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Biological Control of Fusarium Wilt and Crown Rot of Tomato

Principal Investigator: Dr. Alex Sivan, The Institute for Applied Biosciences
Grantee Institution: Ben Gurion University of The Negev, Beer Sheva, Israel

Collaborator: Dr. Sanobar Khodijibaeva, Institute of Microbiology,
Academy of Science of Uzbekistan, Tashkent, Uzbekistan

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ES&T Counselor, American Embassy, Tel-Aviv, Israel

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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) 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.Growth and development of FORL in saline soils

The following soil samples were used in the researches:

Djizak region:

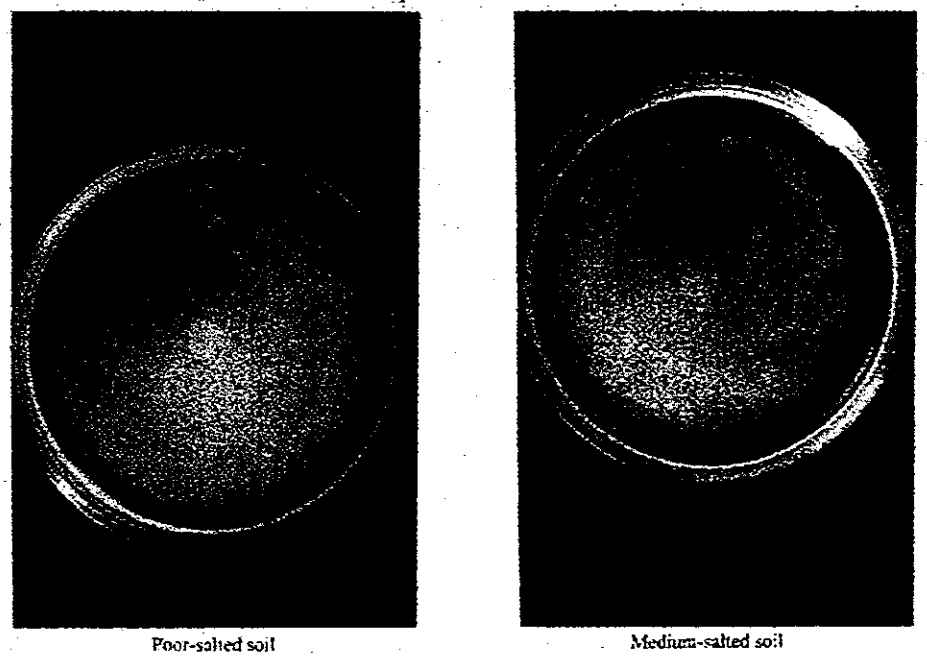
Soil Depth (cm)	Cl^-	SO_4^{2-}
30 – 50	0,010	0,065
0 – 30	0,049	0,661

Syr-Darya region:

Soil Depth (cm)	Cl^-	SO_4^{2}
30 – 50	0,014	0,987
0 – 30	0,371	1,251
0 – 22	0,271	1,684

In low and medium salted soils the growth of FORL was not inhibited during 30 days of growth, spore germination was observed 15 – 18 hours after inoculation and the colonies developed aerial mycelium., The results confirm high resistance of FORL to high salinity (Fig. 1).

Growth and development of FORL in saline soils Photo 1



Poor-salted soil Medium-salted soil

Fig. 1. Growth of FORL on low and high salinity

Therefore, the possibility of FORL infection of plants cultivated in salted soils can not be excluded during tomato.

Growth and development of fungal antagonists to FORL in saline soils

The growth and development of two of the most active and saline resistant fungal antagonists of FORL: *Stachybotrys lobulata* and *Stachbotrys chartarum* were examined. The above mentioned cultures revealed their high resistance to high NaCl concentration. Spore germination on high and low saline media was observed on the first day after inoculation without any reduction in the activity against FORL.

Growth and development of antagonistic actinomycetes to FORL in saline soil

The growth and development of two of the most active and saline resistant strains Streptomyces roseoflavus, st. 7 and Streptomyces sp., st. 4/14 were examined. The selected cultures revealed their high resistance to heavy saline concentration (Fig. 2).

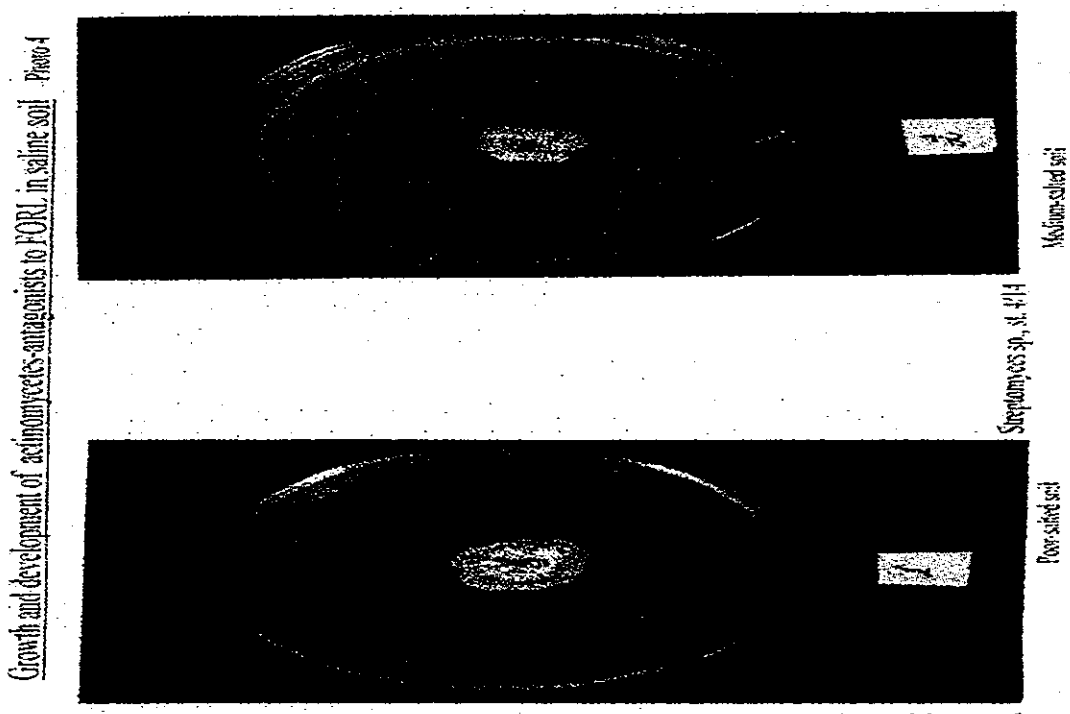


Fig. 2. Growth of an FORL-antagonistic actinomycete on high and low salt concentration.

Spore germination was generally similar on non-salted, poor-salted or medium-salted soils. However, some inhibition in germination of the antagonists was observed on the medium-salted medium. However, this did not affect the antibiotical activity of the actinomycetes against FORL.

2.Antibiotic substances producing by Streptomyces roseoflavus, st. 7 – the antagonist of FORL.

We tried to extract FORL-inhibitory substances from the bacterial culture filtrate by n-butanol. The butanol extract was evaporated, dissolved in chloroform- methanol (1:1) and fractionated on a silicagel column. . Fractions each containing 100 ml were collected, evaporated, dissolved again in chloroform:methanol and separated on TLC. After developing theextracted substances, similar fractions were collected and their antifungal activity was tested. Two of the combined samples were found active and they were combined, and separated again on a silica gel followed by TLC using chloroform : methanol : water (65:30:6) in both systems. Similar fractions were combined and antifungal activity was tested. One of the combined fractions (#16). This fraction was further extracted by acetone and then by ethylacetate.

The antibiotic activity of this extract against FORL was the greatest inducing an inhibition zone of 7 mm (Table 1).

Table 1. Antifungal activity of culture filtrate extracts of *Streptomyces roseoflavus*, st. 7.

Extract	Radial inhibition zone (mm)	Control	Radial inhibition zone (mm)
Cultural filtrate	3,0	Nutrient medium	0
Butanol extract	4,0	n-butanol	0
Fraction 2 (43-49)	5,5	Chloroform – methanol (1:1)	0
Fraction 12 (50-58)	5,5	Chloroform – methanol (1:1)	0
Fraction 13	6,0	Chloroform – methanol-water, (65:30:6)	0
Fraction 16	7,0	Chloroform – methanol (1:1)	0

The potential of active fractions to protect tomato plants against FORL will be further studied.

3. Biological control of FORL under greenhouse conditions

Germination of tomato sees was observed 6 – 8 days after sowing.

Various microbial-antagonists showed growth promoting influence on tomato plants, including an increase of the root system .
Estimation of FORL infection rate was carried out 60 days after sowing. The results in Table 2 show significant reduction in FORL infection rate by several antagonistic strains.

Table 2. The effect of application of actinomycetes and a fungous, under greenhouse conditions, on FORL infection on tomato plants.

Treatment	**Infected plants (%)**
Control	4.0
FORL	70.0
FORL+ *Streptomyces roseoflavus*, st. 7	9.0
FORL+ *Streptomyces* sp., st. 4/14	11.0
FORL+ *Stachybotrys lobulata*	14.0
FORL+ *Stachybotrys chartarum*	17.0

Thus, in some degree actinomycetes-antagonists are more active inhibitors of disease development than the fungus of genus *Stachybotrys*. But tested strains reduced significantly the FORL infection (Table 2).

4. Biological control of FORL under greenhouse conditions

The efficiency of microbal antagonists (actinomycetes and fungi) in protecting tomato from FORL infection was confirmed under open soil conditions. Both their active stimulating influence on the growth and development of tomato plants and reduction in disease incidence was observed. Application of the microbial antagonists resulted in a shorter germination time (6-7 days)

compared with the FORL control (9-10 days). The first plants infected in the control with FORL and in the uninfected control appeared on the 17th and on the 20th day after contamination, respectively, compared with the microbial antagonists treatment where FORL infection was obseved on the 30th day.

Table 3. The effect of application of actinomycetes and a fungous, under field conditions, on FORL infection on tomato plants. Disease incidence was recorded two months after sowing.

Treatment	**Infected plants (%)**
Control	3.5
FORL	44.0
FORL+ *Streptomyces roseoflavus*, st. 7	7.0
FORL+ *Streptomyces* sp., st. 4/14	12.0
FORL+ *Stachybotrys lobulata*	13.0
FORL+ *Stachybotrys chartarum*	15.0

Israeli group

1) Screening for chitinolytic bacteria and actinomycetes

Chitin is a major component of fungal cell wall. Therefore, microorganisms capable of degrading chitin have the potential to serve as effective biocontrol agents. We have conducted a major screening for soil bacteria and actinomycetes capable of utilizing chitin as the sole carbon source in the medium. Strains that showed growth in this medium were further analyzed for the presence and specific activities of their chitinolytic enzymes. Chitin is a polysaccharide composed of N-acetyl-glucoseamine (NAGA). Two major enzyme (i.e. chitinase and chitobiase) are taking part in the enzymatic hydrolysis of chitin. The data in Table 4 show the selected chitinolytic

bacterial isolate sand the specific activity of chitinase and chitobiase. Chitinase was measured using two methods: 1. quantitative analysis of NAGA released from chitin and 2. Degradation of pNp-NAGA (a synthetic substrate which produce a color upon its degradation. This color was measured colorimetrically). Chitobiase activity was measured colorimetrically using the synthetic substrate pNp-chitobiose. Production of the chitinolytic enzymes was carried out by growing the isolates in a synthetic medium containing chitin (2%;w/v) as a sole carbon source. The culture filtrates from various time points during incubation were dialyzed and lyophilyzed to serve as a crude enzymatic preparation.

Table 4. Specific activity of chitinolytic activity of screened bacterial isolates.

1- Specific activity was determined as μmol NAGA/mg protein/hr
2- One unit of specific activity was determined as the amount of protein which increases the O.D. (410nm) by 0.1 per min

Bacterial isolate	Chitinase activity Release of NAGA[1]	Chitinase activity Degradation of pNp-NAGA[2]	Chitobiase activity. Degradation of pNp-chitobiose[2]
800	0	9.06	1.47
807	0	7.28	7.18
812	1.4	7.55	7.73
816	2.29	3.21	8.62
817	0.95	4.72	8.17
819	1.51	7.46	6.50
825	4.58	3.01	9.48
826	4.67	2.19	8.16

Of the above list of bacterial strains showing chitinolytic activity 3 strains were selected for studying the kinetics of the various chitinolytic enzymes (Fig. 3-5).

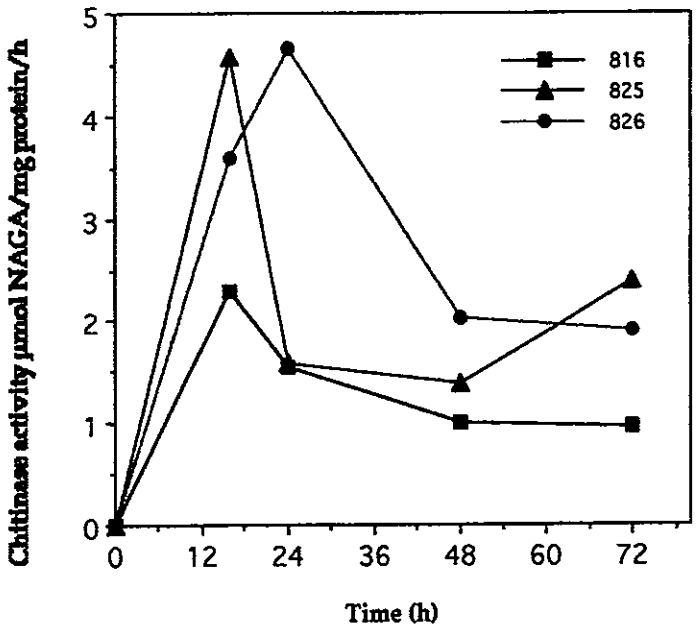


Fig. 3. Specific activity of chitinase produced by bacterial isolates based on release of NAGA from chitin. Specific activity was determined as µmol NAGA/mg protein/hr

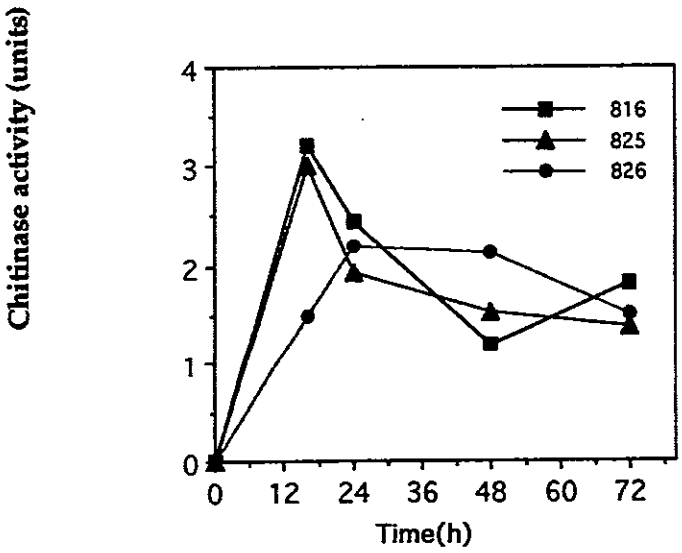


Fig. 4. Specific activity of chitinase produced by bacterial isolates based on degradation of pNp-NAGA. One unit of specific activity was determined as the amount of protein which increases the O.D. (410nm) by 0.1 per min

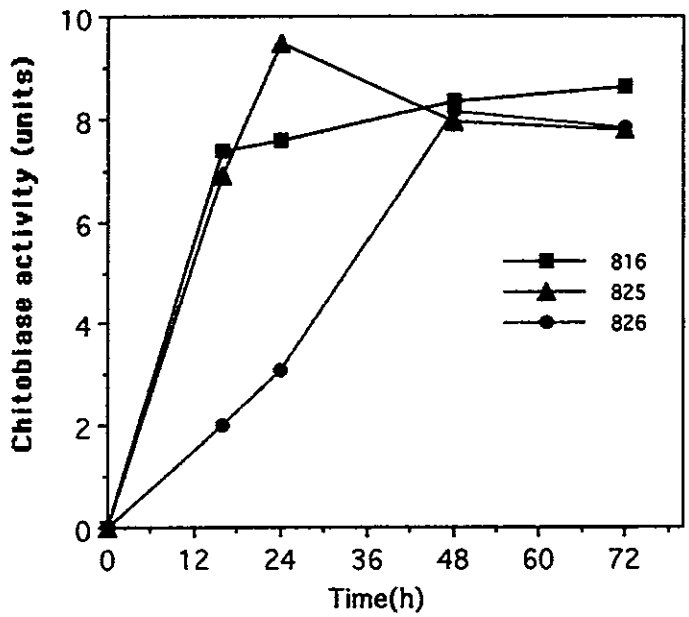


Fig. 5. Specific activity of chitobiase produced by bacterial isolates based on degradation of pNp-chitobiose. One unit of specific activity was determined as the amount of protein which increases the O.D. (410nm) by 0.1 per min

All three bacterial isolates (816, 825 and 826) showed high activity of chitinase and chitobiase. Maximal activity for both enzymes was observed 16 –24 hr after inoculation. A positive correlation was found between the two methods used for the estimation of chitinase.

Table 5. Specific activity of chitinolytic activity of screened actinomycetes isolates.
1- Specific activity was determined as μmol NAGA/mg protein/hr
2- One unit of specific activity was determined as the amount of protein which increases the O.D. (410nm) by 0.1 per min

Actinomycetes isolates	Chitinase activity- Release of NAGA[1]	Chitinase activity Degradation of pNp-NAGA[2]	Chitobiase activity. Degradation of pNp-chitobiose[2]
A801	24.44	2.32	12.12
A803	9.35	30.89	15.40
A804	25.24	30.06	14.69
A805	8.04	32.45	11.88
A806	7.49	35.48	11.64
A808	8.80	23.50	15.90
A809	7.60	39.63	21.50
A810	12.63	46.64	1.34
A811	9.33	36.74	15.00
A814	0	43.14	0
A815	0	42.66	0
A818	12.40	30.31	12.53
A820	0	29.02	3.53
A821	10.35	46.23	4.88
A822	6.98	12.46	4.05
A823	3.76	39.57	5.76
A824	2.62	11.29	3.10

In general, the group of selected actinomycetes showed higher chitinolytic activity compared with that of bacteria. This probably results from higher production of chitinase and chitobiase compared with the bacterial isolates. Four of the best actinomycetes listed above were selected for studying the production of chitinolytic enzymes as mentioned for the bacteria.

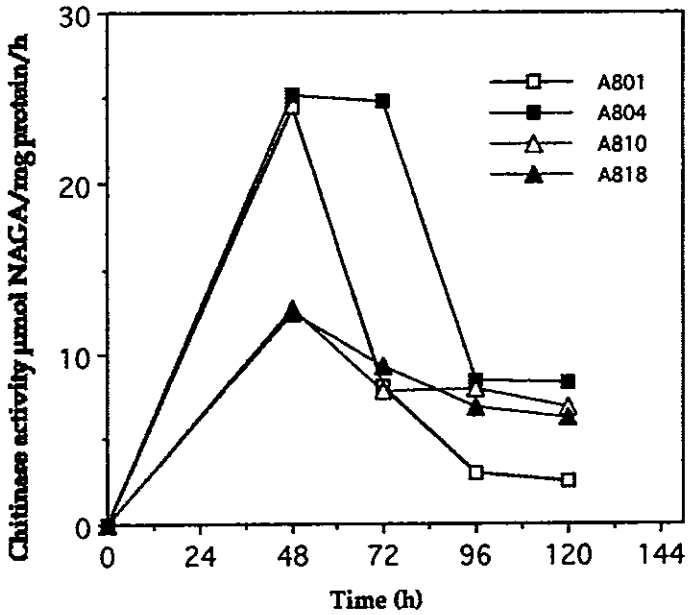


Fig. 6. Specific activity of chitinase produced by bacterial isolates based on release of NAGA from chitin. Specific activity was determined as µmol NAGA/mg protein/hr

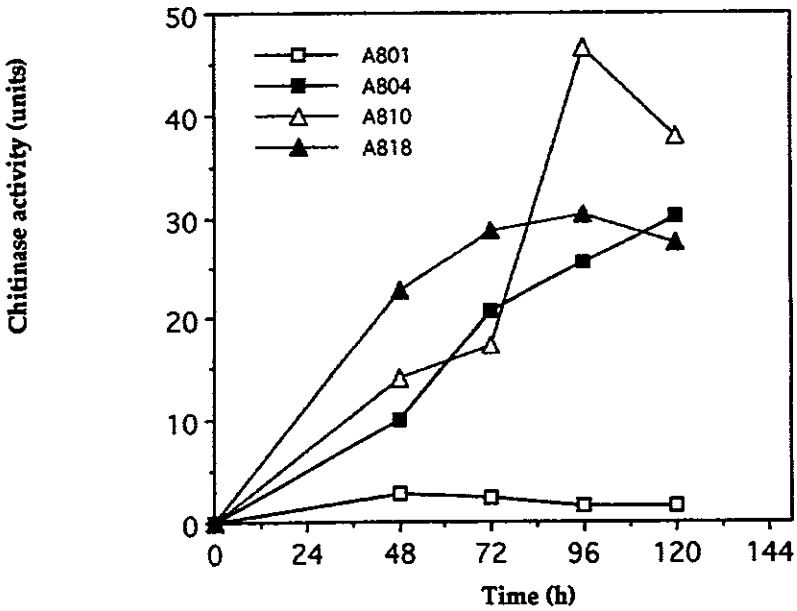


Fig. 7. Specific activity of chitinase produced by bacterial isolates based on degradation of pNp-NAGA. One unit of specific activity was determined as the amount of protein which increases the O.D. (410nm) by 0.1 per min

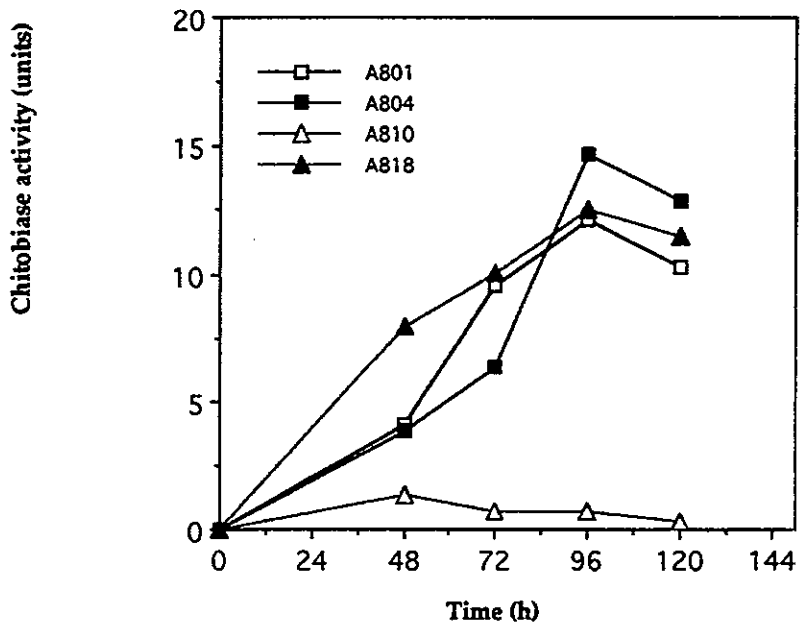


Fig. 8. Specific activity of chitobiase produced by bacterial isolates based on degradation of pNp-chitobiose. One unit of specific activity was determined as the amount of protein which increases the O.D. (410nm) by 0.1 per min

The data in figures 6-8 clearly show the higher chitinolytic activity of the selected actinomyces strains compared with that of the bacteria. For example, the chitinase level in actinomycetes strains A801, A804 and A810 (Fig. 6) was more than 5 folds greater than that of the bestbacterial isolates (825 and 826) (Fig. 3). However, the chitinolytic activity of the actinomycetes reached its highest level only after 48 96 hr of incubation compared with 16-24 hr in the bacteria.

We plan also to test the potential of these chitinolytic strains to utilize and degrade cell wall of FORL. For this purpose we have produced large amounts of cell walls that are free of cellular sugars and proteins. These experiments will be done shortly.

C) Scientific Impact of Collaboration

During the third year of the research the scientific efforts of both research groups were focused on characterization and separation of antibiotic metabolites active against 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 third year is the identification of specific potential antagonists and the preliminary characterization of their mode of action (lytic enzymes and antibiotics). During this research period Prof. S. Khojibaeva and Dr. Kh. Karimova visited the laboratory of Dr. A. Sivan in Beer sheva. This meeting focussed on study of the basic features of FORL in culture and on diseased plants and analyzing 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).

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

During June 2001 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. 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 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, 2001 –June 2002 – Sudabe Mamedova from the Uzbekistanian institute trained for one year in the laboratory of Dr. A. Sivan.

A visit of the Uzbekistanian collaborator is scheduled for mid September 2002.

No publications have yet came out from the project.

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

No particular request.