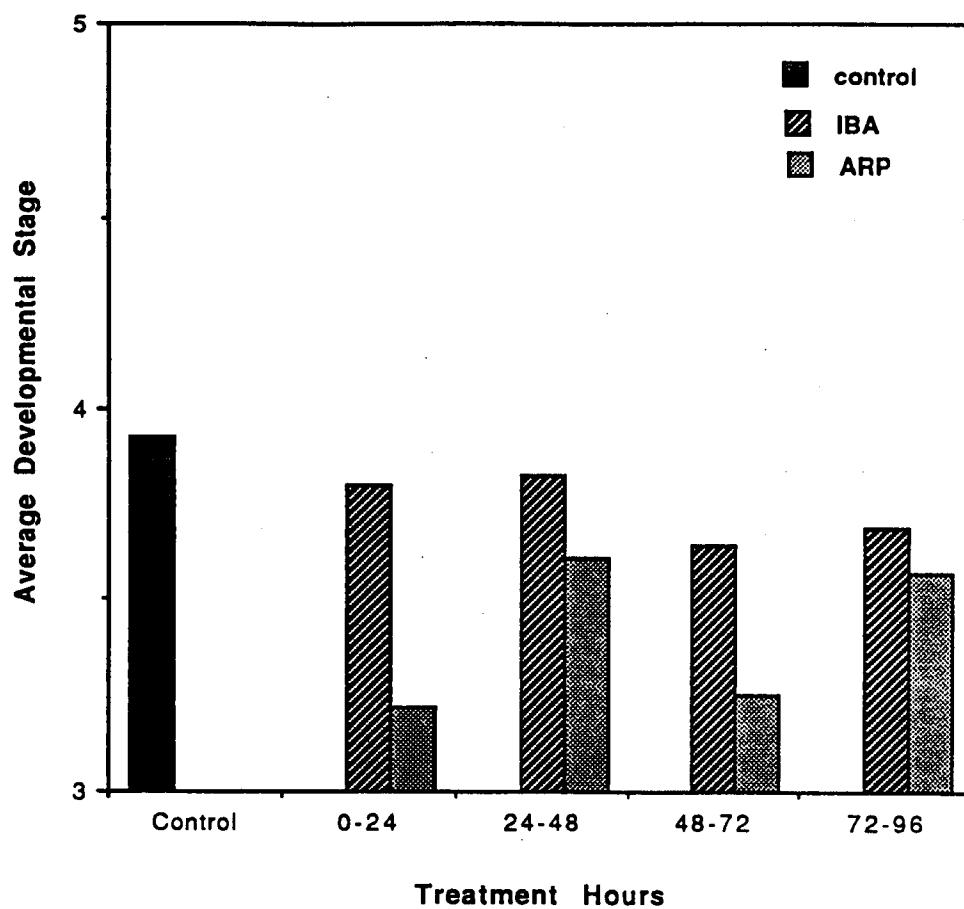


Figure 14. Effect of IBA or ARP pulse (treatment hours) on average developmental stage of root primordia in mung bean hypocotyls harvested 96 hours after cutting.

2.h. ARP translocation in plant tissues

K.W. Mudge, N.J. Glassbrook, M. Raviv, Sh. Medina

2.h.I. Objective

At present, there is no direct evidence as to whether ARP is mobile in plant tissues. Although its water solubility is enough to account for its activity in the mung bean bioassay, it seems necessary to prove its actual translocation in the vascular system of avocado. The suggested experimental approach to this subject depends upon the completion of both synthesis and radioactive labeling of ARP which will enable its detection by autoradiography or other radioactive techniques.

2.h.II. Introduction

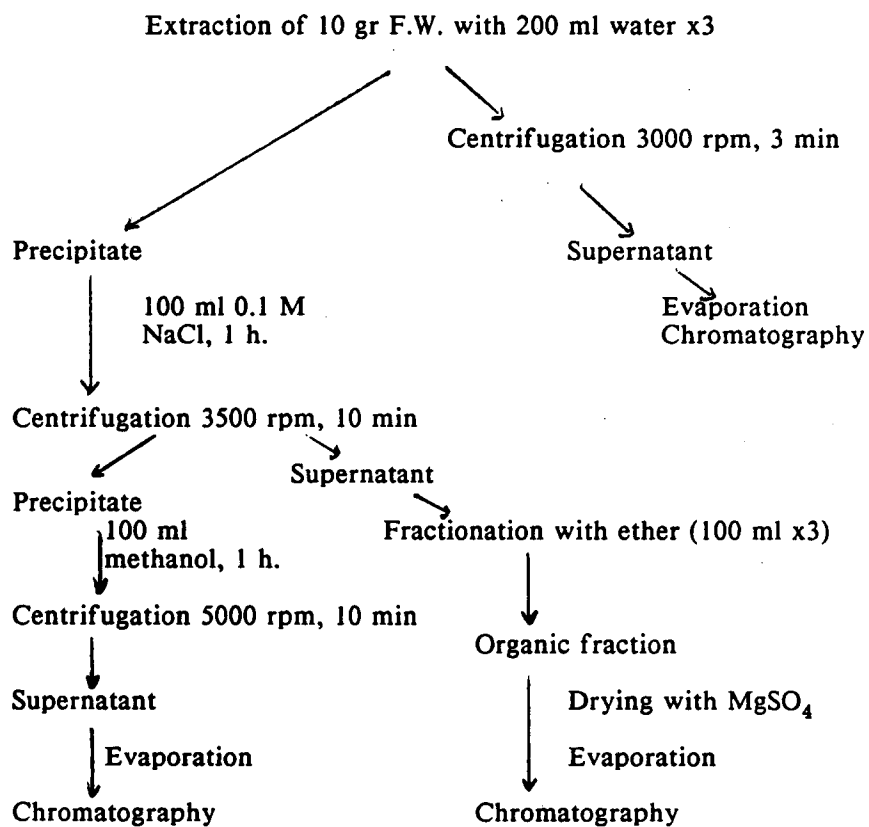
In a previous study (Raviv, 1981), we showed that leafless or girdled juvenile avocado cuttings which normally will root readily cannot root even if supplemented with optimal concentrations of IBA and/or sugar. Since ARP is mainly found in leaves and buds (Raviv *et al.*, 1986) it suggests an ARP translocation from the cutting's tops to their bases until a threshold amount is accumulated that induce root formation. Levels of ARP in cortex of the bases of avocado cuttings were analyzed using mung bean bioassay and indeed it was found that ARP accumulates there to large concentrations until a few days before root emergence. Since no radioactively-labeled ARP was available to us, we had to address the issue in an indirect manner by searching for response to ARP rather than tracing radioactive ARP. In addition, we applied an extraction technique enabling the distinction among compounds which are part of a membrane, are adsorbed on a membrane or not connected to membranes.

2.h.III. Results and discussion

ARP's ultra-structural localization

Ten gr. F.W. of young avocado leaves were extracted and fractionated according to flow sheet no. 2.

Flow Sheet no. 2



The three resulted fractions were chromatographed on whatman paper 3mm with isopropanol water = 8:2 (v:v) and the last section (R_f 0.9 - 1.0) served for mung bean rooting bioassay. Equivalents of 0.01, 0.05, 0.1, 0.2 and 0.5 gr F.W. were tested against control (chromatographed paper strip without plant material). Using this procedure, the aqueous fraction contains a compound which were not part of or attached to membranes. The NaCl causes removal of compounds previously adsorbed on membranes. These compounds will later be preferentially fractionated into the etheric fraction. Methanol will finally cause disintegration of membranes. As can be seen in Table 17, almost all the rooting activity was not extractable by water or 0.1 M NaCl and it therefore can be concluded that under normal conditions ARP is mostly a part of the membranes in cells of avocado leaves.

Table 17. Number of roots/mung bean cutting as affected by various concentrations of 3 fractions extracted from avocado leaves.

Concentration (gr. eq. cutting ⁻¹)	Fraction		
	Aqueous	NaCl	Methanolic
0	6.2 a	6.2 a	6.2 a
0.01	5.5 a	5.9 a	10.4 b
0.05	5.8 a	6.1 a	18.1 c
0.10	7.4 a	6.0 a	23.5 d
0.20	6.8 a	7.6 a	25.1 d
0.50	11.7 b	9.0 ab	24.9 d

The existence of some activity in the aqueous fraction suggests, however, that ARP can be transported to other parts of the plant at least to small extent.

To explain (in part) the promotive effect of leaves on rooting, Jarvis (1986) has proposed that auxin-stimulated rooting is facilitated by acropetal transport of auxin through the transpiration stream (xylem) from the base of a cutting to the leaves where it is loaded into the auxin transport system and retransported basipetally back to the cutting base where it exerts its effect on rooting. To test if leaves were in some way involved in ARP-stimulated rooting the following three experiments were performed.

Foliar application of ARP

Leaves of cuttings from seven-day-old mung beans were injected with approximately 25 to 50 μ l of 0 or 40 μ M ARP in nutrient solution. The cuttings were then placed in vials containing nutrient solution as noted above. Cuttings were harvested after eight days and the roots counted.

The injection of ARP 2II had no effect on the number of roots on the mung bean hypocotyls. Control roots had an average of 25.4 roots per cutting; cuttings injected with ARP 2II had 24.8 roots per cutting.

In another experiment intended to detect ARP transport from mung bean leaves to the cutting's base we applied it via lanolin paste smeared on one of the leaves. In this experiment, too, there was no increase in rooting over the control. However, in the same experiment, we applied IBA over a wide range of concentrations (up to 10 milimols) and no root stimulation was detected either. It can be therefore concluded that translocation of exogenously applied PGR in this system is too slow to be manifested in the time frame of the bioassay.

Effect of ARP and IBA on rooting of decapitated mung bean hypocotyl cuttings.

The lack of a rooting response to foliar application of ARP 2II suggests that translocation of ARP 2II to the leaves is not necessary for rooting at the base of the hypocotyl cutting. However, the experiment is somewhat inconclusive given the difficulty of injecting a controlled amount of material into the leaves. In order to further explore the question of the involvement of ARP or auxin translocation on subsequent rooting, a mung bean hypocotyl rooting bioassay was performed using decapitated mung bean cuttings. Under these conditions it is assumed that acropetal transport would be much reduced due to absence of transpirational uptake of solution from the base of the cuttings.

Mung bean cuttings from eight-day-old seedlings were selected and prepared as in previous experiments, except that the cuttings were decapitated to leave 3 cm of epicotyl above the cotyledonary node. In each vial, two cuttings were placed in the normal physiological orientation (epicotyl up) and one cutting was placed inverted (epicotyl immersed in the treatment solution). There were five vials in each row (rep) and five rows of each treatment for a total of 50 upright cuttings and 25 inverted cuttings for each treatment. The treatment solutions consisted of 0, 20, and 40 μ M ARP 2II in nutrient solution. A positive control consisted of 0.5 ppm IBA in nutrient solution. After ten days, the cuttings were harvested and evaluated for the total number of roots on each cutting.

ARP 2II significantly enhanced the rooting of decapitated mung bean hypocotyl cuttings that were in the normal upright orientation, as did IBA. ARP 2II did not enhance the rooting of the inverted cuttings. Almost none of the inverted zero control or ARP 2II treated cuttings rooted. On the other hand, all of the inverted cuttings treated with IBA rooted, with root numbers comparable to that of

the upright cuttings IBA-treated cuttings, although the roots did not elongate well. Results are shown in Figure 15.

In the mung bean hypocotyl system, ARP 2II appears to act at the site of application. Unlike IBA, ARP 2II does not translocate basipetally in sufficient quantity to enhance rooting of inverted cuttings.

2.h.IV. Conclusions

The indirect evidence presented in the introduction suggests the translocation of ARP in avocado cuttings. Accumulation of ARP can be detected in the rooting region of avocado cuttings 10 days from excision. During the time preceding root emergence, there is a flow of assimilates from the leaves to the region which is closely associated with rooting (Reuveni and Raviv, 1981). A similar trend can be observed in the cortical region of Rangpur cutting's bases (Table 12). In the mung bean system, we were not able to demonstrate ARP translocation.

In the future, the use of immunoassay technique developed during this project will enable us to study ARP's translocation in a critical manner.

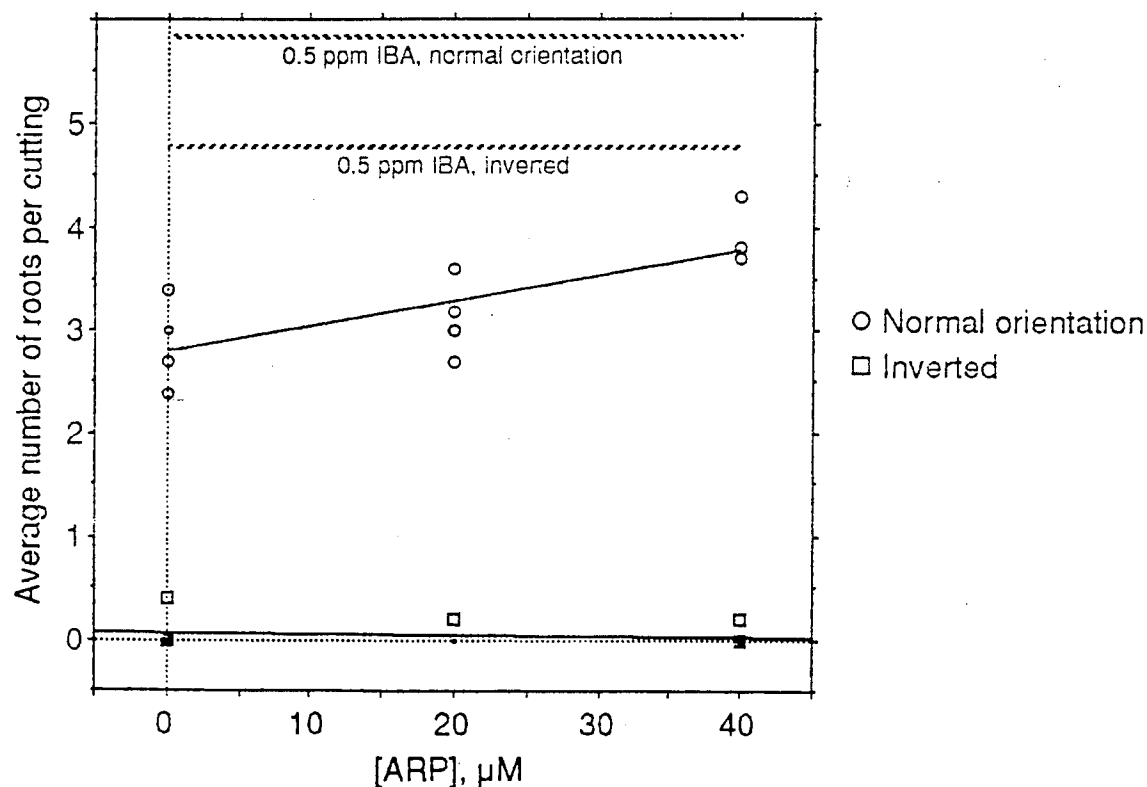


Figure 15. Rooting response of decapitated eight-day-old mung bean hypocotyl cuttings to ARP 2II and IBA.

References

- Jarvis BC (1986) Endogenous control of adventitious rooting in non-woody cuttings. In: New Root Formation in Plants and Cuttings. Edited by M.B. Jackson. Martinus Nijhoff Publ. Dordrecht.
- Raviv M (1981) Juvenility and rooting in avocado cuttings. Ph.D. Thesis. The Hebrew University, Jerusalem (In Hebrew with English abstract)
- Reuveni O and M Raviv (1981) The importance of leaves retention and their contribution to rooting of avocado cuttings. Amer. Jour. Hort. Sci. 106:127-130.

2.i. ARP's mode of action M. Raviv, Sh. Medina

2.i.I. Introduction

When studying the way through which a plant growth regulator (PGR) affects rooting, one should consider several aspects of the process such as where the PGR is normally synthesized? Is the production site different from the rooting site and if so, how is the PGR translocated and at what rate? At what specific rooting stage the PGR exerts its effect and at what concentration? Which morphological events occur during this stage? How it interacts with other PGRs? All these questions were not asked in our proposal since our knowledge at that time was of a preliminary nature. However, as more knowledge gathered and especially when pure material became available, it became possible to deal with some of these questions.

Previous data suggested that the beneficial action of ARP in rooting of mung bean cuttings occurred sometime before IAA exerts its effect (Raviv *et al.*, 1986). Since there is no doubt about the central role of IAA in adventitious root formation, we focused our attention to some possible interactions between IAA and ARP.

2.i.II. Results and discussion

In a previous study (Raviv *et al.* 1986a) we demonstrated the dual nature of the relationships between ARP and IAA. Similar phenomenon was found for PCIB (Kefford, 1973, Raviv *et al.* 1986a) and for naturally-occurring compounds (Tognoni, 1972).

The ARP tested in the above-mentioned study was at this time a partly purified mixture, proved to contain at least 4 different compounds. One of these (II) is identical to the growth inhibitor which was previously found by Gazit and Blumenfeld (1970) and identified by Bittner *et al.* (1971). This fact prompted us to test again ARP's activity in the most direct and sensitive auxin bioassay (Straight growth of wheat coleoptiles) with the pure compounds.

The test was conducted by Prof. E.E. Goldschmidt in the Faculty of Agriculture and we are grateful for his help.

Three compounds were tested - II, 2I, and 2II.

In the first experiment 1.10^{-5} M of the three compounds were tested either alone or with 2.10^{-7} M IAA. The results are shown in table 18.

Table 18. IAA and ARP effects on growth of wheat (CV. Miriam) coleoptiles.

Treatment	Coleoptile length (mm)
Buffer control	14.78 d
II	14.69 d
2I	14.39 d
2II	14.33 d
IAA	20.47 a
IAA + II	18.08 bc
IAA + 2I	18.56 ab
AI + 2II	17.42 c

A second experiment was carried out in order to verify these results and to obtain some dose response data. Its results are shown in table 19.

Table 19. IAA and ARP effects on growth of wheat (CV. Miriam) coleoptiles.

Compound*	Concentration (M)		
	2.10^{-6}	1.10^{-5}	5.10^{-5}
1 I	18.40ab	19.10a	18.63a
2 I	18.07ab	17.83ab	15.53c
2 II	17.00b	15.03c	10.87d

IAA alone - 18.37 ab

Buffer control - 15.70 c

*In the presence of 2.10^{-7} M IAA.

From these two experiments, it can be concluded that although ARP does not affect the straight growth of wheat coleoptiles on its own, it clearly and efficiently antagonized the auxin promotive effect in this bioassay and it does so in the same efficiency order of its activity as a rooting promoter. These results are different from those obtained by Bittner *et al.* (1971) and may indicate that they used a mixture of compounds 1I and possibly 2II and not a pure 2I as suggested there.

In a recent review, Gaspar and Hofinger (1988) show that in a large number of plant species, IAA in freshly-excised cuttings is first oxidized and only at a later stage starts to accumulate, just prior to the beginning of the primordium formation.

Bhattacharya (1988) in reviewing the status of IAA oxidizing enzymes show a parallel trend of an initial peak of activity before returning to normal levels at a later stage.

It was thus hypothesized that in the normal course of adventitious root formation, the decline in IAA level is an event of physiological significance. It was therefore postulated that ARP might affect IAA metabolism. Prof. Y. Riov of the Hebrew University, Faculty of Agriculture, Rehovot, tested this hypothesis for us. He found no effect of ARP on IAA metabolism or conjugation pattern, therefore this hypothesis can be ruled out. Another explanation for the IAA/ARP interaction is by ARP acting as an auxin transport regulator.

ARP as an auxin transport regulator

It was found recently that auxin polar transport can be inhibited by naturally occurring flavonoids. These compounds exert their effect by binding to the same receptor as naphthylphthalamic acid (NPA) - a known synthetic IAA polar transport inhibitor (Jacobs and Rubery, 1988). Wounding often causes a localized increase in flavonoid synthesis in cells adjacent to those directly damaged (Rengel and Kordan, 1987). Jacobs and Rubery (1988) suggested that this reaction is highly adaptive "serving to hold auxin within cells near to the wound by inhibiting the efflux carrier while not affecting auxin transport in unwounded portions of the plant."

Although chemically ARP is very different from flavonoids we checked the possibility that ARP acts in the rooting process of mung bean cuttings through a similar mechanism as quercetin - the most effective polar auxin transport inhibitor.

All possible combinations of 4 quercetin concentrations (0, 10^{-7} M, 10^{-6} M, 10^{-5} M), 3 IAA concentrations (0, 10^{-5} M, $5 \cdot 10^{-5}$ M) and 2 ARP concentrations (0, $2 \cdot 10^{-5}$ M) were tested as to their effect on rooting of mung bean cuttings. The results appear in Figure 17. These results suggest that

inhibition of polar auxin transport, if indeed occurring in mung bean, cannot offer any advantage for the process of adventitious root formation. The addition of quercetin did not affect rooting of control cuttings, of cuttings treated with IAA, ARP or their combination.

Another approach which was studied as a possible mechanism enables this IAA/ARP interaction was that ARP may act as a modifier of the tissue's sensitivity to auxin. In order to test this hypothesis, a plant reaction should be used which is highly sensitive to auxin and especially to the native auxin IAA. Rooting bioassays are not suitable for this purpose due to their low sensitivity to IAA and their long duration.

The gravitropic response of maize roots was chosen since it is rather quick (4 h.) and very sensitive to IAA.

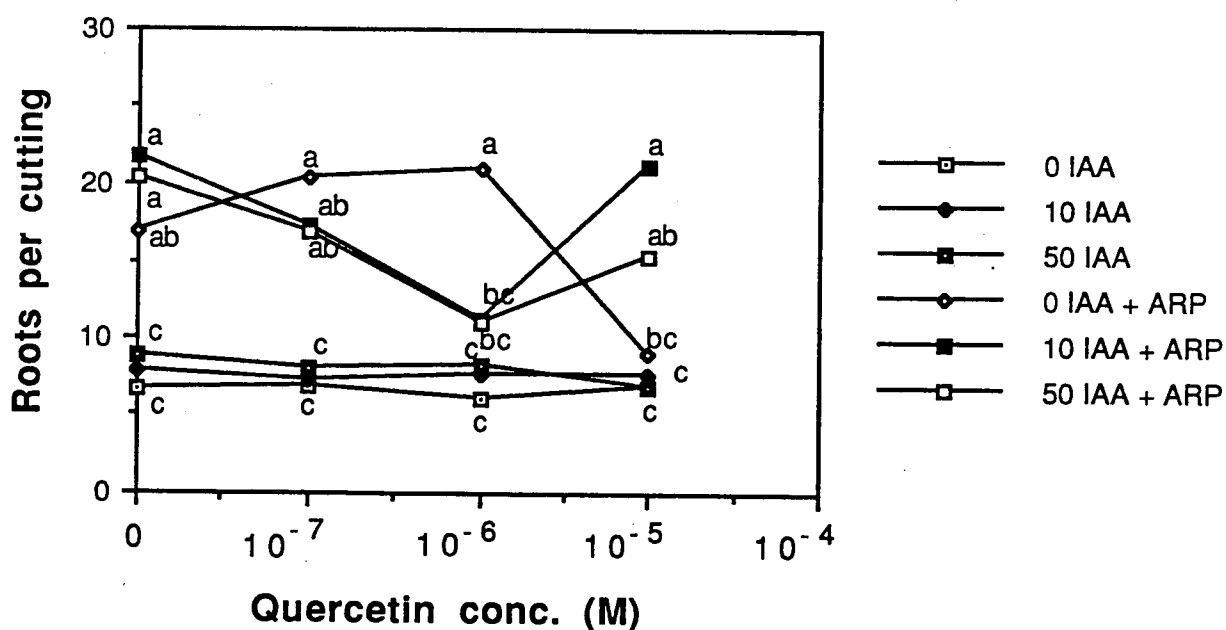


Figure 17. Rooting response of mung bean cuttings to various combinations of Quercetin, IAA and ARP.

Materials and methods

The bioassay is based upon the ability of gravitropic-neutral dark-grown maize radicles to respond to light pulse by initiating gravitropic bending. This reaction is elicited by immediate redistribution of IAA following the light pulse. IAA migrates to the lower part of the root, causes there a growth inhibition. When the upper part of the root continues its normal growth, bending occurs.

Growth regulators (GR) applied to the elongating zone (just behind the root apex) can modify this reaction at very low concentrations.

Maize roots were germinated under complete darkness for 3 days. At this stage, when their primary roots (radicles) are 2-4 cm. long they were exposed to brief light pulse during which the GR were applied via tiny agar blocks. The curvature angle is measure 3-4 hours after the light pulse and is greatly affected by the nature and concentration of the growth regulator applied. The available literature on the methods applied to this bioassay was incomplete and we had to spend some time to refine the working procedures.

Seed germination medium.

Any physical pressure applied to the root abolishes its responsiveness. In one experiment we tested this effect and found that even very gentle touch caused a reduction of the bending angle from $41.0^\circ \pm 3.4^\circ$ in control to $10.8^\circ \pm 9.0^\circ$.

Vermiculite is usually mentioned in the literature as a germination medium. Seed exposure and cleaning is somewhat inconvenient and time consuming when vermiculite is used but the problem of thigmotropic response interfering with the gravitropic one is by far more disturbing. Since some physical contact cannot be avoided when seed are embedded in the vermiculite we decided to test the use of filter paper, arranged in layers as a germination medium and found it better (and, of course, easier to handle) than vermiculite.

Physical support during the test itself.

Practically nothing is mentioned as to this detail in the literature. Small rockwool slabs found to be ideal for this purpose.

Light intensity

Light is essential for the induction of the gravitropic response. Large variations can be found among different laboratories as to the details of light intensity and duration. We found that a short (10 min.) pulse of light emitted from cool-white fluorescent tubes with fluence rate of ca. $10\mu\text{Mole}\cdot\text{sec}^{-1}\cdot\text{m}^{-2}$ saturates the light requirements of the reaction. The subsequent growing period is done under darkness.

Calcium

Calcium is required for the manifestation of the gravitropic stimulus through its effect on auxin transport. In order to avoid possible Ca^{++} deficiency, the filter paper which serves for germination is saturated with a solution of 150 ppm Ca^{++} supplied as $\text{Ca}(\text{NO}_3)_2$.

Plant material

Various cultivars of maize were tested for their sensitivity and for uniformity of germination and bending. The best cultivars were Anjou 210 and LG11. Unfortunately we are very short of their supply so that the work reported here was done using the local cultivar "Halamish."

Results

IAA, ARP and their combination (IAA - different concentrations, ARP - $1\cdot 10^{-5}\text{M}$) were tested. Application was done via agar blocks weighing ca. 2 mg each, applied directly onto the elongating zone of the root, 2-3 mm. beyond its apex. The agar blocks were inserted above the roots which were positioned horizontally. The results appear in figure 18.

ARP acts synergistically with IAA to counteract the gravitropic response of maize roots. Except at very low IAA concentrations ($5\cdot 10^{-9}\text{M}$) the combined effect of IAA and ARP is always stronger than the arithmetical additive effects of IAA and ARP when applied separately and the difference is roughly equivalent to a one order of magnitude of IAA. It can be therefore concluded that ARP did increase the tissue's sensitivity to auxin in this case.

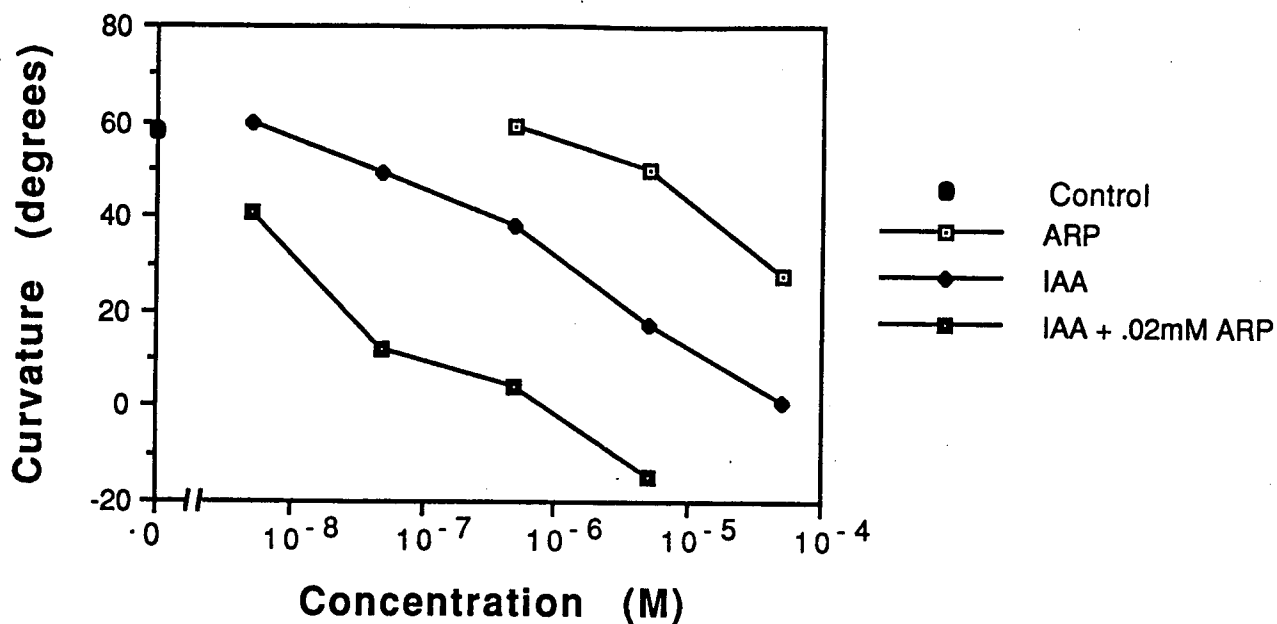


Figure 18. Gravitropic response of maize roots as affected by applications of IAA, ARP and their combination. Concentration of ARP was $1 \cdot 10^{-5} \text{M}$ when given in combination with IAA

An instantaneous result-taking as was done in this experiment does not reflect the dynamic nature of the process. Several observations taught us that both IAA and ARP have only transient effects on growth direction of maize roots. After a while, the normal growth habit (positively gravitropic) resumes even with relatively high concentrations of the growth regulators. It is also of significance to note that prevention of root bending is not a result of an overall growth cessation. To better characterize the process, an experiment was planned to study the process' kinetics.

In this experiment, only one concentration was chosen for each growth regulator. The choice was done so as to achieve ca. 50% inhibition of maximal bending of the control and according to several preliminary tests, the chosen concentrations were $1 \mu\text{M}$ for IAA and $5 \mu\text{M}$ for ARP. In this case, the whole set up was photographed every 1/2 hour and the bending angles were measured on the photographs. The test's duration was 5 hours. The results appear in figure 19.

It is suggested that ARP, by increasing the tissue's sensitivity to endogenous auxin at the application site, counteracted temporarily the gravitropic bending. The initial horizontal growth is the result of the balance between the effect of higher auxin concentration in the lower side of the root and a lower auxin concentration but enhanced response due to increased sensitivity at the upper side. As the root continues its growth the elongating zone escapes from the ARP-affected zone and normal growth pattern resumes.

2.i.III. Conclusions

The fact that ARP's peak of activity was repeatedly found to precede IAA's in root induction of hypocotyl cuttings of mung bean implies its involvement in a certain preparative stage. Based on this preliminary study, it could be hypothesized that such a role might be the increase of the auxin's sensitivity of the target cells - these cells which when later in the process will be subjected to IAA (either endogenous or exogenous) will respond by vigorous cell division and primordia formation.

References

- Bhattacharya NC (1988) Enzyme activities during adventitious rooting. pp. 88-101 in: Adventitious root formation in cuttings. Davis TD BE Haissig and N Sankhla (eds.) Dioscorides Press, Portland.
- Bittner S S Gazit and A Blumenfeld (1971) Isolation and identification of a plant growth inhibitor from avocado. *Phytochem* 10:1417-1421.
- Gaspar T and M Hofinger (1988) Auxin metabolism during adventitious rooting. pp. 117-131 in: Adventitious root formation in cuttings. Davis TD BE Haissig and N Sankhla (eds.) Dioscorides Press, Portland.
- Gazit S and A Blumenfeld (1970) Cytokinin and inhibitor activity in avocado mesocarp. *Plant Physiol.* 46:334-336.
- Jacobs M and PH Rubery (1988) Naturally occurring auxin transport regulators. *Science* 241:346-349.
- Kefford NP (1973) Effect of hormone antagonists on the rooting of shoot cuttings. *Plant Physiol.* 51:214-216.

Raviv M O Reuveni and EE Goldschmidt (1986) Evidence for the presence of a native, non-auxinic rooting promoter in avocado. *Pl. Growth. Regulation* 4:95-102.

Rengel Z and HA Kordan (1987) Effects of growth regulators on light-dependent anthocyanin production in *Zea mays* seedlings. *Physiol. Plant.* 69:511-516.

Tognoni F and R Lorenzi (1972) Acidic root promoting growth inhibitor(s) found in *Picea* and *Chamaecyparis*. *J. Amer. Soc. Hort. Sci.* 97:574-578.

3. General conclusions and suggestions for future research

The biologically active (in terms of rooting stimulation) components of the avocado rooting promoter (ARP) were purified and characterized chemically. It was found that the acetylenic and trihydroxyl compounds are more active than the olefinic and 1 acetoxyl - 2,4-dihydroxy compounds. This information can serve for further research dealing with structure/function relationships of various possible ARP analogues.

The activity is strictly stereospecific. This and some other lines of evidence suggests that this molecule has a true biological activity *per se* and not as a precursor for other active compounds.

A laboratory technique for synthesis of the molecule was developed. A commercial synthesis procedure has yet to be developed. Two identification methods were developed - one based on gas chromatography and the second on immunoassay. Using the first method, we found ARP 2I and/or 2II in plants of various families thus demonstrating its generality. In some cases, we were able to show a putative link between rooting ability, ARP-like activity and relative ARP content in a tested combination of genetically-close pair of cultivars or species.

The GC analysis method can yield at present only relative data. It can, however, be calibrated to generate quantitative results given additional efforts will be devoted to it. The second method should be further developed, too. Its main use will be for ultrastructural localization of ARP using *in situ* hybridization techniques.

During the current research, relatively less effort was directed to the horticultural aspects. We were able to prove ARP's heat stability but its usefulness for *in vitro* propagation protocols is still questionable. We were more successful with its application to commercially important species during cuttage propagation but for both purposes an extensive work, has to be done in order to select

optimal concentrations and mode and time of application before any recommendation for horticultural use can be given. We believe, however, that a better attitude at this stage will be to study ARP's mode of action thus enabling later a more exact definition of the conditions (species, type of plant material, seasonality, etc.) in which ARP might show benefit during regeneration processes. A preliminary work has been initiated during this project toward this aim. This work resulted with a working hypothesis which should be tested in the future that ARP can modify the tissue's sensitivity to auxin thus prepares the target cells to faster response to endogenous auxin.

During the course of the present study, it was found that some other compounds, of closely related chemical nature have also root promoting activity. It is suggested that ARP is merely an example for a new class of plant growth regulators. The study of the other putative members of this class should be an important task to chemists and plant physiologists in the future.

4. Obtained vs. expected results and their contribution to agriculture in Israel and the United States

In our proposal we wrote "None of the commercial rooting mixtures available today overcome the difficulties in rooting cuttings of many difficult-to-root species. It is likely that one of the reasons for this failure is that auxin is not the only limiting factors in some of these cases.

Since in many species, even the earliest stages of root initiation fail to commence, it seems logical to assume that lack of a dedifferentiating factor might be the actual bottle-neck of the process. If ARP indeed can serve as such a factor, it may have a horticultural importance as an aid in rooting of cuttings and tissue cultures both in Israel and in the United States.

If the above-mentioned objectives of the research are fully realized, the result might be a new class of plant growth regulator with a clear horticultural importance and a possible general physiological role in various regeneration processes. Some knowledge about its mode of action is expected as well."

Admittedly, we are still in a stage of having difficulties in rooting difficult-to-root species. ARP probably acts as a dedifferentiating factor in some cases, but not in other. Also not always dedifferentiation is the limiting step to rooting. However, since dedifferentiation is undoubtedly an important limiting step in many "difficult" cases, the search for chemicals having the capability to induce it should continue. Bearing this in mind, we regard the presented report as a working model for such a research.

As per our specific objectives listed in the proposal, except for radioisotopic labeling of ARP, we showed within each section that we reached and probably surpassed our goals.

In order to apply our results to commercial horticulture in the United States and Israel adaptations should be done for the local plant material and conditions. It will be possible to conduct this kind of work only when procedure for commercial synthesis of ARP will be developed.