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**Detecting Activity of Juvenile Phase-Specific
Translocatable Substances that Influence Rooting
Potential Using *in vitro* Rooting Assays and
Expression of a Specific Gene**

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**הספריה המרכזית
למדעי החקלאות
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Title:

Detecting activity of juvenile phase-specific translocatable substances that in-
fluence rooting potential using in vitro rooting assays and expression for a
specific gene.

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Abstract:

Detecting Activity of Juvenile Phase-Specific Translocatable Substances that Influence Rooting Potential Using *in vitro* Rooting Assays and Expression of a Specific Gene. The objectives of the project for which substantial effort was put forth were: 1) Verify the relationship between expression of a cDNA clone (HW103) and the rooting potential of reciprocally grafted cuttings of juvenile and mature lamina and petioles of *Hedera helix* L. 2) Detect rooting promoter fractions in exudates from the juvenile leaves of *H. Helix* by assaying for rooting with leaf petioles of juvenile and mature *H. Helix* plants. 3) Isolate, purify and identify compounds which show activity in assays for rooting potential. Some objectives or aspects of the objectives of the original proposal were not pursued for the reasons put forth in the body of the report. The most significant findings of the project are : 1) The MS medium is a better medium than Romberger medium for performing the leaf petiole rooting assay. 2) HW103 gene expression is cell-type specific with higher levels of expression in mature than juvenile phase *H. helix* petioles as evidenced by *in situ* hybridization which suggests a negative relationship between HW103 expression in specific cells involved in root initiation and the lack of rooting potential in mature petioles 3) HW103 gene expression may be lower in mature petioles which had been grafted to a juvenile *H. helix* lamina than mature petioles that had been grafted to a mature lamina and this putative lower expression is related to formation of a higher number of roots. 4) HW103 gene expression is not related to auxin induced ethylene production. 5) Two distinct compounds that possess root initiation promoting activity can be detected mainly in diffusate of juvenile *H. helix* leaves using mung bean hypocotyls and *H. helix* leaf petioles *in vitro*. 6) *H. helix* diffusate active fractions do not consistently promote rooting in avocado mini-cuttings. 7) Chemical identification of the active rooting compounds was not accomplished because of the death of Prof. Becker, one of the collaborators, and the resultant loss of his data.

These results indicate that these may be a molecular basis for reduced rooting potential in mature *H. helix* petioles and that there are diffusible (translocatable) compounds in juvenile *H. helix* leaves which promote rooting in juvenile and mature *H. helix* petioles and mung bean hypocotyls.

Objectives of the original research proposal:

1) To verify the relationship between expression of a cDNA clone (HW103 or an avocado homolog encoding for a cell wall protein) and the rooting potential of a) reciprocally grafted cuttings of juvenile and mature lamina and petioles of *Hedera helix* L. and b) etiolated and non-etiolated stem cuttings from mature *Persea americana* Mill (avocado) and *H. helix* plants. 2) To detect rooting promoter fractions in exudates from juvenile leaves of avocado and *H. helix* by assaying for rooting with leaf petioles or *in vitro* derived shoots from juvenile and mature plants of these species. 3) To determine the influence of rooting promoter fractions detected in objective 2 and Avocado Rooting Promoter on expression of genes for cDNA referred to in objective 1. 4) To isolate, purify, and identify compounds which show activity in rooting and gene expression assays for rooting potential.

The deviations from the objectives of the original proposal are noted and explained in the section on relevant data, methodology, results and discussion.

Relevant data, methodology, results and discussion:

This section of the report is organized based on the country where the research was carried out. United States:

Initially we concentrated work on optimizing the English ivy leaf cutting grafting techniques and enhancing the rooting response of excised juvenile and mature ivy-petiole segments *in vitro* for use as a rooting assay.

In the leaf cutting grafting work we compared approach and side grafts. We concluded that side grafts are much superior because grafting success is higher, the response is quicker, they're easier to perform and side grafts eliminate the rooting that sometimes occurs on the scion of approach grafts. With the side grafts, a rooting response can be detected as soon as 25 days after healing of the union and treatment of the base of the cutting with auxin. In addition, we found that the grafting process can be simplified and speeded up by using 1/8 or 5/32 inch interior diameter natural latex rubber tubing as a sleeve to bind the scion to the stock.

In the work on optimizing the rooting response of excised juvenile and mature petioles *in vitro*, we've studied the effect of modification of the culture medium. Initially, we found that Murashige and Skoog (MS) basal medium salts gave a superior rooting response as compared to the Romberger basal medium salts that we had used previously.

When we found that MS was superior to Romberger medium for the rooting assay we did several experiments to determine the basis for this superiority. There are two major differences between MS and Romberger medium basal salts. One difference is in total nitrogen with Romberger being much lower than MS and the other being the $\text{NO}_3^-/\text{NH}_4^+$ ratio with MS being 2:1 and Romberger being 1:2.5. Therefore, we did several experiments to compare rooting response when these two parameters were varied. Table 1 shows the results of one of these experiments in which the $\text{NO}_3^-/\text{NH}_4^+$ ratio was varied. Notice that the total N is about two thirds of that in full strength MS. In another experiment we had found this N level optimal for rooting.

Based on these experiments we concluded that MS medium is a better medium than Romberger for the leaf petiole rooting assay. Because of ease of carrying out assays using commercially available MS medium powder we chose or use $\frac{1}{2}$ strength MS ($\frac{1}{2}$ MS) in comparison to Romberger's medium for most experiments. For the *in situ* by hybridization work, which was already underway at this point we used Romberger's medium.

The majority of the remaining work carried out in the U.S. concentrated on assessing the functional significance for adventitious root initiation of expression of a proline-rich protein gene (HW103). One approach we used to do this was *in situ* hybridization. First we developed an *in situ* by hybridization protocol which is described in published paper 3.

Using the *in situ* hybridization protocol, we studied the spatial and temporal expression of HW103 in ungrafted, excised, *in vitro* cultured juvenile, mature and chronologically young mature (CYM) *H. helix* petioles. Cell division and root initiation responses of petioles were also studied to determine whether these processes were correlated with localized expression of the HW103 gene. Petiole cultures showed cell divisions at day 5 after culture in all three types of auxin-treated petioles but not in non-auxin-treated petioles. Juvenile and one population of CYM auxin-treated petioles formed root primordia after nine days of culture. Mature petioles and another population of CYM petioles formed only callus in response to auxin.

The HW103 mRNA was not detectable in petioles of any phase immediately after excision (Tables 2 and 3). In both juvenile and mature petioles the HW103 mRNA preferentially accumulated in the phloem parenchyma, the inner cortex adjacent to the phloem, and cells surrounding ducts (Table 2). Steady state levels of HW103 RNA were much lower in juvenile relative to mature petioles cultured *in vitro*. Auxin reduced the steady state levels of HW103 RNA in CYM petioles but not in mature or juvenile petioles (Table 3). The differences in HW103 gene expression in juvenile and mature

Table 1. Influence of nitrate: ammonium ratio in modified Murashige and Skoog medium on percent rooting of and number of roots on auxin treated *in vitro* cultured segments from different positions of juvenile and mature *Hedera helix* leaf petioles.

$\text{NO}_3^-/\text{NH}_4^+$ <u>(mM)</u>	Juvenile		Mature		Mature	
	<u>Proximal</u>		<u>Proximal</u>		<u>Medial</u>	
	%	Root	%	Root	%	Root
	rooting	no.	rooting	no.	rooting	no.
39.4/0	100	7.3	0	0	10	1.5
32.8/6.6	100	9.8	5	2.0	40	4.0
29.1/10.3	100	10.3	10	1.5	85	7.8
10.3/29.1	90	8.6	0	0	0	0
6.6/32.8	85	5.0	0	0	0	0

Table 2. Expression pattern of HW103 cDNA in juvenile and mature petioles of *Hedera helix*.

TISSUE	GENE	JO	MO	J5-J5A	M5-M5A
Epidermis	Cont	-	-	-	-
Epidermis	103	-	-	+	+
Out Cortex	Cont	-	-	-	-
Out Cortex	103	-	-	+/-	++/-
Duct	Cont	+	+	+	+
Duct	103	+	+	++	+++++
In Cortex	Cont	+	+	+	+
In Cortex	103	+	+	++/-	+++/-
Phloem	Cont	+	+	++	++++
Phloem	103	+	+	++	++++
Xylem	Cont	-	-	-	-
Xylem	103	-	-	-	-
Pith	Cont	-	-	-	-
Pith	103	-	-	+/-	++/-

JO-MO: juvenile and mature petioles after excision

J5-M5: 5 days cultured juvenile and mature petioles

J5A-M5A: NAA pulse-treated. 5 days cultured juvenile and mature petioles

Table 3. Expression pattern of HW103 cDNA in chronologically young mature petioles of *Hedera helix*.

TISSUE	GENE	MyO	My5	My5A	My9	My9A
Epidermis	Cont	-	-	-	-	-
Epidermis	103	-	++	++	++	++
Out Cortex	Cont	-	-	-	-	-
Out Cortex	103	-	+/-	+/-	++/-	+/-
Duct	Cont	+	+	+	+	+
Duct	103	+	+++++	++	+++++	++
In Cortex	Cont	+	+	+	+	+
In Cortex	103	+	++/-	++/-	+++/-	++/-
Phloem	Cont	+	+	+	+	+
Phloem	103	+	+++	++	++++	+++
Xylem	Cont	-	-	-	-	-
Xylem	103	-	-	-	-	-
Pith	Cont	-	-	-	-	-
Pith	103	-	+/-	+/-	++/-	++/-

MyO: chronologically young mature petioles after excision

My5-My9: 5 and 9 days cultured chronologically young mature petioles

My5A-My9A: NAA pulse-treated, 5 and 9 days cultured chronologically young mature petioles.

petioles persisted at day 9 in callus derived from the original cell types (Table 2). These results are also shown in the photomicrographs shown in paper 3.

These results show that HW103 gene expression is cell-type specific with higher levels of expression in mature than juvenile phase petioles. HW103 gene expression is preferentially localized in cells which are involved in root initiation and where the first cell divisions occur in response to auxin. These data show a negative relationship between the expression of HW103 in specific cells and the lack of rooting potential in mature petioles. The repression of gene expression by auxin treatment in specific cells involved in root initiation of CYM petioles coincides with an intermediate rooting potential. The expression of this gene is preferential in specific cells involved in rooting and may be negatively related to the potential of these cells to participate in root morphogenesis in response to auxin. These data are consistent with an inverse relationship between competence to form adventitious root and HW103 mRNA levels in specific cell types.

These results were important for subsequent experiments involving reciprocally grafted juvenile and mature petiole cuttings in that they indicate that differences in HW103 gene expression in juvenile and mature petioles persists in callus cells. It is also important because it is consistent with an inverse relationship between competence to form roots and HW103 mRNA levels in specific cell types. This supports our hypothesis that there will be changes in HW103 gene expression as a result of treatments (grafting) that influence rooting potential. A second approach we used to assess the functional significance for adventitious root initiation of expression of a proline-rich protein gene (HW103) is reciprocal grafting of juvenile and mature petioles of English ivy.

Using the side grafting technique previously described (Geneve, *et al.*, 1991. J. Expt. Bot. 42:65-69), a reciprocal grafting experiment using juvenile and mature leaves was carried out. Petiole bases from successful graft unions were excised and petioles treated with auxin (day zero). Samples for total RNA extraction were taken at 0, 7, 14, 21 and 28 day intervals. Samples were taken of the basal callus excised prior to auxin treatment on day 0, the rooting zone (the basal 5mm) and the scion petiole adjacent to the graft union. Mature leaves were taken from the 1st fully expanded leaf below the actively growing apical meristem. Juvenile leaves were taken from the same position and 2 or 3 nodes below this. Rooting data were taken from designated sample trays. Ungrafted leaves were excised from the mother plant and treated with auxin on day 0.

Rooting assay: The grafting of the juvenile laminae to mature petioles did not increase the rooting percentage in comparison to the mature/mature combination. This finding applies both to the rooting percentage data taken at the termination of the experiment (44 days) and data taken at earlier time periods (Table 4). However, the presence of the juvenile lamina had a significant effect ($p=0.05$) on root numbers per rooted petiole (RNRP) (7.1 for J/M and 3.7 for M/M at 44 days). This effect was correlated with an increase in rooting percentage between 35 d and 44 d from 21% to 73% in the J/M combination. Although a similar increase in rooting percentage was found in M/M grafts during the same time period, this was not accompanied by a similar increase in RNRP. RNRP for J/M, J/J and J at 35 d (ungrafted) were similar to each other and had about twice the number of roots as M/M and M.

Northern analysis of HW103 expression:

Basal callus of grafted stock petioles was collected on the day of auxin treatment and stock laminae excision. There was detectable expression of HW103 in the basal calli of mature petioles but no apparent difference between M/M and J/M (data not shown).

No significant expression of HW103 was found in the rooting zones of any of the graft combinations on day 0 (Figure 1); nor in any of the scion petioles samples at any time. There was similar expression of HW103 in the rooting zones of all three graft combinations at 7, 14 and 21 days. There were some small differences, for example, a higher expression in M/M at 7 days. However, this may not be outside the limits of experimental error. At 28 days, there was less expression in the J/M combination when compared to M/M. This may be correlated with the initiation of rooting primordia. However, the difference in numbers of root primordia was not apparent until after day 35. This raises the question as to whether the lower expression of HW103 in mature petioles grafted to juvenile lamina is associated (and not causative in controlling a change from wound / auxin-induced callus growth to rhizogenesis) with a change in morphogenic pathway or that the lower expression is allowing a change to occur in the morphogenic pathway. This experiment was repeated during the last year of the project with similar equivocal results. A more precise and sensitive way to test the functional significance of the proline-rich protein gene (HW103) expression for adventitious root initiation is to use *in situ* hybridization to analyze expression in a reciprocal grafting experiment. This would have the advantage of being able to assess expression specifically in the tissues that are involved in root initiation (see paper 3).

Table 4. Summary of grafting experiment.

Days elapsed since day zero

	14D		23D		29D		35D		44D	
Graft	Root %	Root nos.	Root %	Root nos.	Root %	Root nos.	Root %	Root nos.	Root %	Root nos.
J/J (n=22)	16%	3.5±1.85	91%	6.1±0.94	95%	6.8±3.6	100%	6.6±0.70	100%	8.2±0.45
J/M (n=24)	0%	0	0.08%	1.5	13%	2	21%	2±0.45	73%	7.1±0.76
M/M (n=22)	0.05%	2	0.05%	2	10%	2	22%	2.3±0.63	65%	3.7±0.36
M (n=34)	0	0	0.12%	1.8	25%	2.2	33%	2.5±0.45	50%	3.4±0.40
J (n=41)	49%	4.1±0.62	100%	7.4±.052	100%	8±0.46	100%	8 ±0.40	100%	Not measured

16 DAY EXPOSURE TOTAL GRAFTING
5th JUNE 1995

DO NOT REMOVE

RZ RZ P P - P RZ P RZ P RZ P
M/M J/S M/M J/M J/M J/M J/S J/S J/M J/M J/M
(0) (0) (0) (0) (7) (7) (7) (0) (0) (0) (0) (0) (0) (0)

RZ P RZ RZ P RZ P RZ P RZ P
J/M M/M M/M M/M J/M J/M J/M J/M J/M J/M
(14) (14) (14) (20) (20) (21) (21) (21) (21) (21) (21) (21) (21) (21) (21)

TOTAL GRAFTING

Figure 1. Northern blot of HW103 expression in the rooting zone (RZ) or proximal scion petiole (P) of juvenile/mature graft combinations during a 28 day period.

A third approach used to test the functional significance for adventitious root initiation of a proline-rich protein gene (HW103) was based on the hypothesis that ethylene plays a role in regulation of HW103 expression. This hypothesis was based on results of an experiment which indicated that venting a culture vessels which lowers ethylene levels and silver thiosulfate (STS, an inhibitor of ethylene action) treatment can lower HW103 expression in ivy petioles. These results indicating that venting and STS treatment lower HW103 expression provide a potential means for modulating

HW103 expression independent from auxin treatment and phase effects and thereby allow us to test the functional significance of HW103 expression for regulating competence for adventitious root initiation.

To test the involvement of ethylene in promoting HW103 expression we performed replicated experiments with juvenile (J), mature (M), and young mature (YM) petioles. In one replicated experiment we tested the effect of auxin and type of medium (1/2 MS or Romberger) on HW103 expression in unvented vessels. As Figure 2 shows, there were differences in HW103 expression due to phase with mature petioles having the highest expression and juvenile and young mature petioles having a lower and similar level of expression. However, auxin did not promote HW103 expression in any of the petiole types with either of the culture medium used. These results can be rationalized relative to ethylene acting as a promoter of HW103 expression only if auxin does not induce ethylene production. However, Geneve *et al.* 1990, (J. ASHS 115:123-127) showed conclusively that auxin treatment does induce ethylene production and we confirmed their findings in results reported in the 1994-95 progress report. Consequently, the replicated results shown in Figure 3 argue against ethylene being involved in regulation of HW013 expression.

To further investigate the possible promotion of HW103 expression by ethylene, a replicated experiment involving auxin, ethephon (ethylene releasing agent) and STS treatments was carried out. As shown in Figure 3, neither auxin or ethephon treatment enhanced HW103 expression over non-treated controls. However, when petioles were pre-treated with (STS) before auxin or ethephon treatment expression of HW103 was decreased markedly in all petiole types. These results indicate that STS is not inhibiting HW103 expression through its activity as an inhibitor of ethylene action,

<u>J</u>	<u>M</u>	<u>YM</u>	<u>J</u>	<u>M</u>	<u>YM</u>	<u>J</u>	<u>M</u>	<u>YM</u>	<u>J</u>	<u>M</u>	<u>YM</u>
$\frac{1}{2}$ MS			Romberger			$\frac{1}{2}$ MS			Romberger		
						+ NAA pulse			+ NAA pulse		

Figure 2. Northern blot of HW103 expression in *in vitro* cultured juvenile (J), mature (M) and young mature petioles on two media with and without an auxin (NAA) pulse.

Figure 3. Northern blot of HW103 expression in *in vitro* cultured juvenile (J), Mature (M) and young mature (YM) petioles treated with auxin (NAA) or ethephon and combinations of auxin or ethephon with silver thiosulfate (STS).

but through some other mechanism. It is known that heavy metals like silver can bind to proteins other than the putative ethylene receptor. So, even though silver is inhibiting HW103 expression, it probably is not doing so through its affect on the ethylen action.

These results taken overall indicate that ethylene is not involved in regulation of HW103 expression. This means, unfortunately, that ethylene treatment cannot be used as means to test the functional significance of HW103 expression, independent of auxin and phase, for regulating competence for adventitious root initiation.

Based on our results overall, the best approach for testing the functional significance of HW103 for regulating competence for adventitious root initiation is probably reciprocal grafting of juvenile and mature ivy leaves combined with *in situ* hybridization to assess expression of HW103 in tissue from which adventitious roots are initiated. However we were not able to test the *in situ* hybridization approach because of the termination of the project. A second approach for testing the functions significance of HW103 is to use the *in situ* hybridization approach in mature and juvenile *H. helix* petioles that have been treated with the active diffusate fractions that were isolated by the Israeli groups. Unfortunately, these active fractions were not available until too late in the project duration to carry out such experiments.

Israel:

The work of the Israeli groups was severely affected by 2 major events: the sudden death of Prof. D. Becker and the severe illness of Dr. O. Reuveni. The separation work, planned to be performed by Becker's group, to be used by the other 2 groups, as well as for the U of M group, was later performed by the New Ya'ar group. However, we failed to retrieve most of the chemical data, accumulated by Prof. Becker. As Prof. Becker made use of most of the funds, allocated for the Technion group, Prof. Gutman, who offically took over Becker's role, was not able to complete most of the planned work, including the chemical identification. This report is dedicated to the memory of Prof. Dan Becker - a friend and an able chemist.

a. Plant material

Juvenile and mature ivy cuttings were propagated and established as mother plants in a greenhouse in Newe Ya'ar. These plants are genetically identical to those used by the U of M group. The juvenile and mature plants are genetically uniform. The juvenile plants retained their original phase-related characteristics throughout the whole project period. On the other hand, mature plants tended to undergo rejuvenation. This may be a result of the cultural practices: ample water and nutrition frequent prunings and constant removal of flowers. Repeated removal of juvenile offshoots coming from mature plants was performed.

b. Diffusate collection

Numerous (~100) runs of diffusate collection from juvenile and mature ivy leaves were conducted as planned in the proposal. The collection of diffusates was conducted as described in the research proposal, in a collection tank, especially designed by us for this purpose. Only fully expanded leaves were used. Fresh weight of the leaves was determined and the activity was later evaluated in relation to the initial FW of the diffusing leaves. The collection was performed over a 7-day period under controlled conditions of temperature, relative humidity, irradiance and photoperiod. The collected diffusates were evaporated under vacuum and were used for various purposes.

c. Separation - General

For most of the research period the Technion group was not able to conduct the separation work, as planned in the proposal and the Newe Ya'ar group took the responsibility for this part of the project. Several separation methods were used. Routinely conducted blank runs showed consistently that the activity in the active fractions are not artefacts resulting from either the solvents, the carriers or interactions among them.

Separation methods that were used included:

- High Pressure Liquid Chromatography (HPLC) based on reverse-phase C_{18} and a linear acidified water-ethanol gradient (95% ethanol increased from 30% to 100%).

- Column chromatography, based on the carrier and solvent system of the HPLC runs. This method enables separation of large quantities of diffusate and was the most important method, used to supply purified active fractions. The rooting activity is consistently found in the fraction that contains 50% ethanol. Most of the separated material from this active fraction was sent, after validation of rooting activity, to the Technion for chemical identification (see in the following). Additional quantity was sent to the Volcani group, to be used in avocado rooting experiments, *in vitro*. Additional quantity was sent to the University of Minnesota group, to be used in conjunction with the molecular biology work.
- Dynamic solid phase extraction on mini columns (Sephadex G-25 and Cellufine GCL-1000), followed by electrophoresis on SDS polyacrilamide gel, stained with Coomassie blue R. 250 and using molecular weights (MW) markers.
- The protein separated with the previous method, was then applied to various ultrafiltration membranes of different cut-off sizes, associated with different MWs.
- Gel permeation chromatography with Biogels P-2 and P-6, another MW-based separation method.
- Anion exchange column (DEAE-Sephadex+DEAE-cellulose)
- c.1. Results of the separation work
- The results of the separation work, described below, are mainly based on activity tests. However, for the sake of continuity the main results will be described in the following while most of the results related to root promoting activity will be described in the next section.

From the various separation methods based on MW it appears that 2 distinct compounds that possess root promoting activity can be detected, mainly in the diffusate of the juvenile leaves: a small, non-protein molecule which is eluted by 50% ethanol in water and 2) a larger ~6KD peptide. Both activities are not absorbed by anion exchanger.

d. Activity tests

The most commonly used activity test was the mung bean rooting bioassay. Other rooting assays were:

- Rooting of avocado minicuttings *in vitro* (conducted by the Volcani group).
- Rooting of ivy petioles *in vitro*.

d.1. Mung bean rooting bioassay

As mentioned above, most of the activity which was found in the crude diffusate, could be recovered, after column chromatography in the fraction eluted by 50% ethanol. Root promoting activity of both crude diffusate and the ivy active fraction (IAF) were usually higher in diffusate taken from the juvenile leaves than that of the mature leaves. The increased activity was expressed as shorter period to root emergence and more roots formed mung bean per cutting. However, an interesting phenomenon was apparent whenever mature leaves were sampled after the plants were cut back (for management purposes, as described above) - a somewhat lower activity in the diffusate and at the same locations in the chromatograms as that of the juvenile diffusate could also be found. This normally preceded the rejuvenation processes which were mentioned above.

Several Dose-Response curves were performed using the mung bean rooting bioassay in order to establish the optimal concentration for rooting activity of the crude diffusate and the IAF. Both crude diffusates from the juvenile leaves and IAF revealed optimum activity around 3 gram equivalents (FW) of ivy leaf diffusate.

d.2. Rooting of ivy petioles *in vitro*

The mung bean rooting bioassay is considered as a non-discriminative assay. In order to improve our understanding as to the relevance of the ivy diffusate to real ivy-specific rooting phenomena, ivy petioles were rooted *in vitro* with and without auxin (NAA) and IAF. This assay was repeated several times with various modifications and combinations of the putative root-promoting substances. The conclusions are: Both juvenile and mature ivy petioles do not form roots unless treated with auxin. Mature petioles can survive without auxin for about 18 days while juvenile petioles survived for very long periods (>90 days), formed callus but no roots. Optimal NAA concentration for root formation is 100 ppm. However, when optimal concentration (1 gr. Eq. FW per vial) of IAF is applied a synergistic effect on root formation can be detected. In the presence of IAF optimal NAA concentration drops to 50 ppm.

d.3. Rooting of avocado minicuttings *in vitro*

Rooting of avocado minicuttings was studied in order to find out to what extent the effect of IAF on root formation is general. Avocado minicuttings grown under controlled conditions *in vitro* were selected as test plant material because their low rooting response suggests that some conditions, conducive to rooting are at a suboptimal level.

Several rooting assays were conducted during the project using various combinations of IBA (which was found as the most active form of auxin for root induction of avocado minicuttings) and IAF. Although in some cases IAF (at 0.5-1.0 gr. Eq. FW per cutting) exhibited root promoting activity, the general conclusion is that IAF does not play any significant role in the process of root formation of avocado minicuttings.

e. chemical identification

As mentioned previously, the death of Prof. Becker in the middle of the project and our inability to retrieve his data prevented us from accomplishing the project's goals. The results of extensive GC-MS and 2D-NMR work which was carried out by him were lost.

However, the results obtained by the group of Prof. Gutman will be described briefly. The Technion group conducted two types of analyses: NMR in D₂ and DMSO and Electrospray Mass Spectrometry. The spectra of the two NMR determinations are complex and suggest that no aromatic hydrogens exist in the compound. Some peaks suggest the existence of glucoside moiety. The ES-MS spectra reveal one distinct peak at 413 D and several minor peaks. As yet we have no clue to the nature of the compound.

Detailed U.V.- visible light absorption spectra were determined at Newe Ya'ar using a Kontron UV-visible spectrophotometer. Peaks were found only in the active fraction (50% ethanol) at wavelengths of 235, 268, 277 and 308 nm. None of the other fractions showed any typical light absorption. It is still unclear what compounds absorb light at these wavelengths.

Description of Cooperation:

Professor Raviv made a trip to the United States to learn *in situ* hybridization techniques at the University of Minnesota. However, he was not able to make use of this technique to evaluate the

effect of active diffusate fractions on HW103 gene expression because he had to assume a greater role in separation and identification of active fractions upon the death of Professor Becker. Professor Hackett made a trip to Israel (not funded by BARD) to consult on the ivy petiole rooting assay and to learn the avocado microshoot rooting assay.

Evaluation of the research achievement with respect to the original research proposal and its objectives:

In relation to Objective 1, results indicate that HW103 gene expression may be lower in mature petioles which had been grafted to a juvenile *H. helix* lamina than mature petioles that had been grafted to a mature lamina and this lower putative expression is related to formation of a higher number of roots. Under objective 1, we did not pursue our intention of comparing gene expression in etiolated and non-etiolated cuttings from mature *Persea americana* (avocado).

In relation to Objective 2 and 4, we detected rooting promoter fractions in exudates from juvenile leaves of *H. helix* using the ivy petiole rooting assay and the mung bean assay. We were not able to identify the chemical nature of these active fractions. The lack of our ability to identify the active fractions was partly due to the death of Professor Becker part way through the project and the loss of his data. Under objective 2 we did to pursue our intention of detecting rooting promoting fractions from exudates of avocado leaves. In relation to objective 3, we did not determine the influence of rooting promoter fractions detected under objective 2 on the expression of the HW103 gene.

Conclusions:

The most significant findings of the project are : 1) The MS medium is a better medium than Romberger medium for performing the leaf petiole rooting assay. 2) HW103 gene expression is cell-type specific with higher levels of expression in mature than juvenile phase *H. helix* petioles as evidenced by *in situ* hybridization which suggests a negative relationship between HW103 expression in specific cells involved in root initiation and the lack of rooting potential in mature petioles 3) HW103 gene expression may be lower in mature petioles which had been grafted to a juvenile *H. helix* lamina than mature petioles that had been grafted to a mature lamina and this putative lower expression is related to formation of a higher number of roots. 4) HW103 gene expression is not

related to auxin induced ethylene production. 5) Two distinct compounds that possess root initiation promoting activity can be detected mainly in diffusate of juvenile *H. helix* leaves using mung bean hypocotyls and *H. helix* leaf petioles *in vitro*. 6) *H. helix* diffusate active fractions do not consistently promote rooting in avocado mini-cuttings. 7) Chemical identification of the active rooting compounds was not accomplished because of the death of Prof. Becker, one of the collaborators, and the resultant loss of his data.

These results indicate that there may be a molecular basis for reduced rooting potential in mature *H. helix* petioles and that there are diffusible (translocatable) compounds in juvenile *H. helix* leaves which promote rooting in juvenile and mature *H. helix* petioles and mung bean hypocotyls.

List of Publications:

The following three papers were published as a result of the research carried out under this project:

1. Murray, J.R., M. Sanchez, A.G. Smith and W.P. Hackett. 1993. Differential competence for adventitious root formation in histologically similar cell types. In: Biology of Adventitious Root Formation. T.D. Davis and B.E. Haissig (eds.). Plenum Press. New York and London.
2. Woo, H.-H., W.P. Hackett, and A. Das. 1994. Differential expression of a chlorophyll *a/b* binding protein gene and a proline rich protein gene in juvenile and mature English ivy (*Hedera helix*). *Physiol. Plant.* 92:69-78.
3. Sanchez, M.C., A.G. Smith and W.P. Hackett. 1995. Localized expression of a proline-rich protein gene in juvenile and mature ivy petioles in relation to rooting competence. *Physiologia Plantarum* 93:207-216.

Patents:

None