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Biological Control of Fusarium Wilt and Crown Rot of Tomato

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EXECUTIVE SUMMARY

Fusarium crown and root rot of tomatoes caused by the soil fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) is one of the most devastating pathogens of tomatoes in Israel and worldwide. In Uzbekistan heavy tomato yield losses are attributed to *Fusarium* spp. including FORL. On the other hand, the massive use of synthetic pesticides in the past two decades in Uzbekistan has caused a grave ecological problem.

In Uzbekistan the problem of environmental protection is particularly acute because of prolonged utilization of chemicals in cotton fields at rates tens fold higher than the normal dose, sometimes with irreversible negative consequences. Therefore, in the last years special attention is given to biological control of plant pathogens, The overall goal of the proposed research is to develop a biological control system for fusarium wilt and crown rot of tomato based on the recently isolated bacterial, actinomycetes and fungal strains. Biological control of plant pathogens based on naturally occurring microorganisms may serve as an environmentally-friendly alternative to the polluting chemical pesticides.

Moreover, in Uzbekistan there is an acute problem of saline soils. According to the data of the Ministry of Agricultural and Water Economy of Uzbekistan (2001), from 4,3 million hectares of irrigating soils 52,1 % - are salted, 252 thousand hectares of heavy salted soils are not used and the annual growth of salted soils is 1,5 %. Therefore, the effect of salinity on FORL and on the physiological activity of potential antagonists against FORL has to be studied.

The present report consists of: 1) study of the influence of temperature, illumination, pH and salinity, on growth of FORL and of fungal and actinomycetes that are antagonists to FORL; 2) isolation of bacteria which are resistant to salinity; screening these bacterial isolates for mycoparasitic or antibiotic interactions against FORL; 3) Testing biological control of FORL under greenhouse conditions using isolated bacterial strains; 4) Screening for chitin (a polysaccharide constituent of fungal cell walls) degrading bacteria for selection of FORL mycoparasites.

SECTION I

A) Research objectives

The overall long-term aim of this research is to develop a biological control system for fusarium wilt and crown rot of tomato in Uzbekistan based on newly isolated microbial antagonists. This goal will be pursued in several stages, each dedicated to a different specific objective:

1. To test the biocontrol efficacy of the recently isolated actinomycetes and bacteria against various strains (Uzbekistan and Israeli) of fusarium wilt and crown rot of tomato under greenhouse conditions.
2. To study methods of application of selected antagonists under greenhouse conditions in order to improve biocontrol efficacy.
3. To study the biological control mechanisms of selected antagonists (i.e.. mycoparasitism, antibiosis and competition) which may be involved in this biocontrol.
4. To evaluate culture media based on agricultural and industrial wastes and growth conditions suitable for large-scale production, formulation and application of selected microbial biocontrol agents.
5. To study the survival of selected antagonists in soil and in the rhizosphere of tomatoes.
6. To test the biocontrol efficacy of various microbial preparations under commercial greenhouse and field conditions in Uzbekistan.

B) Research Accomplishments

Effect of ecological factors on *Fusarium*, isolated from wilt and crown rot of tomatoes

1. Influence of temperature

The temperature is one of the factors causing changes in fungal activity and affects the onset, development and prevalence of plants diseases. Under conditions present in Uzbekistan no data is available on temperature influence on growth of *Fusarium* species (wilt and crown rot of tomatoes).

Therefore, we tested the growth of FORL and of other species of *Fusarium* at various temperatures (Table 1). The data showed that maximal growth for most of the Fusarium strains was obtained at thee range of 27 C-30C.

Table 1. Influence of temperature on growth of various species of *Fusarium*

Species	Radial growth after 6 days (mm)					
	5-10 °C	15 °C	20 °C	27 °C	30 °C	35 °C
FORL	0	12	20	35	45	2
F. semitectum	0	18	25	45	45	1
F. solani	0	7	12	30	30	4
F. gibbosum v. bullatum	0	9	12	40	45	1
F.moniliforme v subglutinans	0	6	13	35	40	12

Indeed, field observations have shown, that the high temperatures in June and July (38-40 °C) exert heat pressure on disease development and the decrease in temperatures up to (25-30 °C) usually increases the incidence of wilt infection.

2. Influence of medium acidity

It is well established that wilt pathogens differ in their demand for medium acidity. The change of pH during cultivation of various *Fusarium* species and their ability to change medium reaction (data not shown) indicate a high plasticity of infection, and its large adaptability to infect plants, particularly tomatoes.

Fungal growth on nutrient medium with various pH levels has shown that the growth on acid medium is remarkably slowed down but under medium alkalisation the intensity of mycelium growth is increased. Hence, cultivation of *Fusarium* should be carried out at pH level close to neutral.

After 30 days of cultivation on medium with various pH levels the mycelial weight was detrmined (table 2).

The data showed that all *Fusarium* species isolated from tomatoes infected with wilt and crown rot of can grow at a wide range of initial acidity by changing it to alkaline during their activity. However, the optimum pH for their vital function ranges

between 4.4 and 6.6 – a level that corresponds to tomatoes pH. The study of the fungus biology has allowed to make the conclusion, that optimum conditions for fungus development coincide with the condition of tomatoes development. This circumstance may explains the high susceptibility of tomatoes to FORL.

Table 2. Dry weight (mg) of mycelium at various pH levels

Species	Medium pH				
	2.8	4.4	4.8	6.6	7.8
F. oxysporum	200	1,100	1,500	800	100
F. solani	100	600	900	700	50
F. gibbosum v. bullatum	200	1,300	1,400	600	230
F. semitectum	150	1,000	1,200	800	100
F. moniliforme v. subglutinans	250	1,400	900	800	200

3. Screening of antibiotic effects of various isolaes against FORL

In order to search for effective antagonists which inhibit the growth and development of wilt and crown rot of tomatoes (FORL) we tested the antibiotic activity of fungal and actinomycetes isolates from the rhizosphere of healthy and infected tomatoe plants. Three fungal genera were studied: *Aspergillus, Acremonium* and *Stachybotrys* (Aspergillus 44 strains; *Acremonium* - 20 strains *Stachybotrys* - 7 strains). Experimental studies showed variable antibiotic activity among fungal strains against FORL. Among *A. niger* only two strains have demonstrated antagonism activity against FORL. After 6 days of the incuabation their zone of suppression was 6-7 mm. Among *A. ochraceys* 4 strains showed a suppression zone of 6-10 mm. Only one strain of *A. terreus* showed a suppression zone of 6 mm (Table 3).

Table 3. Antibiotic activity of fungus strains of genus *Aspergillus* Micheli against FORL. Data show Distance between fungal cultures (mm)

Species	Incubation time (Days)						
	0	1	2	3	4	5	6
Aspergillus niger v. Thieng							
Aspergillus niger 2	45	35	24	15	10	7	7
Aspergillus niger 1	45	35	25	17	10	8	6
Aspergillus ochraceus Wilhelm							
Aspergillus alliaceus	50	45	37	30	17	6	6
Aspergillus ochraceus 1077	45	40	35	30	22	12	10
Aspergillus ochraceus 1079	45	40	34	23	13	6	6
Aspergillus ochraceus 513	45	40	33	23	12	7	6
Aspergillus ochraceus 4	45	40	30	22	14	6	6
Aspergillus ochraceus 48	50	45	37	30	17	6	6
Aspergillus terreus Thom							
Aspergillus terreus 26	45	40	32	25	17	11	5
Aspergillus terreus 29	45	40	32	25	20	10	5
Aspergillus terreus 30	55	40	35	25	20	10	6

Five strains of *Acremonium* genus showed antibiotic activity showing 6-7 mm of inhibition (Table 4).

Table 4. Antibiotic activity of fungus strains of genus *Acremonium* against FORL. Data show distance between fungal cultures (mm)

Species	Incubation time (Days)						
	0	1	2	3	4	5	6
Acremonium polychromum	55	50	45	33	14	12	6
Acremonium strain 6	50	45	40	35	24	15	6
Acremonium strain 7	50	50	45	32	25	10	5
Acremonium strain 9	45	40	37	25	17	6	5
Acremonium strain 12	45	40	30	20	13	7	7
Acremonium strain 49	55	50	45	35	15	13	7

Among strains from the genus *Stachybotrys* 4 strains showed 7-10 mm inhibition zone (Table 5). Thus, only representatives of *Stachybotrys* genus and *Aspirgillus ochraceys* group demonstrated the highest antagonist activity .

The representatives of actinomycetes – *Streptomyces* genus (20 isolates) and *Streptoverticillium* (4 isolates) - have been isolated from the rhizosphere of tomatoes and studied. Seven strains showed strong antibiotic activity against FORL. Their zones of suppression reached 12-14 mm.

Table 5. Antibiotic activity of fungus strains of genus *Stachybotrys* against FORL. Data show distance between fungal cultures (mm)

Species	Incubation time (Days)						
	0	1	2	3	4	5	6
Stachybotrys chartarum	35	30	28	20	10	8	7
Stachybotrys parvispora	35	30	26	18	9	6	5
Stachybotrys lobulata	35	30	25	20	12	10	10
Stachybotrys atra	34	28	20	11	8	7	7
Stachybotrys Zanzevariae	35	29	19	12	8	6	5
Stachybotrys 12	36	30	26	20	13	10	10
Stachybotrys 45	35	28	20	15	10	3	3

4. Effect of salinity on growth of FORL

FORL was cultured on agar plates containing Capec medium supplemented with increasing concentrations of NaCl. The data is presented in table 6 and

Fig.1.

Table 6. Growth of FORL on various concentrations of NaCl.

NaCl (mM)	Radial growth (mm) of FORL					
	1	2	3	4	5	6
58	5	11	17	25	36	43
116	5	10	16	24	33	40
232	5	8	13	20	30	33
348	4	7	12	18	25	30
464	2	6	11	15	24	30
580	2	5	9	15	22	25
696	0	4	7	11	15	20
812	0	4	6	8	10	12
928	0	2	3	4	5	8
1044	0	1	1	2	2	4
1160	0	0	0	0	0	0
Control	5	9	17	25	30	45

FORL seems to be capable of growing at relatively high levels of salinity. Significant growth was noted at concentrations higher than 0.5M NaCl.

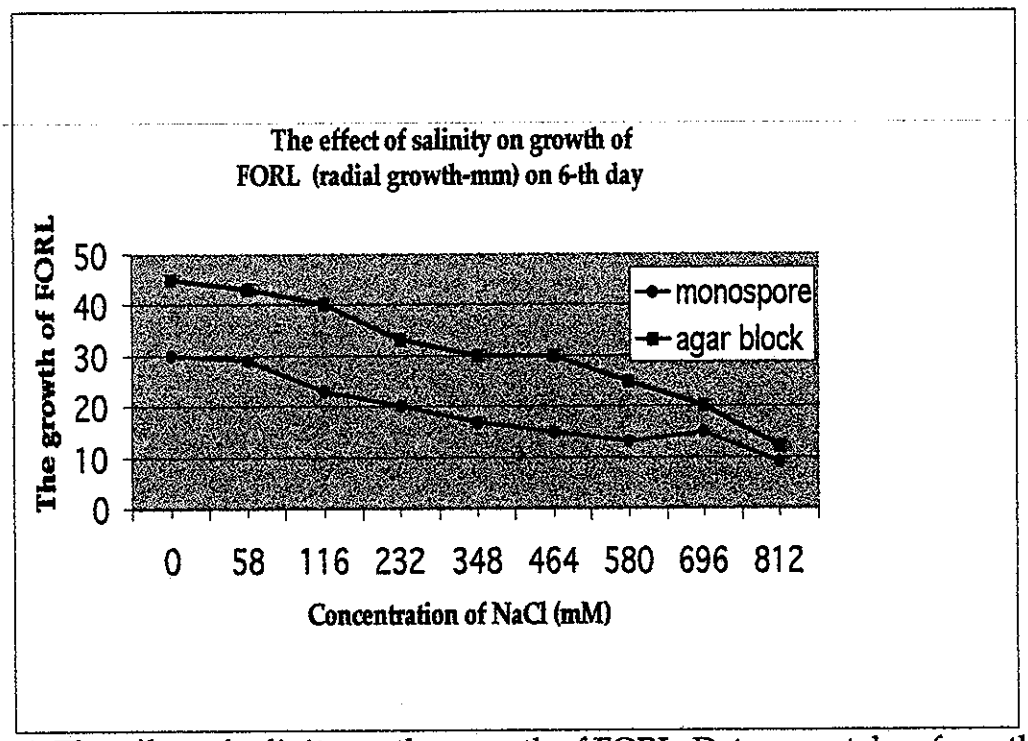

Figure 1. The effect of salinity on the growth of FORL. Data were taken from the 6th day of growth.

6. Resistance of potential antagonists to salinity

The high salinity of soils in Uzbekistan and the adaptive nature of FORL pathogens to high salinity requires that potential antagonists will tolerate these levels of salinity. Few strains of actinomycetes that inhibited FORL in dual culture tests were screened for tolerance to salinity (Fig. 2).

Table 7. Radial growth of actinomycetes (mm) on various concentrations of NaCl on 5th day

Strain	Concentration of NaCl (mM)									
	58	116	232	348	464	580	696	812	928	1044
4 / 1	6	6	6	4.1	3.2	2.4	0	0	0	0
4 /14	7,1	7	7	6.4	5.9	4.2	0	0	0	0
4 / 4	8	7.3	5.9	5.1	4.3	3.8	0	0	0	0
247	7.2	7.1	7.1	6.2	6	4	2,3	0	0	0
7	7.5	7.5	6	4.2	3.5	3	3,1	0	0	0
3 / 1	9.5	9.4	7.9	7	5.7	4.2	3,1	2	0	0

The data show that most of these strains are capablle of growing on high levels of NaCl. Three of these strains could grow on 696 mM NaCl (Table 7).

However, some fungal isolates showed a remarkable tolerance to salinity. Surprisingly, the growth of some strains of *Aspergillus* significantly enhanced by concentrations of up to 1.2 M NaCl (Table 8).

Few strains of *A. ochraceus* (7, 513 and 1079) and of *A. niger* (1 and 2) seem to have the potential to be suitable biocontrol candidates as they combine high resistance to salinity and are also effective inhibitors of FORL in dual culture tests. In the near future we will test these strains in biological control tests under greenhouse conditions.

Table 8. growth of fungal strains at various concentrations of NaCl

Species	Control	*Concentration of NaCl (mM)*									
		58	232	464	580	696	928	1044	1160	1276	1392
Stachybotrys sp stain 12	4	4	3	3	2,5	2	0	0	0	0	0
Stachybotrys sp stain 45	5	5	5	5	4	2	0,5	0	0	0	0
S. chartarum	4	4	4	3	2,5	2	0	0	0	0	0
S. lobulata	4	4	4	3	2,5	2	0	0	0	0	0
Stachybotrys atra	4	4	4	3	2,5	2	0,5	0	0	0	0
A. ochraceus stain 48	5	6	7	9	10	9	6	6	5	5	4
A.. ochraceus stain7	4	5	7	9	10	9	6	6	6	6	5
A. ochraceus stain 513	4,5	6	7	10	10	10	7	7	7	7	5
A. ochraceus stain 1079	4,5	5	7	9	9	9	6	6	6	6	5
A. polychromum	4	4	3	3	2,5	0	0	0	0	0	0
Aspergillus.sp strain 49	4,5	4,5	4,5	4	3,5	1	0,5	0	0	0	0
Acremonium sp strain 12	4,5	4,5	4	3	3	1	0	0	0	0	0
Acremonium sp strain 6	3	3	3	2	3	0,5	0	0	0	0	0
Asperg. Terreus	12,5	12	12	11	11	7	5	4	3	3	1
Asperg. Niger stain 1	9	9	9	9	9	7	4	3	2	1	1
Asperg. Niger stain 2	12	12	12	14	14	10	8	5	4	4	2

Virulence of FORL strains under greenhouse conditions

Strains of FORL isolated from various sites (and even from the same site) may manifest virulence variability against tomato plants. An example of such variability is presented in Fig. 2. Data show that FORL isolates incorporated into the soil induced disease incidence in tomato plants (scored as dead plants) that ranged between 10 and 42% 40 days after planting. FORL strain CRNK #9 was chosen for future biocontrol tests.

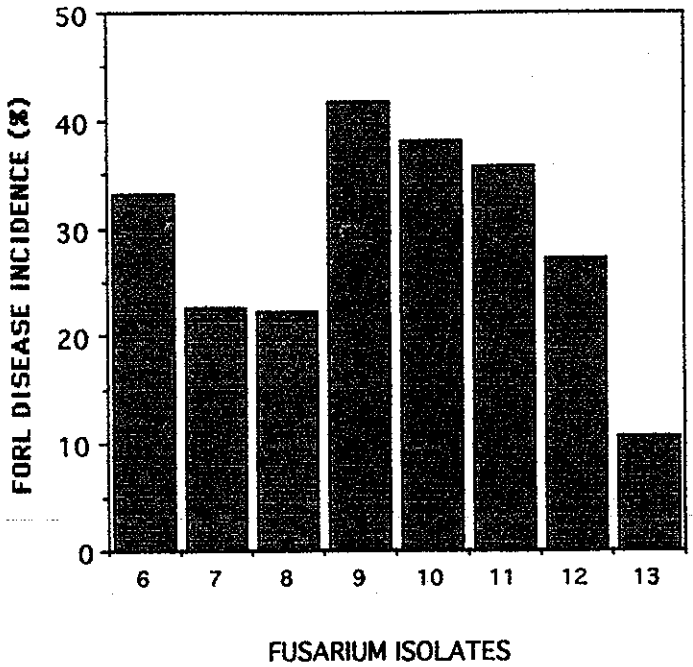

Figure 2. Disease incidence of tomato plants induced by various isolates of FORL. Conidial suspensions prepared from each isolate were used to inoculate a peat:vermiculite:perlite mixture ($5X10^8$ microconidia per litre mixture). Data represent dead plants counted 40 days after planting.

Biological control of FORL under greenhouse conditions

Several bacterial strains that were isolated during the first year of the research and were shown to produce antibiotic substances that inhibited radial growth and biomass production of FORL were chosen for bioassays under greenhouse conditions. Microconidial inoculum was prepared as described above. Each of the bacterial strains was cultured in nutrient broth medium for 18 hr, bacterial cells were washed and suspended in saline solution. These suspensions were added to the FORL inoculated soil mixture. Tomato seeds were planted and emergence was recorded after 7-10 days.

The data is presented in figures 3 and 4. Three bacterial isolates were found to

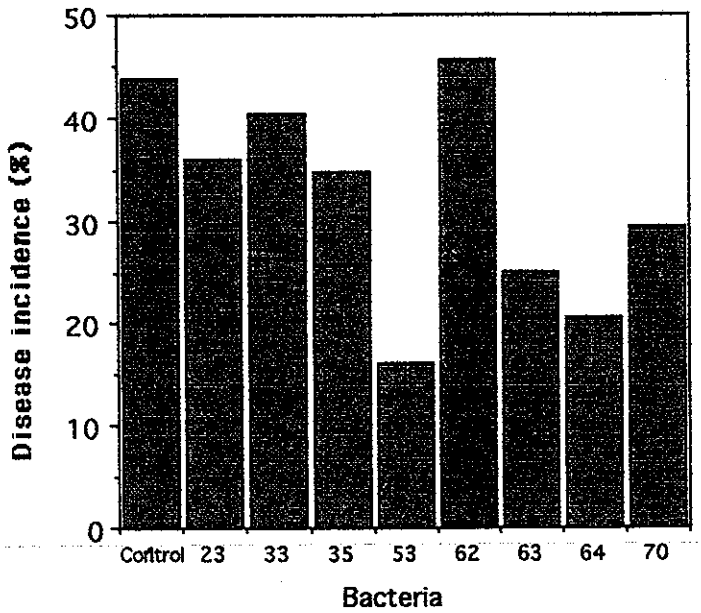

Figure 3. Biological control test of FORL using bacterial isolates. Soil mixture was inoculated with microconidial suspension ($5x10^8$ microconidia per litre) and supplemented with either one of the bacterial suspensions.

be effective antagonists reducing the disease incidence by up to 65% (strain 53) (Fig. 3&4).

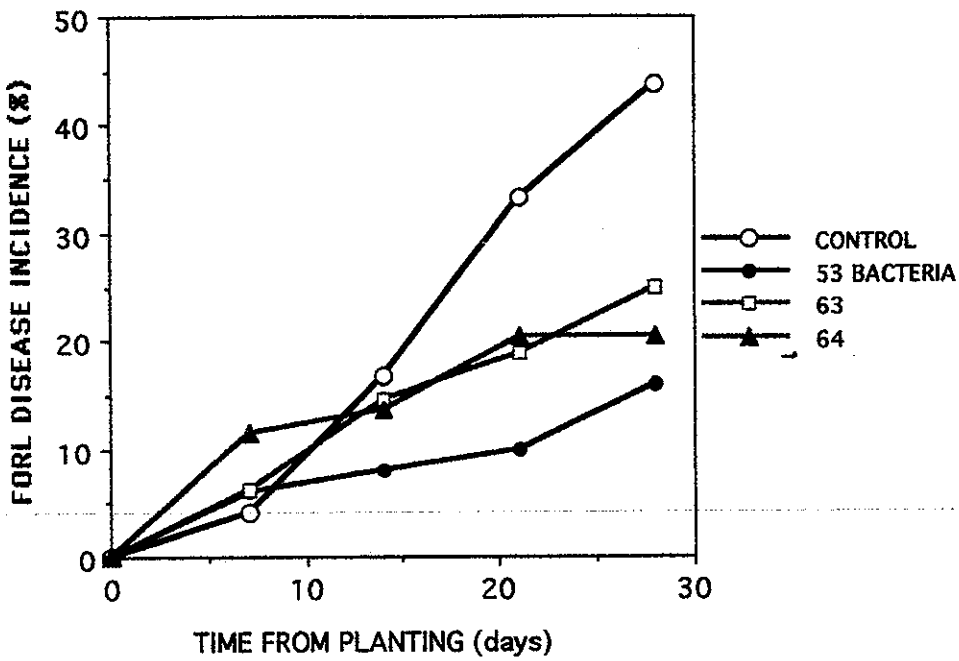

Figure 4. Biological control test of FORL using bacterial isolates. Soil mixture was inoculated with microconidial suspension ($5x10^8$ microconidia per litre) and supplemented with either one of the bacterial suspensions.

Isolation of chitinolytic bacteria

Chitin (a polimer of N-acetyl-glucosamine) and laminarin (ß – 1,3 glucan with 1,6 branching) are the major polysaccharides in fungal cell walls. We have started a large screening for soil bacteria capable of degrading chitin. Such bacteria have the potential to serve as mycolytic biocontrol agents acting as mycoparasites. We have produced colloidal chitin by partial hydrolysis of crab shell chitin. The colloidal chitin was introduced to synthetic medium as the sole carbon source. In order to detect chitinolytic bacteria agar plates containing the chitin medium (look opaque because the chitin is insoluble) were inoculated with soil suspensions made with more than 50 soil sampels.

Recently, we obtained several chitinolytic bacterial strains that were identified by their ability to produce clear degradation zones around the colonies on the opaque chitin containing plates.

These isolates will be further screened for chitinase activity and for the ability to utilize FORL cell walls as a sole carbon source. The effective bacterial strains will be further tested in biological control tests under greenhouse conditions.

C) Scientific Impact of Collaboration

During the second year of the research the scientific efforts of both research groups were focused on characterization of eco-physiological traits of FORL such as temperature range for growth and the effects of medium pH and salinity on the growth and development of FORL. In addition we have revealed that certain antagonists can adapt to the high salinity of soils prevailing in Uzbekistan. The major impact of the research in the second year is the identification of specific potential antagonists and the preliminary characterization of their mode of action (mechanisms of biocontrol). During this research period Dr. A. Sivan visited Uzbekistan and met with Prof. S. Khojibaeva and Dr. Kh. Karimova. This meeting focussed on study of the basic features of FORL in culture and on diseased plants and analyzing the data of the second year of research. We also discussed future directions of the research. Specific directions included focussing on biocontrol mechanisms of potential antagonists (i.e. antibiosis and mycoparasitism).

D) Description of Project Impact

The strength of this project is, by no means, the collaboration between the two research teams. The transfer of knowledge yielded the isolation of some promising strains for biocontrol. The results obtained thus far in the project are yet premature to be used commercially. However, we believe that the recently isolated bacterial and fungal antagonists (in Uzbekistan and in Israel) will provide a solid ground for development of a biological control system for

FORL based on naturally occurring soil bacteria. Such biocontrol agents may safely substitute the use of polluting chemical fungicides.

E) Strengthening of Developing Country Institution

During the second year of research the Uzbekistan team has purchased some basic equipment which include a refrigerator, an incubator and a shaker for cultivation of microorganisms.

Two months ago Dr. A. Sivan visited the laboratory of Prof. S. Khojibaeva and Dr. Kh.Karimova. This visit was utilized to study of the basic features of FORL in culture and on diseased plants and analyzing the data of the second year of research. We also discussed future directions of the research. Recently, a trainee (Sudabe Mamedova) from the Uzbekistan Institute started a six month training program in the laboratory of Dr. A. Sivan.

F) Future Work

The research is being carried out according to the scheduled program. Future work will focus on the following tasks:

Testing the biocontrol efficacy of the recently isolated actinomycetes and bacteria against various strains (Uzbekistan and Israeli) of fusarium wilt and crown rot of tomato under greenhouse conditions.

Studying methods of application of selected antagonists under greenhouse conditions in order to improve biocontrol efficacy.

To study the biological control mechanisms of selected antagonists (i.e.. mycoparasitism, antibiosis and competition) which may be involved in this biocontrol.

To study the survival of selected antagonists in soil and in the rhizosphere of tomatoes.

To test the biocontrol efficacy of various microbial preparations under commercial greenhouse and field conditions in Uzbekistan.

SECTION II

A) Managerial Issues

NO special managerial issues for this period were noted.

B) Budget

No major changes in the budget have been made.

C) Special Concerns

Special concern protocols have not been changed.

D) Collaboration, Travel, Training and Publications

The collaboration has been facilitated through frequent e-mail communication and during the visit of the Israeli P.I. to the Uzbekistanian lab:

June 12, 2001 - Eight days visit of Dr. A. Sivan to Uzbekistan.

June 4, 2001 – Sudabe Mamedova from the Uzbekistanian institute starts six months training in the laboratory of Dr. A. Sivan.

A visit of the Uzbekistanian collaborator is scheduled for mid September 2001.

No publications have yet came out from the project.

E) Request for American Embassy Tel Aviv or A.I.D. Actions

No particular request.