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Isoflavonoid Regulation of Root Bacteria

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Final report for Project IS-2388-94R

ISOFLAVONOID REGULATION OF ROOT BACTERIA

A. Abstract

The overall objective of this project was to develop a conceptual framework for enhancing root colonization by beneficial bacteria. To accomplish this aim we tested the hypothesis that production and excretion of the plant phytoalexin medicarpin can be used for creation of a special niche along the legume roots, where beneficial microorganism, such as rhizobium, will have a selective advantage. On the Israeli side it was shown that higher medicarpin levels are exuded following the application of *Rhizobium meliloti* to the rhizosphere but the specific biochemical pathway governing medicarpin production was not induced significantly enough to support a constant production and excretion of this molecule to the rhizosphere. Furthermore, pathogenic bacteria and chemical elicitors were found to induce higher levels of this phytoalexin and it became important to test its natural abundance in field grown plants. On the US side, the occurrence of flavonoids and nucleosides in agricultural soils has been evaluated and biologically significant quantities of these molecules were identified. A more virulent *Agrobacterium tumefaciens* strain was isolated from alfalfa (*Medicago sativa* L.) which forms tumors on a wide range of plant species. This isolate contains genes that increase competitive colonization abilities on roots by reducing the accumulation of alfalfa isoflavonoids in the bacterial cells. Following gene tagging efforts the US lab found that mutation in the bacterial efflux pump operons of this isolate reduced its competitive abilities. This results support our original hypothesis that detoxification activity of isoflavenoids moleculs, based on bacterial gene(s), is an important selection mechanism in the rhizosphere. In addition, we focused on biotin as a regulatory element in the rhizosphere to support growth of some rhizosphere microorganisms and designed a bacterial gene construct carrying the biotin-binding protein, streptavidin. Expressing this gene in tobacco roots did not affect the biotin level but its expression in alfalfa was lethal. In comclusion, the collaborative combination of basic and applied approaches toward the understanding of rhizosphere activity yielded new knowledge related to the colonization of roots by beneficial microorganisms in the presence of biological active molecules exuded from the plant roots.

B. Objectives of the original research proposal

This project sought to increase our understanding in the regulatory effect of plant signal molecules on soil bacteria in the rhizosphere. We have hypothesized that plant has evolved the capacity to control soil microorganisms that colonize roots and to promote colonization of beneficial microbes. Defining the basis for this phenomenon will improve methods for enhancing beneficial bacterial populations in the soil and reduce the undesirable disease induced soil pathogens.

Beneficial bacteria are important on roots not only for their particular biological activity that supports plant growth, but also for reducing the present of pathogenic microorganisms in the rhizosphere. We hypothesize that beneficial bacteria colonizing alfalfa roots grow well in the presence of isoflavonoids phytoalexins that are known to reduce growth of soil microorganisms. Other molecules are also exuded from alfalfa roots and contributes to the significance of this niche. Therefore, any relevant mechanism induced for adaptation will be supported by the genetic characteristics of the resistant microorganism.

We identified four specific objectives:

1. Define environmental factors affecting medicarpin release by alfalfa roots.
2. Isolate medicarpin-inducible bacterial genes (med)
3. Isolate bacterial genes that regulate medicarpin exudation by alfalfa
4. Test diverse root bacteria for homologous genes and assess their contribution to rhizosphere colonization.

C. Accomplishments

Individual scientific reports of work supported wholly or in part by this grant are contained in the following pages.

Evaluation of alfalfa phytoalexin (Medicarpin).

To accomplish this aim our original idea was to enhance the production and excretion of the plant phytoalexin medicarpin and to study the role of this molecule in the rhizosphere. Our view was that medicarpin is an important ecochemical factor, which is known to be present in the alfalfa rhizosphere and which could function as a general inhibitor or regulator of bacterial growth in that region.

Dr. Phillips' lab has long studied how alfalfa (*Medicago sativa*) roots are colonized by the N₂-fixing symbiont *Rhizobium meliloti*. Although this bacterium elicits exudation of medicarpin from all alfalfa roots, it was felt that a pathogenic bacterium might induce higher levels of this phytoalexin and thus offer insight into mechanisms by which medicarpin may function in the rhizosphere. For this reason, at the first phase of the project we initiated a set of experiments to evaluate the potential of the alfalfa roots to accumulate or exude medicarpin.

Medicarpin exudation from alfalfa roots

Based on the initial observation that symbiotic *Rhizobium* cells elicit the exudation of isoflavonoid phytoalexins from alfalfa roots, we established a transgenic (Ri) root culture from alfalfa leaf (see below), tested the capacity of this root culture to exude medicarpin to the growth medium.

Figure 1. Medicarpin levels in alfalfa root exudates (a) and extracts (b) from 6 days old alfalfa plants treated with *R. meliloti* 1021 (Rm1021) or yeast extract (YE). Plants were grown hydroponically as described in the text.

A significantly higher level of medicarpin aglycon was detected in exudates of root cultures incubated with *R. meliloti* 1021 or yeast extract (YE) (Fig. 1). However, the measured absolute difference between untreated controls and *Rhizobium*-treated culture was small. Monitoring of the glycoside forms of the medicarpin revealed a significantly lower level in the *Rhizobium* treated cultures, but not in those of the YE. It can be concluded that the amount of medicarpin exuded from the root cultures to the medium is relatively small and may affect our ability to identify mutants with the original proposed screening protocol.

Induction of medicarpin accumulation in cultures of alfalfa roots

In this set of experiments we assessed the possibility that upon elicitation of the roots (by different biological and chemical elicitors) medicarpin content will be elevated (accumulated) in the root tissue. Such an elevation might be attributed to increased activity of key enzymes involved in medicarpin biosynthesis pathway. In the first set of experiments we monitored the medicarpin content in alfalfa root cultures and alfalfa seedlings (Tables 1-4 and 5-6, respectively) in which isoflavonoid production had been elicited. At the next set of experiments further evaluation of the steady-state mRNA level of isoflavone reductase (IFR) and chalcone isomerase (CFH) was tested in respect to *Rhizobium* inoculation.

All experiments were carried out in 250-ml flasks containing 60 ml of sterile medium as indicated in the various experiments. Cultures for the experiments described in this report were incubated for 7 days in fresh SH (Schenk and Hildebrandt) medium before transferring them to the specific treatment. Incubation of the cultures during both the pre-Incubation period and the experiments was at 25°C. Experiments were continued for 72 h and cultures were frozen after the end of the incubation time. The samples were kept below -80°C until analyzed. *Rhizobium* cell suspensions were incubated at a final concentration of 10^8 cells per ml.

In an effort to determine the optimal conditions for culture growth and medicarpin production, the SH and MS (Murashige and Skoog) media were compared with/out vitamins. (1) Both SH and MS media resulted in similar medicarpin levels and the ratio of formononetin to medicarpin (F/M) were the same. (2) The formononetin levels at both media were significantly lower than the levels obtained in non-nutrient condition (H₂O treatment). (3) Supplementation of vitamins in SH medium resulted in a reduced level of medicarpin production. (4) Addition of sucrose to vitamin containing medium was found to reduce the medicarpin level in SH medium but not in MS.

Table 1. The effect of growth medium (SH or MS) on medicarpin (M) and formononetin (F) production in *Medicago sativa* hairy root culture (clone 232). V- vitamins (Sigma), S- sucrose.

	Medium tested					
	H ₂ O	SH-V	SH+V	SH+V+S	MS-V	MS+V+S
F/M	29.0	15.8	40.3	33.2	16.7	27.0
M (µg/g FW)	0.32±.08	0.30±.07	0.20±.20	0.23±.04	0.28±.03	0.38±.01

In a subsequent experiment we tested the hypothesis that the elicitation of medicarpin may increase when the product of the *nod* genes directs the synthesis of a responding signal in the root tissue. We applied the flavonoid signal molecule luteolin and monitored formononetin and medicarpin accumulation in alfalfa root cultures. Luteolin at a concentration of 1 μ M induced the accumulation of medicarpin (Table 2), but the rate of medicarpin production relative to that of formononetin did not change significantly. Moreover, inoculation with *R. meliloti* 1021 (in the presence of luteolin) increased the ratio of formononetin to medicarpin contents with no reduction in the medicarpin content. It can be concluded that following the elicitation by *Rhizobium* significantly more formononetin was produced with no subsequent induction of the medicarpin synthesis pathway.

Table 2. The effect of luteolin (L) and *R. meliloti* 1021 (Rm) on formononetin (F) and medicarpin (M) production in *M. saliva* hairy root culture (clone 232) grown in SH.

	Medium used			
	SH	SH+L	SH+RM	SH+L+RM
F/M	13.2	14.4	24.1	25.8
M (μ g/g FW)	0.9 $\pm$.3	3.0 $\pm$.6	0.7 $\pm$.2	0.9 $\pm$.1

In the following experiment we tested the possibility that the root culture medium might have influenced the ability of the culture to respond to the *R. meliloti* inoculation (Table 3). Changes that were induced in the culture medium (using MS medium) revealed that medicarpin induction could be obtained following the application of YE and that although the formononetin/medicarpin accumulation rate was not altered, YE elicited the biochemical pathway. No elicitation could be observed for the *Rhizobium-treated* roots

Table 3. The effect of MS medium, YE and Rm 1021 on formononetin (F) and medicarpin (M) production in *M. saliva* hairy root culture (clone 232).

	Medium used			
	H ₂ O	MS	MS+YE	MS+RM
F/M	13.2	27.1	12.4	22.1
M (μ g/g FW)	0.80 $\pm$.3	0.44 $\pm$.1	3.0 $\pm$.1	0.50 $\pm$.12

A significant reduction in medicarpin concentration was obtained when root culture was exposed to exopolysaccharide mutant strain 7055 (*exoF* mutant of *R. meliloti* 1021) or Nod factor (Table 4). In all treatments formononetin concentration was higher than in the control, indicating that the full length of the biosynthesis pathway was not induced all the way (down to the medicarpin).

Table 4. Genotypic effect of *R. meliloti* 1021, *exoF* mutant 705 5 and modulation factor on medicarpin (M) and formononetin (F) accumulation in *M sativa* hairy root culture (clone 232).

	Medium used			
	Control	Rm 1021	Rm 7055	Nod factor
F/M	13.0	28.1	22.9	22.0
M ($\mu\text{g/g}$ FW)	0.8 $\pm$.3	0.44 $\pm$.1	0.45 $\pm$.3	0.52 $\pm$.09

Medicarpin accumulation in alfalfa roots

In experiments described in this part we used 4-day-old hydroponically grown alfalfa seedlings in 400-ml assemblies containing nutrient solution. Roots were exposed to the various treatments, which were applied via the nutrient solutions, and were analyzed for medicarpin and formononetin contents 72 hours later.

Four-day-old seedlings were treated and were analyzed 3 days later. A higher medicarpin concentration could be detected following the dual application of luteolin and *Rhizobium* (L+Rm 1021), but formononetin accumulated to a higher concentration than the medicarpin, which suggests that the induced biosynthesis was not directed towards medicarpin production. It is important to note that as in the case of the root cultures, the YE treatment led to a significant induction of medicarpin accumulation (Table 5).

Table 5. The effect of luteolin (L), *R. meliloti* 1021 (Rm) and yeast extract (YE) on formononetin (F) and medicarpin (M) production in roots of hydroponically grown *Medicago sativa* seedlings.

	Medium used				
	Control	Rm 1021	L	L+Rm1021	YE
F/M	18.2	20.9	22.5	25.9	8.7
M ($\mu\text{g/g}$ FW)	0.07 $\pm$.001	0.083 $\pm$.07	0.07 $\pm$.01	0.12 $\pm$.01	0.30 $\pm$.05

A similar experiment was conducted to test the possibility that the *exoF* bacterial mutant could elicit the accumulation of medicarpin in alfalfa seedlings. From Table 6 it can be seen that seedlings inoculated with strain 7055 contained a higher concentration of medicarpin. This increase was not statistically significant with respect to the medicarpin level, although the relative formononetin/medicarpin ratio was reduced.

Table 6. Medicarpin Accumulation in alfalfa seedlings exposed to *R. meliloti* *exo*-mutant strains 7055 (*exoF*) and 7154 (*exoH*)

	Medium used				
	Control	Rm 1021	7055	7154	Nod Factor
F/M	15.6	21.8	8.9	11.4	18.8
M ($\mu\text{g/g}$ FW)	0.07 ± 0.003	0.085 ± 0.02	0.11 ± 0.03	0.65 ± 0.02	0.059 ± 0.02

Transcription levels (mRNA) of IFR and CHI

The induction of genes involved in the synthesis of medicarpin may depend on the steady-state mRNA level of key enzymes in the isoflavonoids biosynthesis pathway. Two key enzymes are known: IFR and CHI. In the following experiment we monitored the steady-state mRNA levels of the two genes in *Rhizobium*-inoculated plants and we compare them to YE (as a positive control).

In order to find a correlation between Rm 1021 infection and IFR-gene induction, 3-day old alfalfa (*Medicago sativa* L. cv Gilboa) seedlings were exposed to 10^8 cells/ml of the bacterium in a hydroponic growth system. As a control, two additional systems were established; in one, the alfalfa growth medium contained YE at a concentration equivalent to 50 $\mu\text{g/ml}$ of reduced sugars, which has been reported to induce IFR expression in alfalfa cell suspensions (Paiva *et al.*, 1991). The third system contained the growth medium without any additives. Samples of 20-30 seedlings were collected at 6 and 12 h after Rm or yeast extract applications and at 1-day intervals during one week. Total RNA was extracted from the roots of the seedlings with TriReagent (Molecular Research Inc., USA), and 10- μg aliquots were analyzed by means of RNA-MOPS gel (1.1% agarose) and were Northern blot probed with IFR-cloned fragment (Sambrook *et*

al., 1989) (Fig. 2, upper panel). The same blot was probed with CHI probe, since CHI precedes IFR in the biosynthetic pathway, and with a tomato rDNA probe for normalization of the results (Fig. 2). An immediate induction in IFR and CHI transcription was obtained after application of *Rhizobium*. High transcript levels were maintained for 3 days before decreasing to the basic levels. A high induction of IFR and CHI was obtained with YE after 6 h of application, and was followed by a decline of the transcripts to their steady-state levels at 12 h, as previously reported by Paiva *et al.* (1991) for tissue culture.

To investigate the "symbiotic role" of IFR mini-scale hydroponic systems further, the following treatments were used-

1. Alfalfa growth medium (C). 2. C + 20 ml of a fresh yeast-mannitol medium (*Rhizobium* growth medium) to verify that the induction is *Rhizobium* (Rm) dependent and is not caused by YE residues in the bacterial growth medium. 3. C + yeast-extract to a final concentration equivalent to 50 µg/ml reduced-sugars. 4. C + Rm 1021, 10^8 cells/ml. 5. C + Rm 1021, 10^7 cells/ml. 6. C + Rm 1021, 10^6 cells/ml. 7. C + Rm 1021, 10^5 cells/ml. 8. C + boiled Rm cells, 10^8 cells/ml. 9. C + 5-day-old Rm-culture supernatant. 10. C + *Bradyrhizobium* 280A, 10^{10} cells/ml. 11. C + *Hedysarum coronarium*, 10^7 cells/ml.

Samples 8-9 were to test whether IFR induction resulted from an exposure of the roots to the bacteria cells (8) or to a bacterial elicitor (9). Both *Bradyrhizobium* and *Hedysarum coronarium* serve as negative controls since they do not infect alfalfa.

Three-day-old seedlings were exposed to the above treatments for 24 h, and RNA was extracted and analyzed as above. The results are shown in Fig. 3. The same signal intensities were obtained both for negative controls (samples 1,2, 10, 11) and for all the *Rhizobium* samples.

The blots from both experiments were probed with MsENOD 40 to confirm root infection and symbiotic interaction. No signal was obtained (data not shown). In addition, when modulation was tested, nodules were developed three weeks post infection. These results together with the previously described biochemical contents of medicarpin and formononetin in roots, lead us to the suggestion that the specific biochemical pathway may not be transcriptionally induced by *R. meliloti*. However, the slightly higher medicarpin content in the exudates suggested that the *Rhizobium* may

have the capacity to induce exudation of medicarpin that was already available in the tissue.

Sambrook J, Ffitch EF, Maniatis T. Molecular Cloning, a Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, NY (1989)

P,aiya NL, Edwards R, Sun Y, Hrazdina G and Dixon RA. Stress responses in alfalfa (*Medicago sativa* L.) I 1. Molecular cloning and expression of alfalfa isoflavone reductase, a key enzyme of isoflavonoid phytoalexin biosynthesis. Plant Molec. Biol. 17: 653-667 (1991).

Figure 2: Steady-state mRNA levels of IFR and CHI in alfalfa roots at different sampling time following the application of *R. meliloti* 1021 or yeast extract. Rhizobium was applied at a density of 10^8 c.f.u. Experiment was carried as described in the text

Figure 3: Steady-state mRNA levels of IFR and CHI in alfalfa roots following incubation with different rhizobium strains at different concentrations. 1. Alfalfa growth medium (C). 2. C + 20 ml of fresh yeast-mannitol; 3. C + yeast-extract to a final concentration equivalent to 50 $\mu\text{g/ml}$ reduced-sugars; 4. C + Rm 1021, 10^8 cells/ml.; 5. C + Rm 1021, 10^7 cells/ml.; 6. C + Rm 1021, 10^6 cells/ml.; 7 C + Rm 1021, 10^5 cells/ml.; 8. C+ boiled Rm cells, 10^8 cells/ml.; 9. C + 5-day-old Rm-culture supernatant; 10. C + *Bradyrhizobium* 280A, 10^8 cells/ml.; 11. C + *Hedysarum coronarium*, 10^7 cells/ml.

Defining how isoflavonoids regulate genes in rhizosphere bacteria

Attempts to locate isoflavonoid-regulated root colonization genes led to the study of a formononetin/coumestrol-regulated operon in *Agrobacterium tumefaciens* ID1609. This operon was the subject of graduate student Jeff Palumbo's Ph.D. thesis. Mutations in the gene reduce competitive root-colonization by *A. tumefaciens* more than 90% without affecting growth or colonization in single-strain tests. The genes are not present in any *Rhizobium meliloti* strains tested, so we tried to increase competitiveness of *R. meliloti* by producing recombinant strains containing these genes. Strain ID1609, the first *A. tumefaciens* isolated from alfalfa, may be useful itself for transforming alfalfa because it is more virulent on this plant than other commonly used *Agrobacterium* strains. For further details please see the attached

Unlike the well-characterized *A. tumefaciens* strains C58 and Ach5, ID1609 is naturally resistant to kanamycin and spectinomycin. Tumors formed by ID1609 synthesize octopine which is used by the bacterium as a carbon source, and DNA hybridization tests with Ti plasmid-specific probes show the ID1609 Ti plasmid is a typical octopine-type. Transconjugant strains showed that both the chromosome and Ti plasmid in ID1609 contribute to tumor formation on alfalfa. In addition, the non-dormant 'CUFIO' alfalfa cultivar from which ID1609 was isolated was significantly more susceptible to all *Agrobacterium* strains tested than the dormant 'Agate' cultivar. This study described in more details in a paper entitled

Detecting isoflavonoids in rhizosphere soil

A simple method for extraction of biologically relevant molecules from soil was developed and tested. Flavonoid, isoflavonoids, and nucleosides were detected in various soil samples. An ecologically relevant soil extraction procedure separated two types of molecules important for bacteria: flavonoids and small hydrophilic organic compounds. Two flavonoids, identified previously as inducers of modulation genes in *Rhizobium meliloti*, were detected in rhizosphere soil from alfalfa (*Medicago sativa* L.). In addition, biologically significant quantities ($\mu\text{mole/kg}$) of fibonucleosides and deoxyfibonucleosides were found in all soils tested. Long-term wheat (*Triticum aestivum* L.) plots that had received manure contained elevated amounts of

nucleosides, and in a separate experiment, the presence of legumes in a wheat cropping sequence increased soil nucleosides. Intact bacterial cells accounted for less than 1% of the free nucleosides detected. These results offer new testable hypotheses for molecular ecologists and differ from those obtained with older, harsh techniques.

Characterization of a new *Agrobacterium tumefaciens* strain from alfalfa

Agrobacterium tumefaciens strain 1D1609, reported here as the first field isolate from alfalfa (*Medicago sativa* L.), forms tumors on a wide range of plant species. Unlike the well-characterized *A. tumefaciens* strains C58 and Ach5, 1D1609 is highly virulent on alfalfa and is naturally resistant to kanamycin and spectinomycin. Tumors formed by 1D1609 synthesize octopine which is utilized by the bacterium as a sole carbon source. Its resident Ti plasmid is an octopine-type. Transconjugant strains showed that both the chromosome and Ti plasmid in 1D1609 contribute to tumor formation on alfalfa. In addition, the non-dormant 'CUF101' alfalfa cultivar from which 1D1609 was isolated was significantly more susceptible to all *Agrobacterium* strains tested than the dormant 'Agate' cultivar.

Effect of isoflavonoids-inducible efflux pump on competitive colonization.

In an effort to locate novel chromosomal genes that might contribute to tumor formation and/or root colonization, a transposable promoter probe was used to identify *Agrobacterium tumefaciens* strain 1D1609, which was isolated originally from alfalfa (*Medicago sativa* L.), contains genes that increase competitive root colonization on that plant by reducing accumulation of alfalfa isoflavonoids in the bacterial cells. Mutant strain I-1 was isolated by its isoflavonoid-inducible neomycin resistance following mutagenesis with the transposable promoter probe Tn5-B30. Nucleotide sequence analysis showed the transposon had inserted in the first open reading frame, *ifeA*, of a three-gene locus (*ifeA*, *ifeB*, *ifeR*), which shows high homology to bacterial efflux pump operons. Assays on alfalfa showed that mutant strain I-1 colonized roots normally in single-strain tests but was impaired significantly ($P < 0.01$) in competition against wild-type strain 1D1609. Site directed mutagenesis experiments, which produced strains I-4 (*ifeA::gusA*) and I-6 (*ifeA::Ω-Tc*), confirmed the importance of *ifeA* for competitive root colonization. Exposure to the isoflavonoid coumestrol increased GUS activity in strain I-4 21-fold during the period

when coumestrol accumulation in wild-type cells declined. In the same test, coumestrol accumulation in mutant strain I-6 did not decline. Expression of the *ifeA-gusA* reporter was also induced by the alfalfa root isoflavonoids formononetin and medicarpin but not by two triterpenoids present in alfalfa. These results show that an efflux pump can confer measurable ecological benefits on *A. tumefaciens* in an environment where the inducing molecules are known to be present.

Expression of streptavidin in plant's roots.

To broaden the scope of our activity beyond isoflavonoids commonly found in legumes, Dr. Kapulnik's group took advantage of results obtained in an on-going project carried out in Dr. Phillips laboratory which confirmed the role of biotin as a bacterial stimulant. In this project Dr. Phillips demonstrated that biotin affects bacterial growth not only through its role as a vitamin cofactor but also as a regulatory element in other processes that may affect rhizosphere bacteria. Furthermore, application of the biotin-binding protein, avidin, resulted in a significant reduction of *Rhizobium* colonization in legume rhizosphere.

These results encourage us to support the hypothesis that synthesis and release of biotin-binding protein from plant roots (in transgenic plants) may induce a selective constituent for bacterial colonization in the rhizosphere. For this purpose we decided to evaluate the potential of the bacterial streptavidin (STV) to serve as biotin-binding protein. In the following paragraphs we are specifying our contribution to this intention as well as describing our main technical details.

Isolation of the bacterial core-streptavidin.

PCR amplification was performed using *Bam*HI genomic fragment of *Streptomyces avidinii* (50 ng) as a template, and 10 pmole of each primer; a direct primer 5'-actgaGGCATCACCGGCACCTGGTACAAC and a reverse primer 5'-CTACGGCTTCACCTTGGTGAAG, corresponding to nucleotides 166-190 and 527-508 of streptavidin entry in Genbank X03591, respectively. Following denaturation for 3 min at 95°C, 36 amplification cycles of 30 sec at 94°C, 45 sec at 59°C and 45 sec at 72°C with an 5 min extended elongation step were applied in a PTC-100 Programmable Thermal Controller (MJ Research Inc. USA). PCR product was gel purified, cloned in pGEM^{TA} (Promega) and sequenced.

Construction of chimeric core-streptavidin

The PCR product was re-cloned as *Pst*I fragments in-frame to signal peptide for secretion, originated from wheat gene for α/β -gliadin storage protein (pW 8233) (accession # x02539). The signal peptide-fused core-streptavidin was ligated between CaMV 35S promoter and NOS terminator to create plant expression cassettes. The cassette was cloned into pBin+ vector (Van Engelen et al., 1995) and was used for tobacco transformation mediated by *Agrobacterium* EHA 105 (Hood et al., 1993). Along with this task several constructs have been made using root-specific *Tob* promoter (Yamamoto et al., 1991) and the copper-inducible promoter (Mett et al., 1996).

Transformation of tobacco plants

Agrobacterium mediated transformation technique was used to insert the transgene to tobacco plants. The transformants were identified (PCR and selection markers) and characterized for growth performance. F1 seeds of To were evaluated and homozygous plants carrying the *S. avidinii stv* gene have been isolated.

Secretion of core-streptavidin from roots of transgenic plants

Secretion of core-streptavidin from transgenic plants was tested by growth inhibition of biotin deficient bacteria by root exudates. Thirty transgenic tobacco plantlets from each construct, were grown in modified sterile nutrient solution in controlled growth conditions. After two weeks of growth, rooting media (the liquid nutrient solution that supposed to contain the transgenic protein) was collected and applied onto M-9 plates pre-cultured with a biotin deficient bacterium (BDB) isolated from tomato rhizosphere (data is not shown). Non of the tested transgenic plants, of both constructs, found to inhibit growth of the BDB culture in a bioassay experiments. Further evaluation confirmed that tobacco exude high biotin levels (relative to tomato and alfalfa plants) and levels of core-streptavidin assumed to be secreted from the roots was not high enough to confine or eliminate significantly the biotin from tobacco root exudates.

Transformation of alfalfa plants

In order to overcome the problem of high biotin in root exudates alfalfa plants that were found to contain lower biotin levels, were transformed with the same core-streptavidin constructs carried the PR-1 inducible promoter. Following transformation, regeneration and selection of the *stv* containing transformants, plants

(To plants) were allowed to grow in the greenhouse for further evaluations.

Twenty-six transgenic plants were grown in greenhouse up to flowering, but soon after, were degenerated and lost. From bioassay experiments it became clear that alfalfa contained low biotin levels and it can be that the expression of the core-streptavidin in alfalfa were too toxic for normal plant development.

These set of experiments reinforce our initial assumption that the vitamin – biotin is an essential factor for the development of plants and bacteria. Further efforts to fine-tune the biotin level in planta through the expression of biotin binding domain should be considered in future efforts to alter population of rhizosphere bacteria.

D. Description of the cooperation

The two labs on this project maintained a very close cooperation interaction. Scientific problems and accomplishments were discussed frequently through e-mail and fax communications. Periodically when important decisions were required, Drs. Phillips and Kapulnik had direct telephone conversations.

Cooperation between the two groups was strengthened by personal visit and interactions. Dr. Kapulnik visited Davis in May 1996 and graduate student Hanne Volpin from Volcani took a pot-doctoral position in Davis (supported separately by the BARD post-doc fellowship). These allowed a better progress of each lab during particular stages of the project. Specific laboratory methodology and biochemical reagents used in this project were routinely sent between Davis and Israel. For instance, Dr. Phillips's group purified large quantities of medecarpin from alfalfa roots and sent to Israel for evaluation with rhizosphere microorganisms.

E. Evaluation of the research achievements

The assumption that medicarpin, produced by alfalfa roots, can be used as a bacterial selection factor in the rhizosphere was evaluated at the first phase of the project. In Dr. Kapulnik's lab hydroponically grown seedlings or transformed alfalfa root culture (obtained following transformation with *Agrobacterium rhizogenes* Ri-DNA) were developed as experimental system. It became clear that the amount of medicarpin being exuded from the roots is lower than originally assumed (0.05 µg/g FW). A higher medicarpin level (2 to 3 times) could be observed in root exudates following elicitation with yeast extract or *Rhizobium* culture. However, no much de-novo production could be seen in root extracts. These results were supported by expression analysis of two genes (CHI and IFR) involved in medicarpin production. The steady state mRNA levels of the two genes did not alter significantly upon elicitation with *Rhizobium meliloti* 1021. Our conclusion was that the slightly higher medicarpin level detected in root exudates resulted from induction in medicarpin exudation process(es) rather than induction in its biosynthesis. These results lowered the potential to accomplish objectives 2 and 3 of the original proposal.

Dr. Phillips's group looked for the occurrence of flavonoids and small hydrophilic organic compounds in agricultural soil. These efforts offered new technique for molecular ecologists and support our original hypothesis that plant rhizosphere can be characterized by specific molecules exuded from the roots.

The Davis group identified isoflavonoid-regulated root colonization gene in *Agrobacterium tumefaciens*. The formononetin and coumestrol-regulated operon was identified in strain ID1609 (objective 1). Mutations in the gene reduce competitive root-colonization by *A. tumefaciens* more than 90% without affecting growth or colonization in single-strain tests. Yet, the genes were not present in any *Rhizobium meliloti* strains tested (objective 4). To increase competitiveness of *R. meliloti* a recombinant strain containing this gene was produced (objective 3).

Dr. Kapulnik's group worked to define the way to alter plant-rhizosphere quality by promoting biotin deficient conditions in this niche. Although these efforts were not included in the original objectives the modification of biotin level through the expression of the bacterial gene streptavidin seems as an important strategy for future

development with GM organisms. It was concluded that biotin availability in planta can be manipulated but further work is being needed to achieve this goal.

Throughout the course of this work, new knowledge was integrated with existing knowledge in various review articles, and new approaches to defining how beneficial microbes interact with plant roots were identified.

F. List of Publications

1. Phillips, D. A. and Y. Kapulnik. 1995. Plant isoflavonoids, pathogens and symbionts, *Trends in Microbiology* 3:58-64.
2. Dakora, F. D. and D. A. Phillips. 1996. Diverse functions of isoflavonoids in legumes transcend anti-microbial definitions of phytoalexins. *Physiological and Molecular Plant Pathology* 49:1-20.
3. Phillips, D. A. and W. R. Streit. 1996. Applying plant-microbe signaling concepts to alfalfa: Roles for secondary metabolites. In: B.D. McKersie and D.C.W. Brown, eds. *Biotechnology and Improvement of Forage Legumes*, CAB International, Wallingford. Pp.319-342.
4. Phillips, D. A., C. M. Joseph and P. R. Hirsch. 1997. Occurrence of flavonoids and nucleosides in agricultural soils. *Applied and Environmental Microbiology* 63:4573-4577.
5. Palumbo, J. D., C. I. Kado and D. A. Phillips. 1998. An isoflavonoid-inducible efflux pump in *Agrobacterium tumefaciens* is involved in competitive colonization of roots. *Journal of Bacteriology* 180:3107-3113.
6. Palumbo, J.D., D.A. Phillips and C.I. Kado. 1998. Characterization of a novel *Agrobacterium tumefaciens* strain from alfalfa (*Medicago sativa* L.). *Archives for Microbiology* 169:381-386.
7. Ganon, D., S. Galili, D. A. Phillips, H. Volpin, I. Chet and Y. Kapulnik. 1999. The induced defense response following *Rhizoctonia solani* infection is suppressed in mycorrhizal alfalfa roots. Submitted to *Plant Physiology*.