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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 the following topics: 1) Growth and development of FORL in saline soils; 2) Antibiotic substances producing by *Streptomyces roseoflavus*, st. 7 –the antagonist of FORL; 3) Biological control of FORL under greenhouse and conditions; 4) Physiological characterization of the production of chytinolytic enzymes

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

Uzbekistan team:

1) Optimization of biomass and antibiotic production of the antagonist *Streptomyces roseoflavus*

2) Induction of synthesis of defense compounds (phytoalexins) in tomato by microbial metabolites.

Introduction

It is known that the dynamics of microorganism growth and the intensity of physiologically active compounds biosynthesis depend on the components of nutritional medium, pH, temperature, aeration and other conditions of cultivation.

By optimizing the conditions of cultivation of microorganisms it is possible to achieve the significant increase in biomass yield and in microbial production of a variety of useful substances. Moreover, the use of different local industrial wastes as components of nutritional media can lead to the optimizing the culture growth, to achieving the active synthesis of valuable compounds and to subsequent reduce the price of their production.

Besides the facts that lipids are the structural components of tomato cell membrane and, that they are actively involved in interactions with toxic compounds of Fusarium oxysporum . very little is known about the importance of lipid substances in forming of plant resistance to *Fusarium oxysporum* f.sp.radicis-*lycopersici*. Special priority should be given to investigations of the substances which possess the wide spectrum inducing activity. This direction probably, is extremely promising because of the possibility to utilize the whole spectrum of the defense capacity of plants

Cultivation of *Streptomyces roseoflavus*, strain 7.

Different methods have been used to preserve the culture in most active form: 1) in tubes containing liquid medium and on agar amended with the sterile mixture of 5% gelatin and 50% sucrose (1:3), 2) freeze dried, 3) in sterile sand or soil, 4) under the mineral oil. The greatest viability and activity of *Streptomyces roseoflavus*, strain 7 was found when the spores were preserved in sterile sand or soil and as a freeze-dried culture.

Different mineral and organic nutritional media have been used for optimization of the culture growth. They were Gause #1 and #2, Czapek with glucose, starch-ammonium, glycerol-nitrate, peptone-glucose and potato media. Cultivation was performed on liquid and agar media. At these conditions, the maximal accumulation of biomass and the most active synthesis of antibiotics were found on peptone-glucose and glycerol-nitrate media. Over the submerged fermentation the antifungal activity of the culture against FORL ranged between 729 to 2187 unit/dilution on the 5th-6th day of cultivation. The biomass yield was in a range from 7,5 up to 8,9 g/l, and the sum of antibiotics of glycoside and izoprenoide nature was determined as 500-600 mg/l. To optimize the nutritional media, peptone-glucose medium was used as a basis. Further the sources of carbon, nitrogen and phosphorus were changed. As carbon sources we used glucose, sucrose, mannose, lactose, fructose, maltose in concentration 20 g/l. The maximal antibiotic activity was observed on glucose , sucrose and

maltose. The best nitrogen source was found to be ammonium salt $(NH_4)_2 SO_4$ in concentration 3,5 g/l. Over any decrease as well as increase of the concentration we found the inhibition of the culture growth and the production of antibiotics by *Streptomyces roseoflavus*, strain 7.

On the medium with high phosphorus content (K_2HPO_4, 0,7-1,0 g/l) the culture growth and antibiotic production decreased. The optimal phosphorus concentration was determined as 0,4-0,5 g/l. For enrichment of the medium we used cheap local waste of plant raw materials: vine and apple pressed skins, rice and wheat bran (20 g/l of the medium). It was found, that in variants with vine pressed skins and with rice bran the biomass and antibiotic yield was increased. The biomass yield was 9,8-11,2 g/l compare to 8,9 g/l in control. The antibiotic yield was 750-800 mg/l compared with 600 mg/l in control. Thus, we have worked out the optimal composition of the medium for Streptomyces roseoflavus, strain 7 cultivation as follows: glucose –20 g; $(NH_4)_2 SO_4$ - 3,5 g/l; K_2HPO_4, 0,5 g; $MgSO_4$ - 0,5 g; NaCl – 0,5g; $FeSO_4$ – 0,01g; vine pressed skins – 20 g; Water – 1,0 l at pH – 7,2.

To clarify the influence of aeration intensity on the culture growth and the antibiotic production, *Streptomyces roseoflavus*, strain 7 was grown by sub-merged cultivation on a shaker and stationary. We determined that 5-6 days after the inoculation the maximal activity was in the culture grown on a shaker (180 rotation /min). In the stationary grown culture the activity was a half on that on the shaker and reached its maximum only 9-10 days after the beginning of cultivation.

Optimal pH of the medium is 7,0-7,4. It was determined , that on acid media the culture growth was effective, but the antibiotic production was low. Temperature optimum for culture growth is 26-30^{0}C . The temperatures above or below this range inhibited the culture growth and antibiotic production.

Our laboratory regulations of cultivation were further tested at a semi-industrial scale in 100-liter fermenters (the volume of nutritional medium - 50 l) the medium pH developed to 7,2; t^{0}C – 28^0 C on a shaker with 180 r/min. Five percent (5%) of inoculant (2,5 l) was used. As a result , biomass yield reached 9,0 g/l.

Induction of synthesis of defense compounds (phytoalexins) in tomato by microbial metabolites.

We have tested native and diluted (1:1 ; 1:2 ; 1:3 ; 1:4 ; 1:5) cultural broth (liquid) of Streptomyces roseoflavus, strain 7. As a result it was found that the cultural broth (liquid) diluted 1:4 was non-toxic for tomato plants but time it was effective in induction of production of defense compounds . Antibiotics were applied in concentrations 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} mg/ml. At 1 mg/ml some toxic effect was observed on the tomato plants. Positive effect was observed when antibiotics were used in concentrations 10^{-1}, 10^{-2}, 10^{-3} mg/ml, but the most effective was solution with 10^{-2} mg of antibiotics in ml.

Experiments showed that the culture of *Streptomyces roseoflavus*, strain 7 itself and antibiotics of glycoside and izoprenoid nature from this culture possessed the inducing activity and elicited the synthesis of neutral lipids in tomato plants, intensified synthesis and activity of oxy - and epoxyglycerines, which are able to inhibit the growth and development of FORL. It is thought that these effects can be based on activation of peroxidase systems of tomato. The total lipid fraction from experimental tomato plants was more fungitoxic that the same fraction from the control plants.

Table 1. Fungitoxicity of several lipids from tomato plants to *Fusarium oxysporum radicis-lycopersici*.

Class of lipids	Amount of the substance on the disk, mg	FORL inhibition zone (radius, mm)
Carbohydrates	10	0
Triacylglycerines	10	0
Epoxyacylglycerines	10	3,15 ± 0,73
Free fatty acids	10	1,47 ± 0,48
Oxyacylglycerines	10	4,81 ± 0,57
Diacylglycerines	10	0
Sterines	10	0

Table 2. Induction of the synthesis of defense compounds by microbial metabolites.

#	Inductors	Concentration mg/ml	Epoxyacyl-glycerines (g)	Oxyacylgly-cerines (g)
1.	Cultural liquid of *S. roseoflavus*, strain 7	Dilution 1:4	2,01	0,85
2.	Glycoside antibiotic	10^{-2}	2,35	1,01
3.	Izoprenoide antibiotic	10^{-2}	2,43	1,20
4.	Control (water)		0,87	0,35

Earlier experiments under greenhouse and field conditions have revealed that *S. roseoflavus*, strain 7 stimulates tomato resistance to Fusarium wilt and Crown rot. Experiments on the induction of the defense compounds synthesis gave an idea about the nature of the substances which condition resistance of tomato to Fusarium and confirmed our results of the field trials.

Israeli Team

During the last year we have made progress in two related issues

1) Analysis of induction and activity of chitinase of selected bacterial and actinomyctes strains
2) Biological control of FORL by chitinolytic and antibiotic producing strains.

Analysis of induction and activity of chitinase of selected bacterial and actinomycetes strains

Several bacterial and actinomycetes strains were selected based on their ability to grow on synthetic medium containing chitin as a sole carbon source. Growth on agar plates resulted in a clear zone around the bacterial colony indicating the degradation and utilization of the colloidal chitin. Thus, the radius of the clear zone could serve as an initial screening tool of chitinolytic bacteria and actinomycetes. The second step included biomass production of the selected strains (from the first screening) in a liquid medium

containing chitin as the sole carbon source. The third step included activity assays on pnp glucose amine as an analogue of chitin. The most active strains (bacteria and actinomycetes) were further selected for conducting a thorough study comparing the induction of chitinase by chitin compared with the induction by cell walls of FORL . Furthermore, we have also compared the chitinase activity by following the release of NAGA from colloidal chitin and from cell walls of FORL which served as substrates.

a) Induction and activity of bacterial chitinases

Data in figures 1 – 3 show the induction and activity of chitinase in assays where chitin or fungal cell walls served either as inducers of chitinase during bacterial growth or as the substrates for testing the activity of the induced enzyme. The tested strains were selected as mentioned above. The selected strains showed a relative high and consistent chitinase activity (figures 1 – 3). The data clearly show the degradation of the chitin in cell walls of FORL by bacterial chitinases (Fig. 1&3). Induction of chitinase by chitin or by cell walls of FORL showed a similar pattern but a higher activity level was obtained when chitin served as the substrate compared with cell walls (Fig. 2&3). This is not surprising since the cell walls contains a variety of polysaccharides and proteins which may affect activity of the enzyme or interfere with its accessibility to the substrate (i.e. chitin).

All three tested strains showed a similar induction and activity patterns; the most consistent induction and activity was obtained when FORL cell wall served as inducer and as a substrate (Fig. 3). This result may be relevant biological control of FORL in the natural environment where the chitin is found as a component of the fungal cell walls and not as a pure polymer.

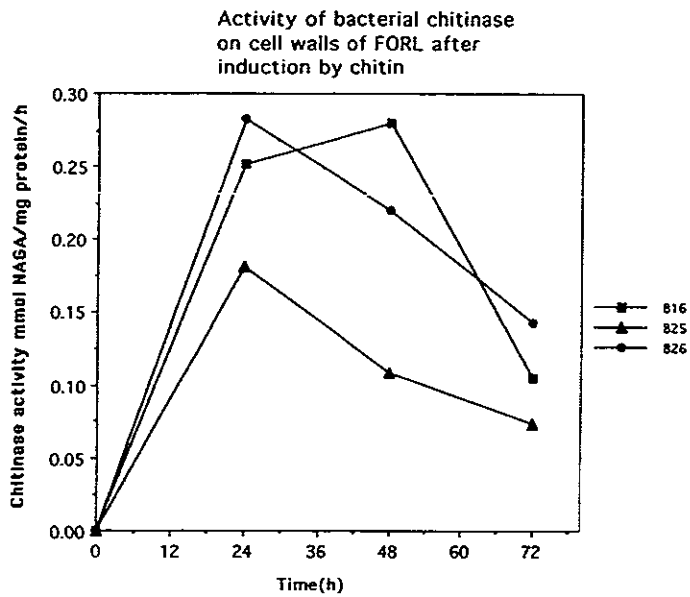

Fig.1. Chitinase activity of selected bacterial strains. Data show the specific activity of the enzyme measured by analyses of the release of N – acetyl D glucose amine from cell walls of FORL. (C-W)

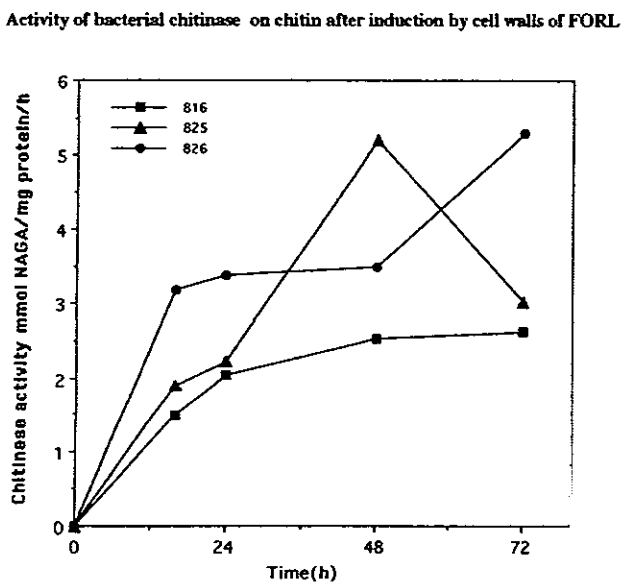

Fig. 2. Chitinase activity of selected bacterial strains. Data show the specific activity of the enzyme measured by analyses of the release of N – acetyl D glucose amine from chitin after growth on cell walls of FORL. (W-C)

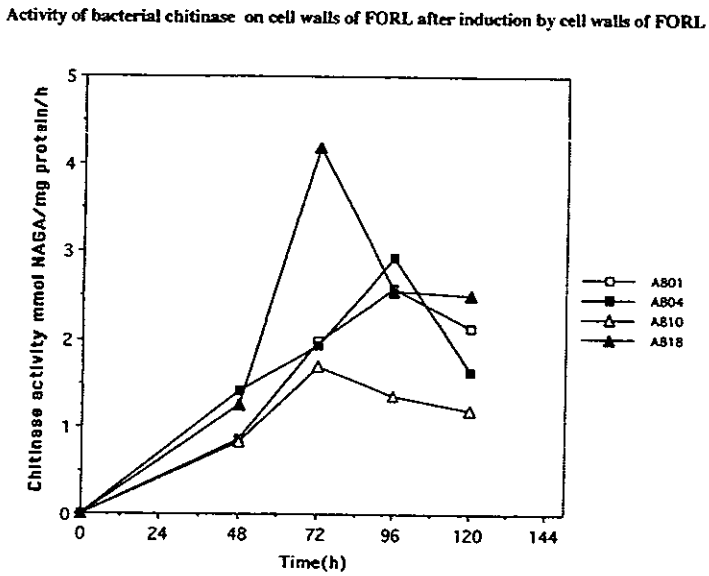

Fig. 3. Chitinase activity of selected bacterial strains. Data show the specific activity of the enzyme measured by analyses of the release of N – acetyl D glucose amine from FORL cell walls after growth on cell walls of FORL. (W-W)

b) Induction and activity of actinomycetes chitinases

Data in figures 4 – 6 show the induction and activity of chitinase - of the selected actinomycetes strains - in assays where chitin or fungal cell walls served either as inducers of chitinase during growth or as the substrates for testing the activity of the induced enzyme. The tested strains were selected as mentioned above. The data show a different pattern of chitinase induction compared with that of the bacterial strains (Figures 1 - 3). The induction of chitinase in most of the actinomycetes strains (either by chitin or by cell walls of FORL) sharply increased with time up to a peak 72 – 96 h after inoculation. However, this peak is followed by a sharp drop (in most strains) in the induction level of the enzyme (Figures 4 – 6). Interestingly, the chitinase activity on chitin after induction with cell walls of FORL was up to 5 folds higher than that obtained with the bacterial isolates (Fig. 5 vs. Fig. 2).

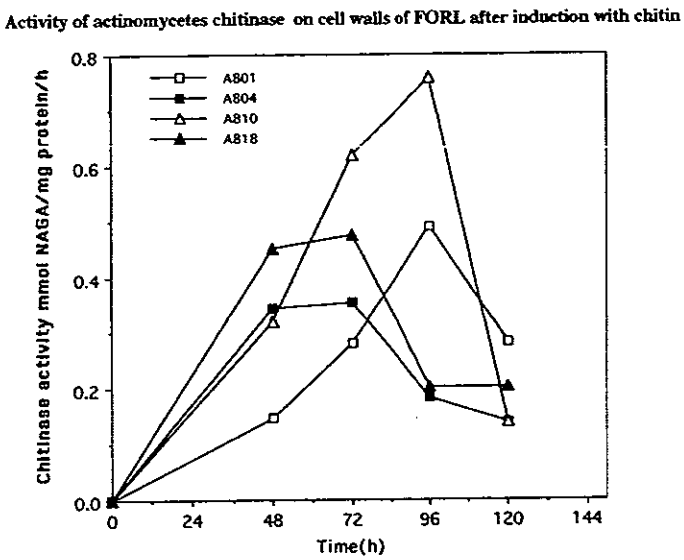

Fig.4. Chitinase activity of selected actinomycetes strains. Data show the specific activity of the enzyme measured by analyses of the release of N – acetyl D glucose amine from cell walls of FORL. (C-W)

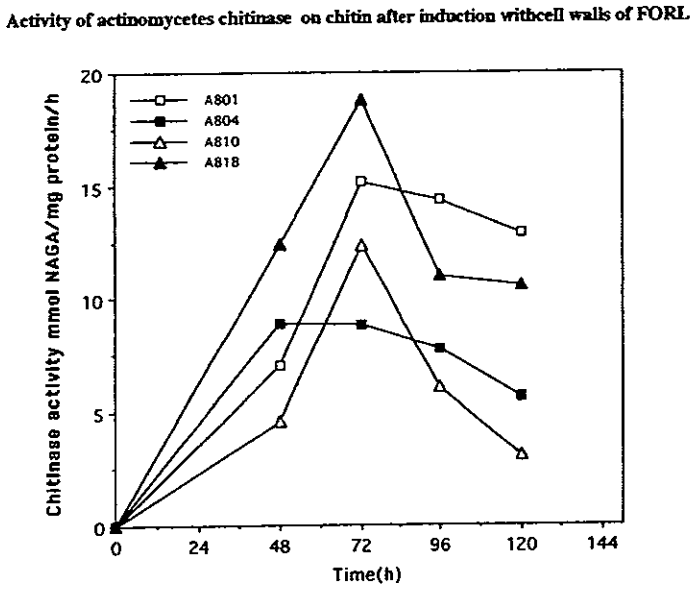

Fig. 5. Chitinase activity of selected actinomycetes strains. Data show the specific activity of the enzyme measured by analyses of the release of N – acetyl D glucose amine from chitin after growth on cell walls of FORL. (W-C)

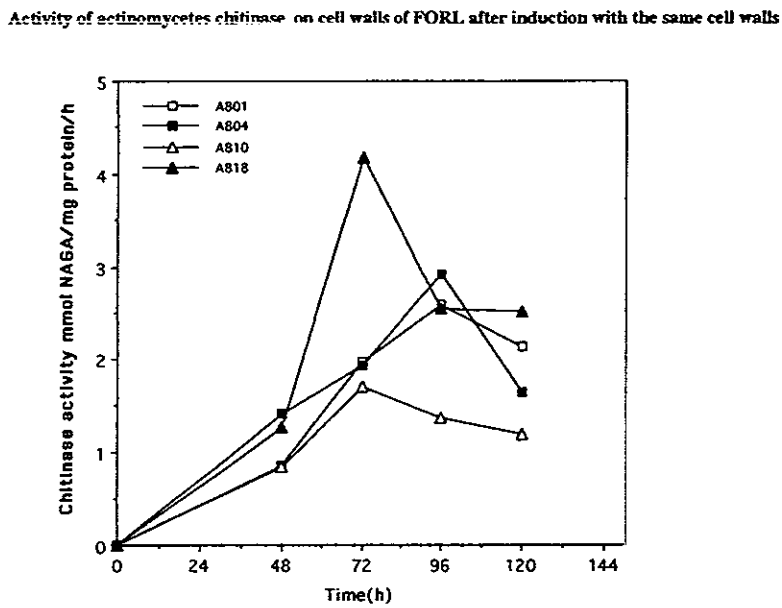

Fig. 6. Chitinase activity of selected actinomycetes strains. Data show the specific activity of the enzyme measured by analyses of the release of N – acetyl D glucose amine from FORL cell walls after growth on cell walls of FORL. (W-W)

The chitinase induction in the actinomycetes strains by cell walls of FORL was up to 9 fold higher that than that in the bacterial strains (Figures 3&6). This may have implication on mycoparasitc interactions between the antagonist and the pathogen higher induction of chitinolytic enzymes may lead to improved biological control.

Biological control assays of chitinolytic and antibiotic producing strains

At this point in our study it was important to evaluate the biological control potential of the selected antibiotic and chitinase producing strains of the tested bacterial and actinomycetes strains. (The screening and selection of antibiotic producing strains was reported in previous reports).

Data shown in figures 7 & 8 demonstrate the biological control of FORL by 2 of the chitinolytic actinomycetes strains, A801 and A803. These isolates reduced the disease incidence of FORL in tomato by ca. 85%. The other two chytinolytic actinomycetes-strains did not reduced thee disease incidence (data not shown). This show the complexity of biological control and the lack of correlation, that sometimes occur, between data obtained in laboratory (i.e. production of chitinase) and the antagonistic ability under natural soil condition.

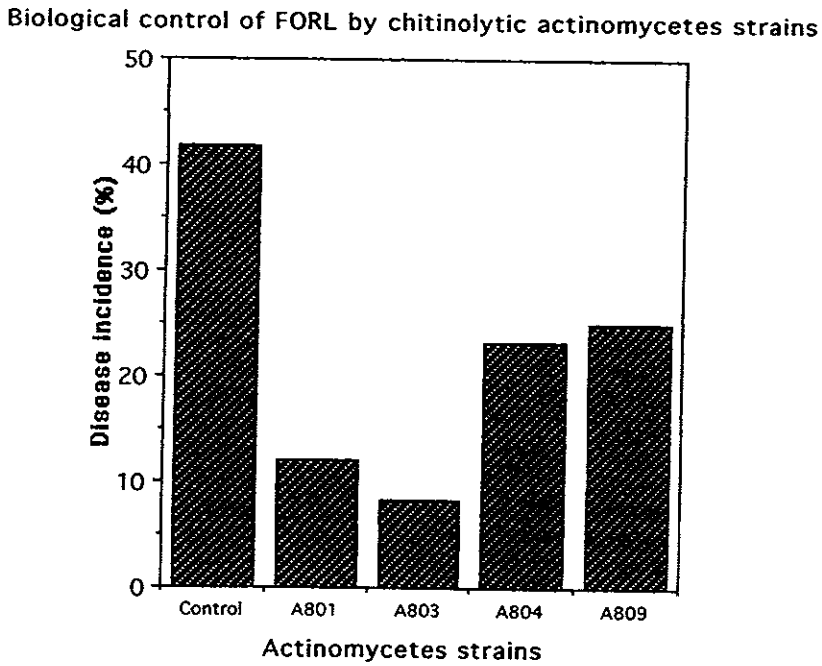

Fig. 7. Biological control of FORL by selected chitinolytic actinomycetes under greenhouse conditions. The disease incidence was assessed 60 days after planting.

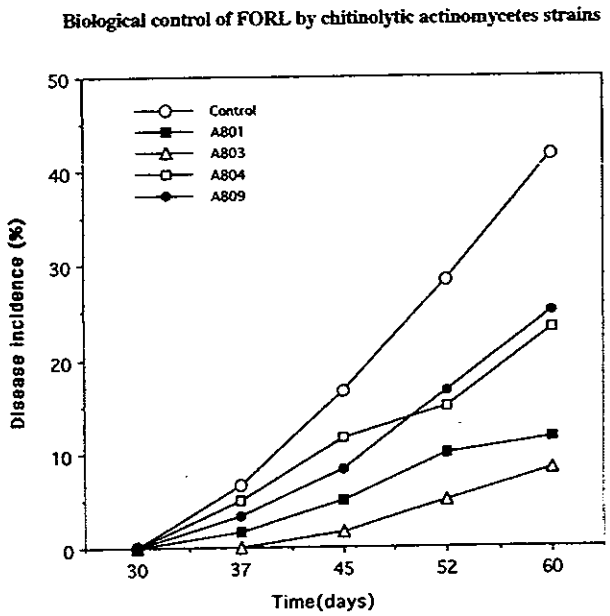

Fig. 8. Biological control of FORL by selected chitolytic actinomycetes under greenhouse conditions.

In an attempt to correlate between the production of FORL inhibitory-substance(s) by actinomycetes strains and their ability to serve as biological control agents we introduced these strains to a soil infested with FORL and planted with tomatoes. Data in Fig. 9 show the biological control of FORL 2 actinomycetes strains that were previously shown to inhibit growth of FORL in cultures. Both antagonists reduced the rate of disease incidence throughout the experiment (60 days) and significantly reduced the number of dead plants at the end of the experiment (Fig. 9).

Biological control of FORL by antibiotic producing strains of actinomycetes

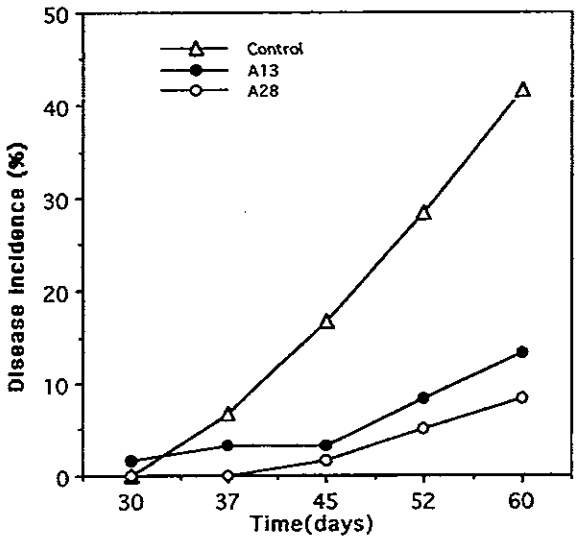

Fig. 9. Biological control under greenhouse conditions of FORL by selected antibiotic-producing actinomycetes strains.

C) Scientific Impact of Collaboration

During the fourth year of the research the scientific efforts of both research groups focused on several research tasks:

1) Optimisation of biomass and antibiotic production of the antagonist *Streptomyces roseoflavus,*
2) Induction of synthesis of defense compounds (phytoalexins) in tomato by microbial metabolites
3) Analysis of induction and activity of chitinase of selected bacterial and actinomycetes strains
4) Biological control of FORL by chitinolytic and antibiotic producing strains.

The major impact of the research in the fourth year is the identification of specific potential antagonists of FORL that show antagonistic properties both in the lab and under natural soil conditions. During this research period Prof. S. Khojibaeva and Dr. K. Karimova visited the laboratory of Dr. A. Sivan in Beer sheva. This meeting focussed on analysing the data of the third 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) and induction of self defence mechanisms produced in tomatoes against FORL.

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 fourth year of research (as well as since the beginning of the project) the Uzbekistan team has gained access to basic method for studying biological control of soil borne pathogens and particularly methods involving techniques for FORL. The Uzbekistan team has purchased some basic equipment, which include a refrigerator, an incubator and a shaker for cultivation of microorganisms.

A trainee (Sudabe Mamedova) from the Uzbekistan Institute worked in the laboratory of Dr. A. Sivan for one year (June 2001-June 2002).

F) Future Work

The project has been extended until December 31 2003 (additional funds were not requested). We are planning to utilize this extension to characterize our most effective

antagonists of FORL. This will include testing methods of application of selected antagonists under greenhouse conditions in order to improve biocontrol efficacy.

We will also study the survival and biocontrol efficacy of our selected antagonists at various levels of soil salinity.

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 Uzbekistanian P.I. to the Israeli laboratory in September 2002..

Training: June, 2001 –June 2002 – Sudabe Mamedova from the Uzbekistanian institute trained for one year in the laboratory of the Israeli P.I.

No publications have yet come out from the project.

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

No particular request.