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The use of symbiotic biodiversity to enhance plant tolerance to environmental stresses

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Executive Summary

The overall objective of this project is to increase crop production and adaptation of shrubs and trees to ecosystems in areas affected by salinity, drought and low soil pH, through the isolation, selection, culture and propagation of stress tolerant soil microorganisms in association with plant roots.

A collection of 219 strains of nitrogen-fixing bacteria isolated from leguminous plants of the Kyzil-Kum desert, Uzbekistan, was created. All isolates were tested for the presence of nitrogen fixation (*nif*) genes, essential for nitrogen fixation, and nodulation (*nod*) genes, required to establish the symbiotic association. These tests allow distinguishing nodule bacteria from rhizosphere bacteria that are frequently obtained during nodule isolation. All isolates were very tolerant to salt, 106 grew at a concentration of 1 M NaCl or higher, and some were able to grow in the presence of up to 2.25 M NaCl, although growth rate at this concentration was significantly inhibited. Finally, 15 strains that gave a positive response for both *nif* and *nod* genes and showed high tolerance to salinity were selected for further study.

Seeds of the plant species from which the nodules were obtained had a deep dormancy that had to be broken by different means. All these plants are now growing and will be used in further studies on their stress tolerance mechanisms, and for reinfection by the selected salt tolerant strains of nitrogen-fixers.

The effects of *Azorhizobium caulinodans* (nodule bacterium) and *Glomus mosseae* (mycorrhiza) on growth and development of wheat were studied in pot and field experiments. Inoculation with the bacterium and with the mycorrhiza brought about increases in yield of different varieties of wheat, and the highest yields were observed when both inocula were applied.

All work carried out during this first year was in compliance with the objectives of the project. Collaboration between the two groups has been close and fruitful. The Israeli investigators took part in a collecting expedition to the Kyzil-Kum desert, in June 1999. The Uzbek PI's have been working in the Israeli laboratory since April 2000.

Section I

A) Research Objectives

Overall aim and specific objectives

Overall aim: To increase crop production and adaptation of shrubs and trees to ecosystems in areas affected by salinity, drought and low soil pH, through the isolation, selection, culture and propagation of stress tolerant soil microorganisms in association with plant roots.

Specific objectives:

(1) To select and propagate stress resistant strains of nodule bacteria and mycorrhiza to enhance crop production in areas affected by salinity and drought.

(2) To study the mechanisms by which symbiotic microorganisms enhance mineral nutrition and growth of plants in nutrient poor soils and in soils affected by salinity and drought.

(3) To study the effect of symbiotic microorganisms on the adaptation processes of plants to salinity and drought.

(4) To test under pot and field conditions the possibility of creating new microorganism/plant associations to increase stress tolerance of the symbiotic associations.

B) Research Accomplishments

Glutamine synthetase activity of Ammodendron conollyi, Astragalus villossimus and Astragalus unifoliolatus and their corresponding nodule bacteria as affected by salinity stress.

Glutamine synthetase is known to be a key-enzyme on the synthesis of key nitrogenous metabolites. GS is subjected to strict regulation both at the transcription and translation level as well as to post translational regulation. Ammonium plays an important role in regulation of the synthesis and activity of GS. Ammonium in the plant root is a product of nitrogenase reduction of atmospheric N_2 or from direct uptake from the soil.

Plants grown in medium with 100 mM NaCl changed enzyme activity of N-assimilation enzymes in their shoots and roots. GS activity in controls (no salinity) was higher then in the stress (+ NaCl) treatments (Table 1). GS activity in the presence of 100 mM NaCl decreased between 16% to 26% as compared with control plants. GS activity of leaves was three times higher then in roots. The same glutamine synthetase activity was observed in plants grown in salinity with comparative high GS-activity in shoots of Astragalus villossimus (1,3mmol glutamine/ mg protein / min). Astragalus unifoliolatus (1,47 mmol product / mg protein / min). On the whole, salinization inhibited GS-activity.

Study of GS – activity in nodule bacteria: Ammodendron conollyi , Astragalus villossimus and Astragalus unifoliolatus under stress conditions showed slight differences in GS activity in all treatments (Table 2).

Table 1: Glutamine synthetase activity of *Ammodendron conollyi, Astragalus villossimus,* and *Astragalus unifoliolatus*

Plants	Specific activity mmol product · mg^{-1} protein min^{-1}		
	Control	100 mM NaCl	% inhibition
Ammodendron conollyi , shoot	0.82	0.69	16
Ammodendron conollyi , root	0.24	0.19	21
Astragalus villossimus , shoot	1.30	0.96	26
Astragalus villossismus , root	0.30	0.25	17
Astragalus unifoliolatus, shoot	1.47	1.09	26
Astragalus unifoliolatus, root	0.40	0.30	25

Note: GS product was g-glutamilhydraxamic acid formed in the course of the reaction.

The green algae Ankistrodesmus braunii grown for 7 hours in medium without nitrogen, was able to produce glutamine synthetase activity 4 fold higher then the corresponding GS activity in algae growing in N containing medium (11) . When 1M NaCl was added to the medium containing nodule bacteria during 24 hours, the activity of glutamine synthetase was inhibited on the average by less than 8 % . Stress conditions did not essentially affect the activity of nodule bacteria. This observation confirms the idea that nodule bacteria of desert plants have a high stress tolerance facilitating plant survival under very harsh desert conditions.

Table 2: Total activity of glutamine synthetase in nodule bacteria

Nodule bacteria	Specific activity: mmol GH mg^{-1} protein min^{-1}		
	+ N (% inhib)	- N	+ N 1 M NaCl
V1	2.37	2.51	2.18
V3	2.43	2.71	2.26
V6[1]	1.98	2.17	1.81
V8[1]	2.70	2.81	2.50
V9	2.54	2.85	2.45
• V9[1]	2.59	2.93	2.37
V30	2.19	2.39	2.10
C8[1]	2.21	2.39	2.00
C11	2.28	2.59	2.37
• C15	1.87	2.09	1.90
C21	2.20	2.32	2.10
• U7	2.14	2.27	2.07
U17[1]	1.66	1.89	1.49
• U30[1]	1.60	1.73	1.51
• U30[2]	1.80	1.87	1.66
CXM1	2.90	3.60	2.62

Study of multiple forms (isoforms) of glutamine synthetase of Ammodendron conollyi, Astragalus villossimus , Astragalus unifoliolatus and their nodule bacteria .

a) Glutamine synthetase of plants.

Different isoforms of glutamine synthetase have been found in a large number of plants and bacteria species. Two forms of glutamine synthetases were discovered in pea leaves (GS_1 and GS_2) . One of the isoforms was located in chloroplasts and the second in the cytosol (12). Later, similar observations were done in pumpkin (13). A study of glutamine synthetase isoforms in

Bradyrhizobium sp cells (lupine) disclosed that N_2 efficient fixing cells of nodule bacteria contain at least 3 isoforms , while inefficient cells have only 2 isoforms (5). Glutamine synthetases with similar catalytic activity frequently differ in amino acid composition, molecular weight and a variety of other properties (specific activity , optimum pH , thermostability etc.). This differences are linked to enzyme location (e.g. cytosol , chloroplast etc.) . Taking this into account, we decided to study the multiple molecular forms (isoforms) of glutamine synthetases in plant and nodule bacteria.

PAGE was used for detection of GS isoforms. Shoot extracts of Ammodendron conollyi and Astragalus unifoliolatus contained two isoforms of glutamine synthetase with different activities and electrophoretic mobility. Only one form of glutamine synthetase was detected in shoots of Astragalus villossimus and in the roots each of the species studied (Fig. 1). The isoform with smaller electrophoretic mobility was designated GS_1, and that with the greater mobility as GS_2.. Practically all glutamine synthetases detected in the zymograms of plant shoots had a high level of electrophoretic mobility and activity, while roots GS displayed low activity.

DEAE – Toypearl 650 M ion-exchanging chromatography was used to confirm the presence of multiple forms of glutamine synthetases in plant shoots. Enzymes elution was conducted with a linear gradient of NaCl (0.0 - 0.3 M NaCl). Two active peaks of glutamine synthetase (GS_1 and GS_2) were found in the course of chromatography(Fig 3). The activity of GS_2 was several times higher than that of GS_1. The first peak eluted with 0,06 M NaCl and the second form at 0,18 M NaCl, respectively. In overground part of Astragalus villossimus very low its activity of GS_1 was observed, which wasnt evident in PAGE zymograms.

Glutamine synthetase of nodule bacteria.

Strains were cultivated for three days in medium containing leguminous broth as a single nitrogen source, in order to study the GS isoforms of nodule bacteria. All the bacteria strains studied had only one form of glutamine synthetase. Only the strain $U17^1$ produced 3 forms of glutamine synthetase (Fig 2.) under the growth conditions used, but in further investigations growing the strains under stress conditions all displayed only one GS form. All GS isoforms had the same electrophoretic mobility. More detailed study of GS isoforms using DEAE confirmed the results obtained with PAGE. Only one peak of glutamine synthetase appeared after DEAE (Fig. 4)

Proteins with GS activity eluted from the column at high concentration of NaCl (0,345 M NaCl). For example : maximum ionic power recorded for the elution of GS of Rhizobium meliloti (CXM1) was 0,24 M NaCl . Strains $U17^1$ and CXM1 had 3 forms of glutamine synthetase. Apparently only two forms of glutamine synthetase (GS_1 and GS_2) are present in shoots of Ammodendron conollyi, Astragalus villossimus and Astragalus unifoliolatus . At the same time the plant roots exhibited only one form of GS that corresponded to shoot GS_1. Finally , all nodule bacteria (except $U17^1$) had only one form of glutamine synthetase.

Physical and chemical properties of glutamine synthetases isolated from legume plants and their nodule bacteria.

A comparative study of physical and chemical properties was carried out between GS_1 and GS_2 of Ammodendron conollyi leaves, leves and roots of Astragalus villossimus and GS of the bacterial strains V1, V3, C81, U7. Maximum activity of Ammodendron conollyi GS_1 was observed at pH 6,2 , while the maximum GS_2 activity of Ammodendron conollyi and Astragalus villossimus (root and shoot) was at 6,4 . (Fig. 5a). Optimum pH of GS isolated from the strains V1 , V3 , $C8^1$ was found to be 6,8 and from $U7^1$ it was 7,0 (Fig. 6.a).

Temperature optimum for GS isolated from plants was 40-43^{0}C (Fig. 5b) while optimal temperature for GS of nodule bacteria was within the range of 40-50^{0}C (Fig. 6b). Plants GS treated at temperatures between 25-50^{0}C, while nodule bacteria GS was treated between 30-80^{0}C. For this purpose different extracts under investigation were subjected to temperature treatmentment for 5

minutes, after which the extracts were cooled down to 4^0C followed by determination of residual GS activity.

Ammodendron conollyi GS_2 and GS of Astragalus villossimus shoots lose about 30 - 40% of their activity after heating at 40^0C. While Ammodendron conollyi GS_1 and GS of Astragalus villossimus root lose up 70% enzyme activity under the same conditions (Fig. 5c). In this respect, GS isolated from nodule bacteria is the most stable. Insignificant activity was lost during heating of bacterial GS enzyme activity at 40^0 and further heating up to 65^0 did not cause enzyme inactivation. On the contrary, GS enzymes of the V1 , V3 , $C8^1$ strains were activated up to 7% in relation to their initial activity (Fig. 6c).

The same picture resulted when plant GS was heated at 37^0C and respectively when GS from nodule bacteria was heated at 50^0C, depending on time. After 50 minutes plant GS loses completely its activity (Fig. 5d) nodule bacteria GS diromg 1.5 h of heat treatment was found to be practically unchanged (Fig. 6d). On the other hand, GS activities of strains V1 , V3 and V8 considerably increased in relation to control treatments.

Glutamine synthetases isolated from plants and nodule bacteria exhibit a considerable difference in physical and chemical properties.

2. Nitrogen fixation of free-living state cultures of nodule bacteria of Ammodendron conollyi, Astragalus villossimus and Astragalus unifoliolatus

At the present time there is a little information about nitrogen fixation of free-living state cultures of nodule bacteria. There is some information pointing that nodule bacteria of Azorhizobium caulinodans are able to fix molecular nitrogen both out of the plant (14) and in symbiosis. There is no evidence about the ability of other nodule bacteria to fix molecular nitrogen out of the symbiotic state. It may be assumed that symbiosis is the result of prolonged reciprocal influence between plants and bacteria, the conditions and growing medium to determine nitrogen fixation of nodule bacteria in the absence of an host plant have not been found yet. Study of nitrogen-fixing activity of the selected bacteria strains in nutrient media, not containing organic or inorganic nitrogen sources except N_2 (nitrogen-free media) was attempted. The cultures of nodule bacteria were grown on semi-liquid medium with the following compositions (g/L):

General medium: Malate –0.5 ; K_2HPO_4-1.74; KH_2PO_4-9.1; NaCl-0.5; $MgSO_4$x $7H_2O$-0.3; $CaCl_2$ x $6H_2O$-0.1; $FeCl_3$ x $6H_2O$-0.01; agar-agar-4.0; microelements – 1ml/L; pH-7.0.

Microelements (%): H_2BO_3-0.5; Na_2MoO_4 x 2 H_2O-0.5; $MnSO_4$ x H_2O-0.3; KJ-0.05; NaBr- 0.05; $ZnSO_4$ x $7H_2O$-0.02; $Al_2(SO_4)$ x $12H_2O$-0.03.

The nodule bacteria were grown in semi-liquid medium consisting of 5 ml of sterile semi-liquid medium was placed in 10 ml flasks followed by 0.5ml of bacterial culture (in liquid state at a density of 10^8 cells ml^{-1}) and kept at 30^0C. The flasks were hermetically closed with rubber caps and as much as possible air was taken out from these flasks with syringes. After 4 days of cultivation the growth of the cultures acetylene (C_2H_2) was introduced to a final concentration of 10% volume. After 24 hours incubation, acetylene reductase activity was determined using gas-chromatography. All the strains of nodule bacteria grown were able to fix acetylene to some extent (Table 3).

Table 3: Acetylene-reductase activity of nodule bacteria of Ammodendron conollyi, Astragalus villossimus and Astragalus unifoliolatus in free-living state, grown in a N-free medium

Bacteria	Acetylenereduction activity nmol C_2H_4 flask^{-1} hour^{-1}
V1	0.39
V3	0.31
V6[1]	trace
V8[1]	0.52
V9	0.47
• V9[1]	0.39
V30	trace
C8[1]	0.34
C11	0.21
• C15	trace
C21	0.18
• U7	trace
U17[1]	0.34
• U30[1]	0.16
• U30[2]	trace
Control *	140.40

*Azospirillum brasilense sp 7. ATCC 29145

The activity of the examined nodule bacteria was too low as compared to that of Azospirillum brasilense sp 7. ATCC 29145. It was impossible to evaluate the acetylene reductase activity in some cultures (V6 , V30 , C15, U7 [1], U30[2]) due to the extremely low activity of nitrogenase . One may assume that nodule bacteria in the process of evolution acquired ability to fix molecular nitrogen , being in free state and it was the original adaptation for bacteria when in soil is not enough nitrogen for bacteria existence in severe desert conditions .

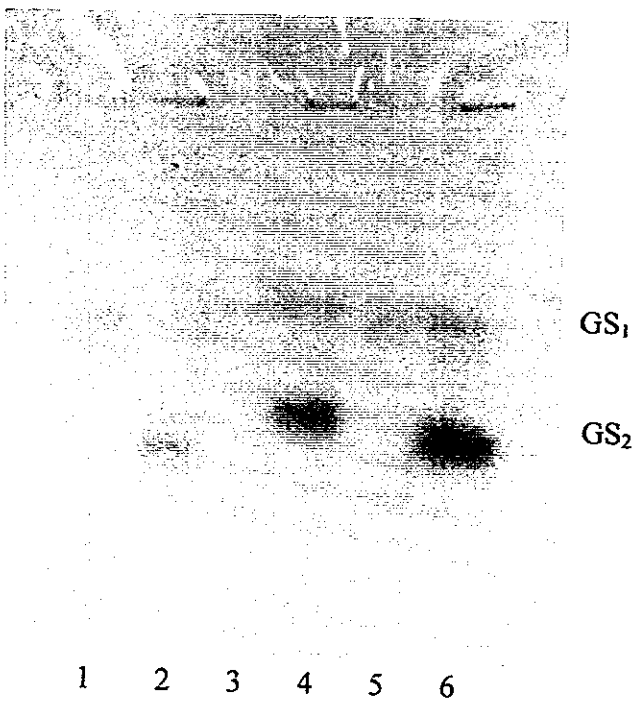

Fig. 1 Zymogram of plants enzyme extracts:
1 – GS root of Astragalus villossimus
2 – GS shoot of Astragalus villossimus
3 – GS root of Astragalus unifoliolatus
4 – GS shoot of Astragalus unifoliolatus
5 – GS root of Ammodendron conollyi
6 – GS shoot of Ammodendron conollyi

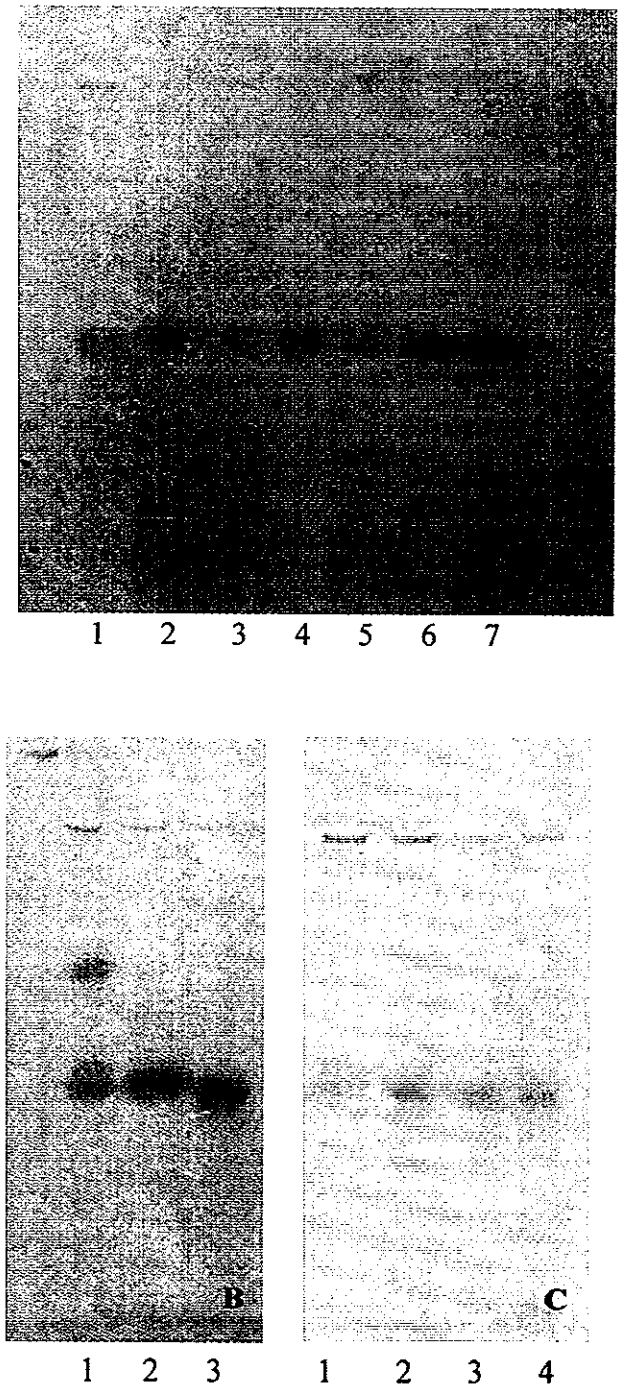

Fig. 2 Zymograms of nodule bacteria enzyme extracts:
A – Nodule bacteria of Astragalus villossimus :
1 – V30 ; 2 – •V9[1] ; 3 – V9 ; 4 – V8[1] ; 5 – V6[1] ; 6 – V3 ; 7 – V1
B – Nodule bacteria of Astragalus unifoliolatus :
1 – U17[1] ; 2 – •U7 ; 3 – •U30[1]
C – Nodule bacteria of Ammodendron conollyi
1 – •C15 ; 2 – C8[1] ; 3 – C11 ; 4 – C21

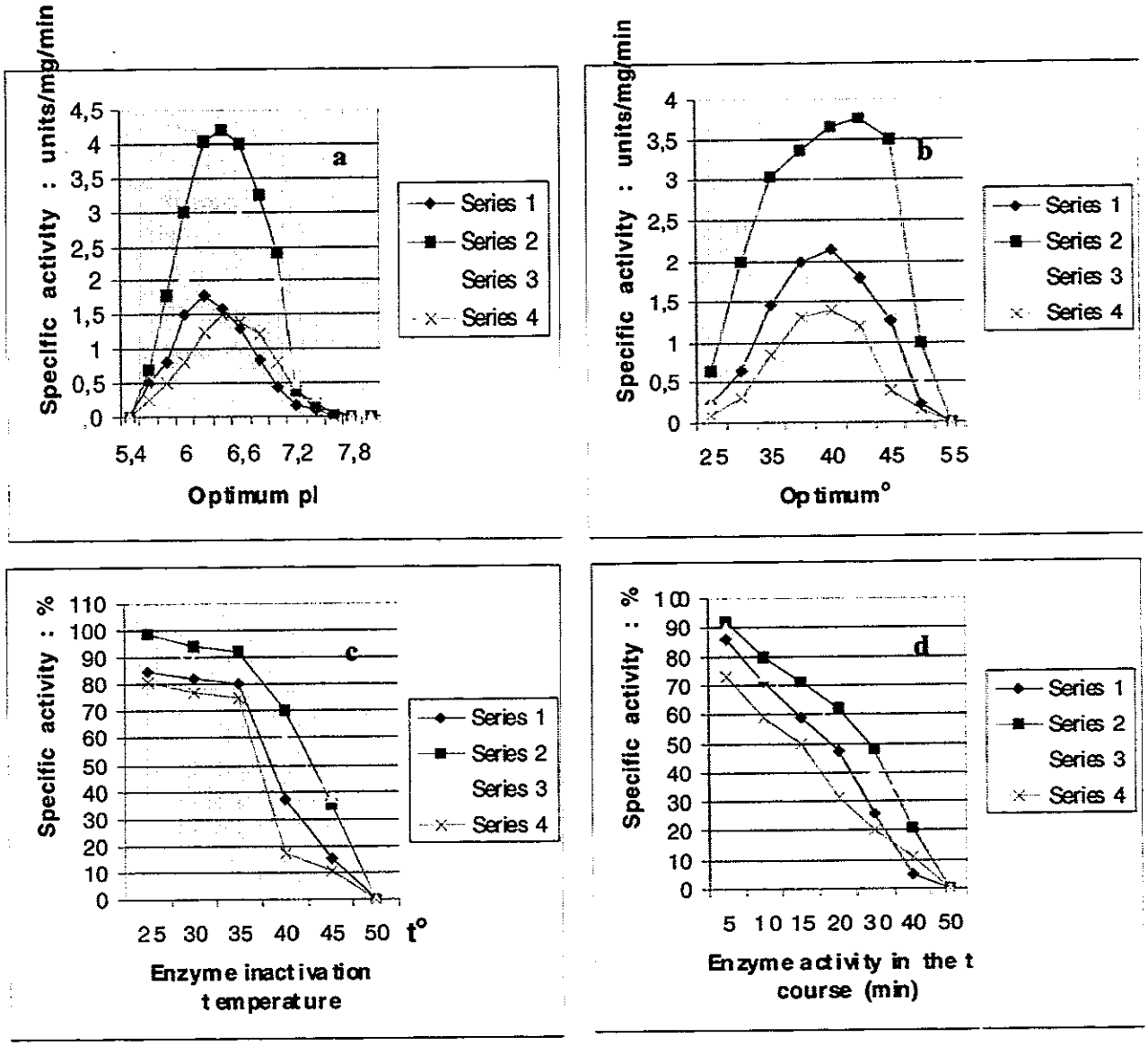

Fig . 3 Physical and chemical properties of plants' glutamine synthetase:
Series 1 – GS_1 Ammodendron conollyi
Series 2 – GS_2 Ammodendron conollyi
Series 3 – GS shoot of Astragalus villossimus
Series 4 – GS root of Astragalus villossimus
Specific activity: the amount of γ-glutamilhydraxamic acid formed in the course of reaction (units/mg/min) .

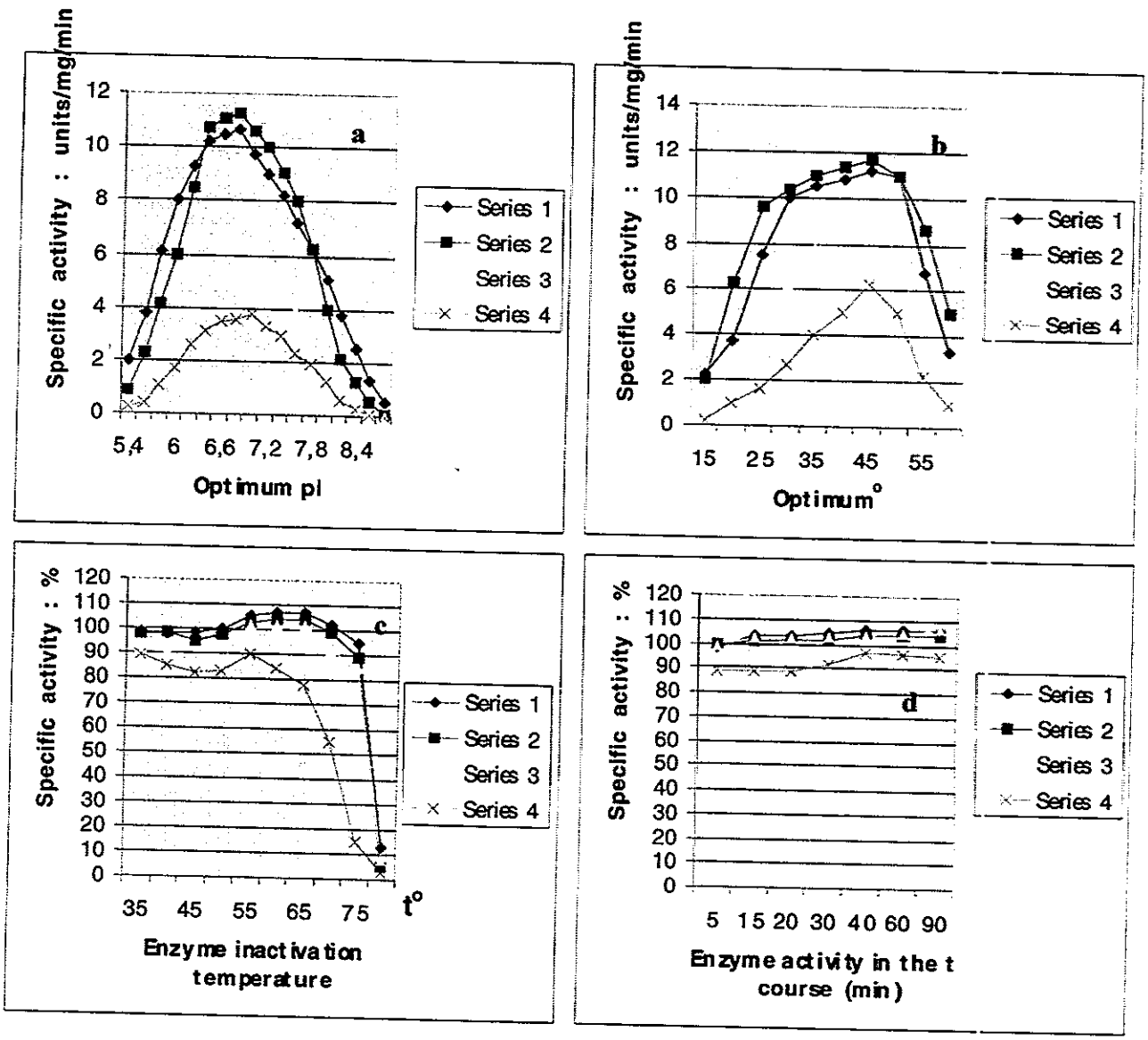

Fig . 4 Physical and chemical properties of nodule bacteria glutamine synthetase:
Series 1 – V1 - Astragalus villossimus
Series 2 – V3 - Astragalus villossimus
Series 3 – C8[1] - Ammodendron conollyi
Series 4 – •U7 – Astragalus unifoliolatus
Specific activity: the amount of γ-glutamilhydraxamic acid formed in the course of reaction (units/mg/min) .

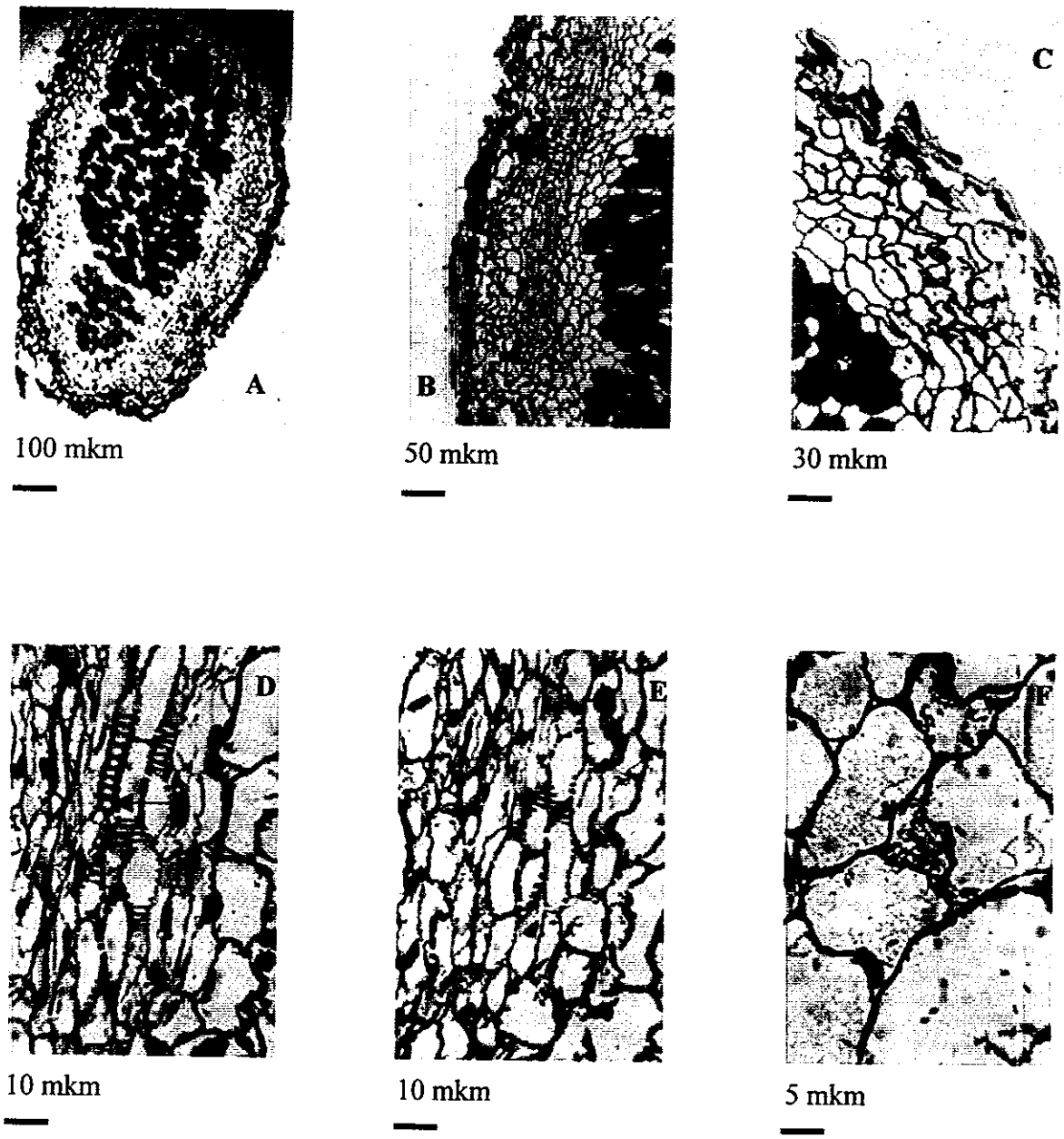

Fig. 5 Light microscopy of Astragalus villossimus nodule sections of plant inoculated with its original Astragalus villossimus nodule bacterium strain V1:

A - Longitudinal section of nodule (magnification x 148).

B – Higher magnification of section (x 315). Visible central nodule plant cells, densely colonized by bacterial cells, and also outer plant cortex nodule cells and the zone of formation and development of infection thread centers.

C – Magnification (x 530) of the same section showing outer bacterial net in the plant cover.

D and E – Higher magnification (x 1974) of the infection thread showing "stair-like" intracellular infection threads (pointed out by arrow) and some elements of intercellular infection threads (globular dark red structures).

F – The highest magnification (x 2835); visible development and expantion of thick infection threads along plant nodule membranes and across nodule cells together with released cells of nodule bacteria.

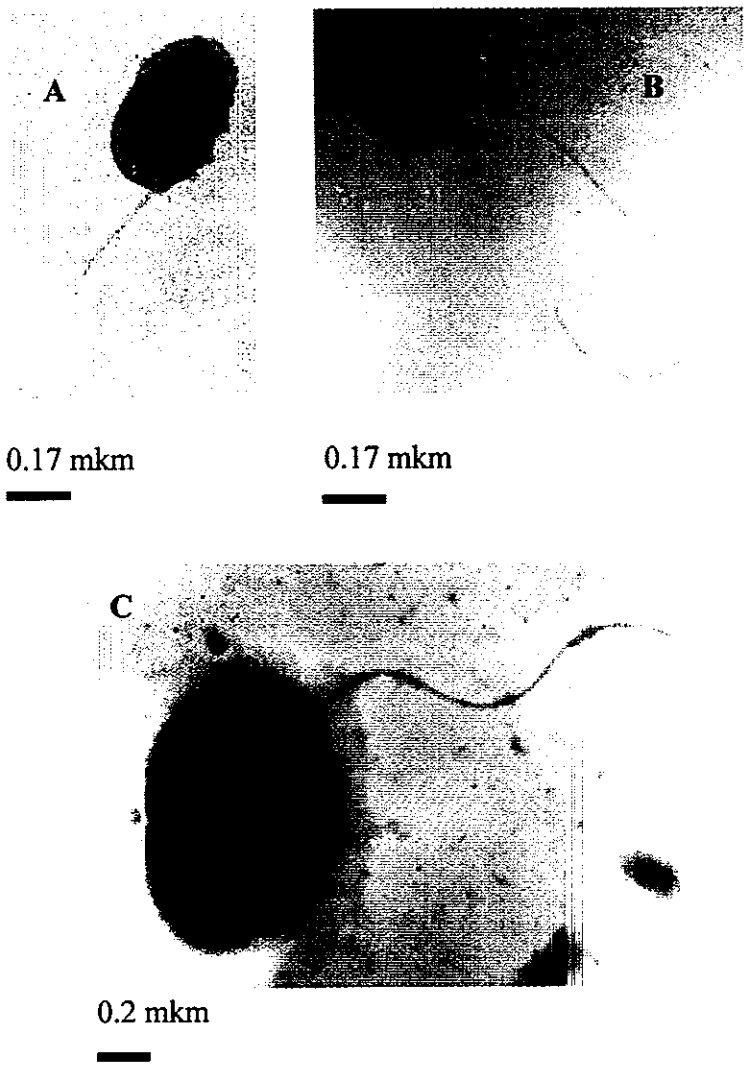

Fig. 6 Desert nodule bacteria in free-living state:
A – Astragalus villossimus`s nodule bacterium strain V1 (magnification is x 5,000);
B – Ammodendron conollyi`s nodule bacterium strain C8[1] (magnification is x 5,000);
C – Astragalus unifoliolatus`s nodule bacterium • strain U7 (magnification is x 6,000).

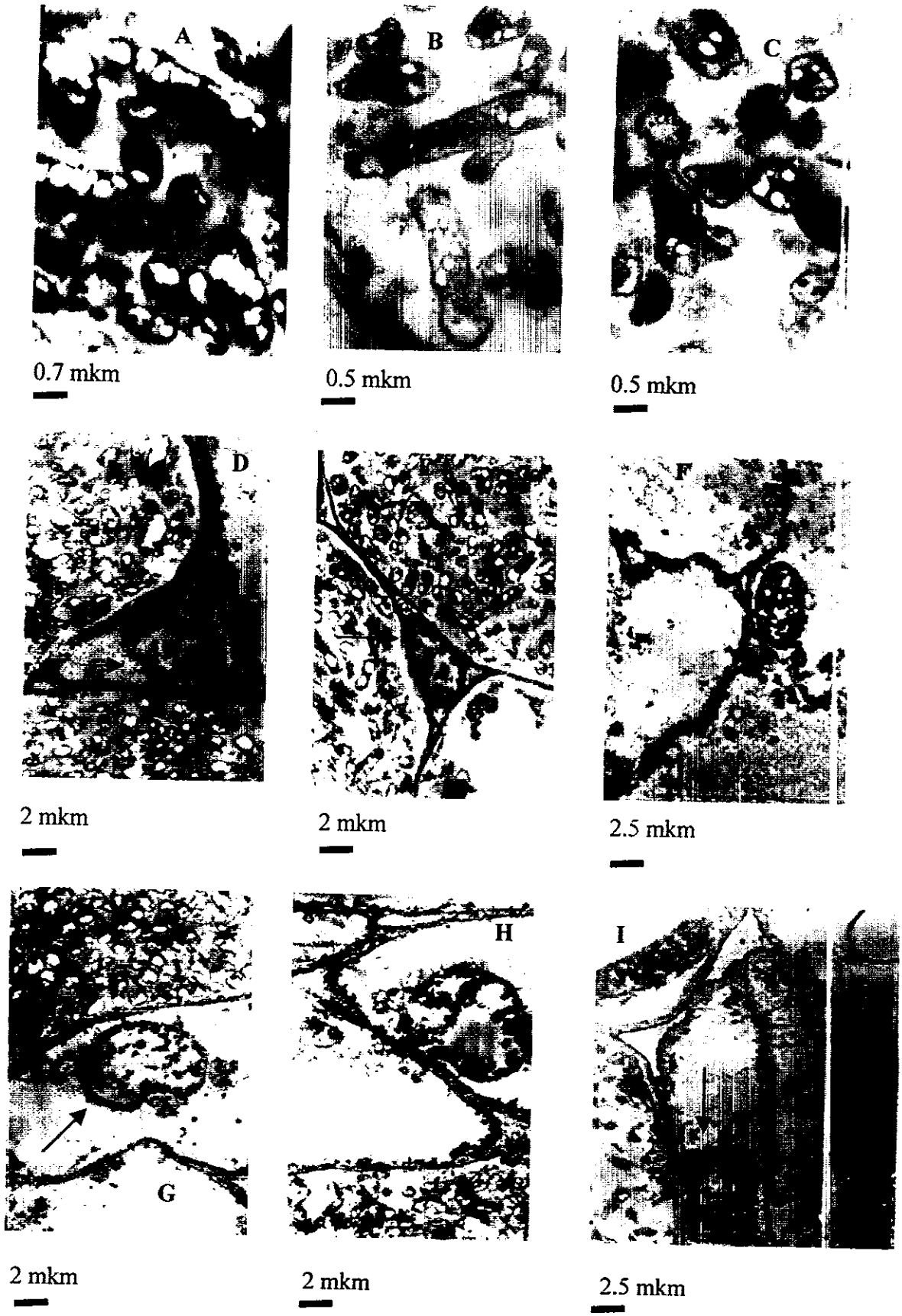

Fig. 7 Electron microscopy of nodule sections of desert leguminous plants: A (V1), B (C8[1]) and C (•U7) – polymorphism of bacteroids. D (V1) and E (C8[1]) – intercellular infection threads. F-I – intracellular infection threads. Localization of infection threads is indicated by arrows.

Biotechnology for the improved adaptation of leguminous trees to stress conditions

One of the overall aim of this project is to increase our capacity to enhance stress tolerance of leguminous species, by means of selection and propagation of stress tolerant microorganisms isolated from ecosystems affected by salinity and draught and capable of forming N_2-fixing associations with native and foreign leguminous plants to improve their biomass production in arid zones. The objectives of the Israeli and Kazak team during this last year of the project included: (a) the identification of stress tolerant nitrogen fixing symbiotic associations and (b) the analyses of those associations showing the highest tolerance, with regards to the efficiency of nitrogen fixation and ammonium provision to the plant.

The 15 bacterial strains tested, initially isolated by the Uzbek investigators, were described in previous reports and had been obtained from nodules removed from the roots of the following woody leguminous plants from the Kyzil-Kum desert of Uzbekistan: *Ammodendron connollyi*, *Astragalus vilosissimus,* and *Astragalus unifoliatus*. All plant seeds were collected from plants growing in the wild in Uzbekistan. Studies were also carried out with *Onobrychis transcaucasica*, a perennial shrub with high growth rate and nutritional value. This plant species is present in hilly regions of the Kyzil-Kum desert and has great potential to be exploited as fodder for valuable karakul sheep.

I. *Ammodendron connollyi*, *Astragalus vilosissimus* and *Astragalus unifoliatus*: effects of salinity and inoculation on plant development.

The salt tolerance of plants germinated and grown under sterile conditions was analyzed. in order to determine the salinity range allowable for plants in the planned inoculation studies. Seeds were pretreated with concentrated H_2SO_4 (20 minutes for A. *vilosissimus* and A. *unifoliatus*, and 40 minutes for A. *connollyi*), rinsed thoroughly with sterile distilled water and imbibed overnight on agar (1%) plates, followed by removal of the seed coats. The seeds where then placed, embryo-side down, on agar plates and incubated at 30 °C for 2-3 days. Single sterile seedlings were planted in 250 ml pots filled with autoclave-sterilized dune sand. The pots were irrigated with 20% Hoagland medium containing 3 mM NH_4NO_3, and from then on NaCl was added up to each of the following concentrations: 0 (control), 50, 100, 200, 300 and 500 mM. There were 10 replicate pots for each treatment. The irrigation prevented drying of the sand between nutrient applications, and the fresh solution volume applied was above field capacity of the pot substrate, thus preventing the accumulation of salts. After four weeks of treatment, plants were harvested, washed with distilled water, blotted on paper towel, separated into roots and shoots, and the length and fresh weight (FW) of roots and shoots were determined. The plant material was then placed in an oven for 48 h at 70 °C, allowed to cool to room temperature, and the dry weight (DW) was determined. Results presented in Figs 8-10 are average values and for each treatment the standard deviations and levels of significance were analyzed by the Student T test using the Microsoft Excel 2001.

Under all conditions tested, root development exceeded by far that of the shoots. Nodules were never found, neither on the roots of controls or plants under salinity treatments.

Among the three plant species studied, *A. connollyi* showed the lowest salt resistance, with significantly reduced growth in the presence of 50 mM NaCl, and no surviving plants in the presence of 100 mM NaCl or above (Fig 10).

In general, the effects of salinity on the development of *A. unifoliatus* (Fig. 8) and *A. vilosissimus* (Fig. 9) were similar and no plants survived at 300 and 500 mM NaCl. The dry and fresh weights of the shoots of plants grown in the presence of 50 and 100 mM NaCl were higher or not significantly different from those of the control plants (Figs 9 and 10).

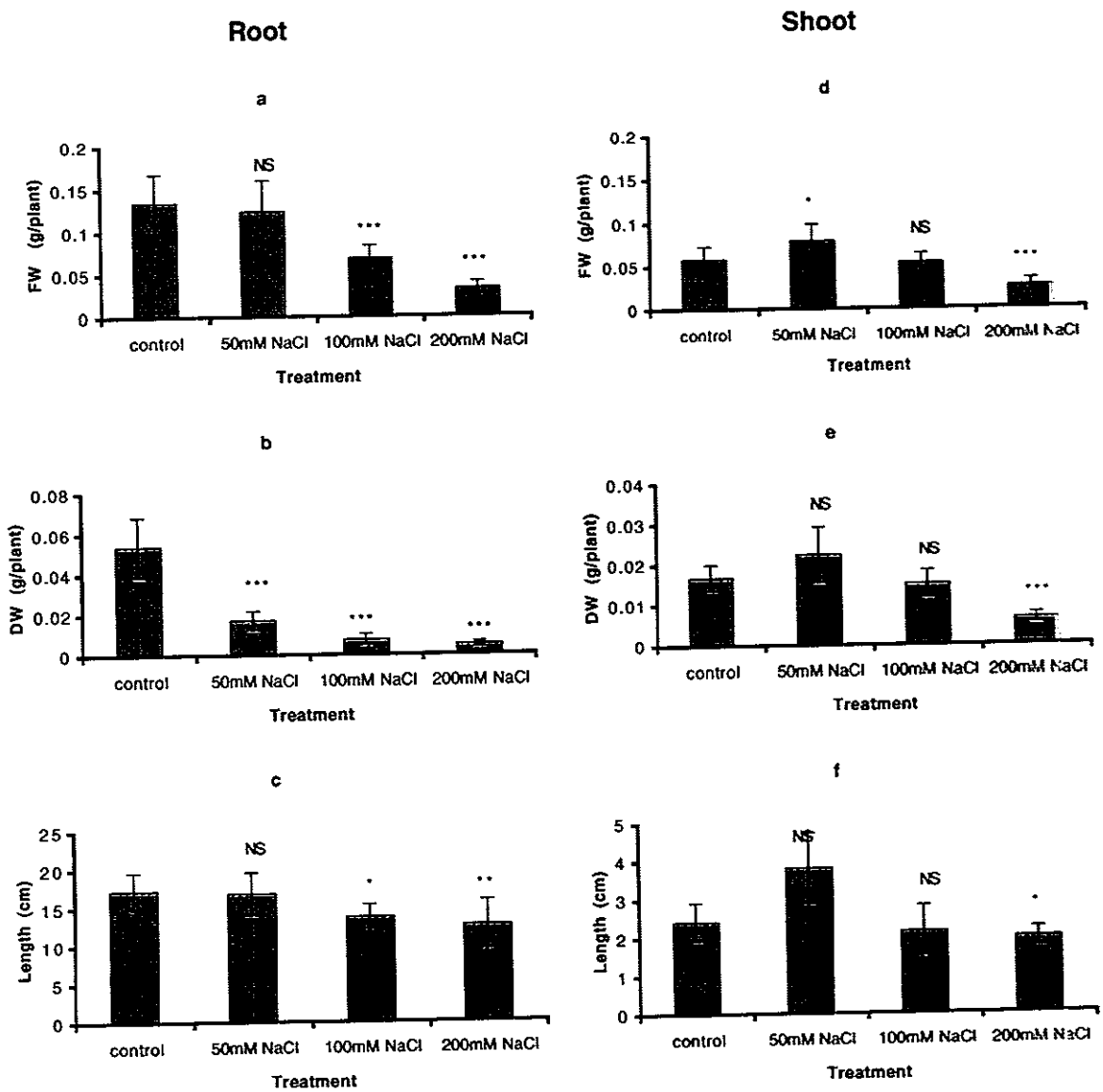

Figure 8: Effect of salinity on the development of five-week old *Astragalus unifoliatus* plants.

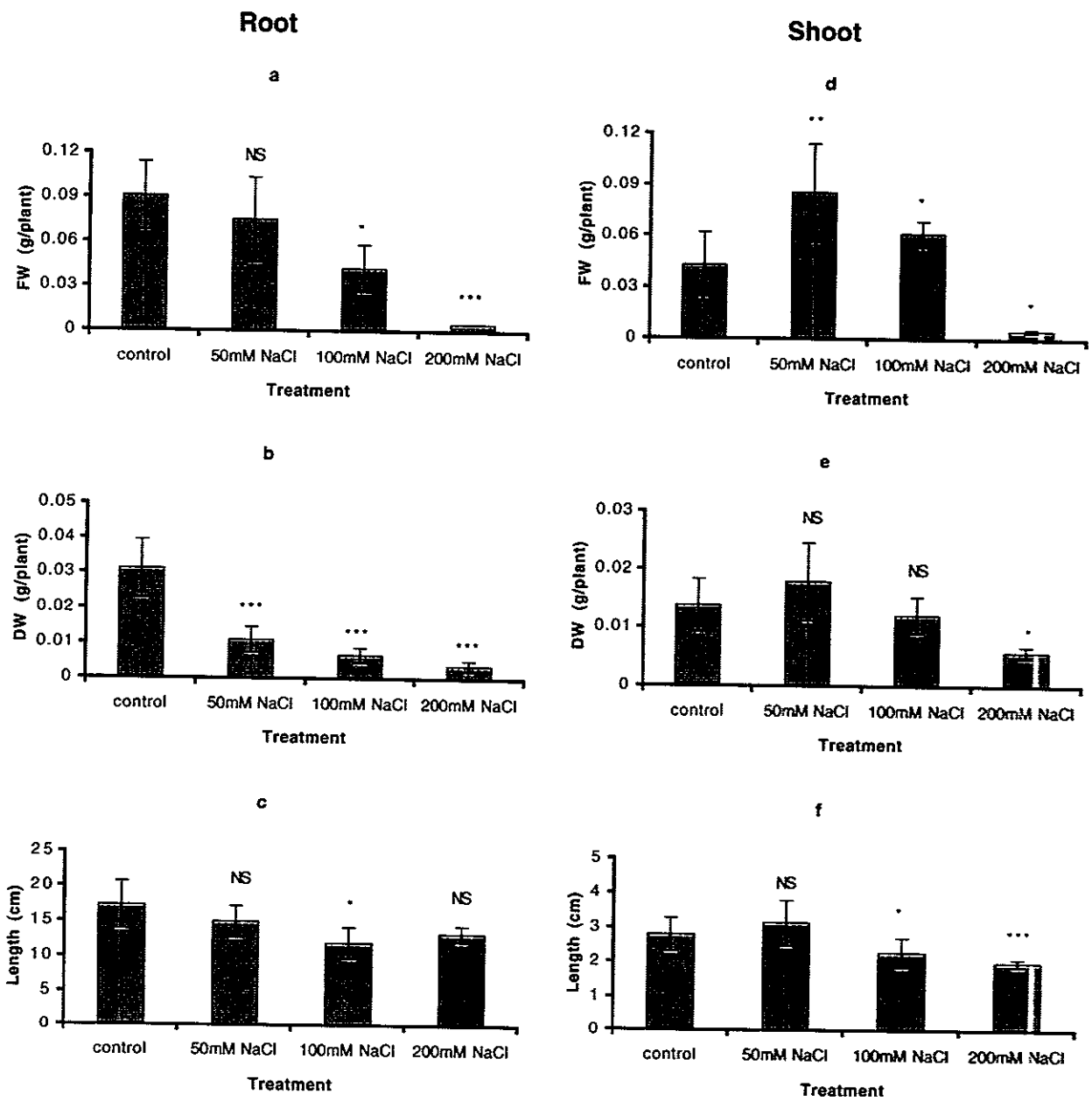

Figure 9: Effect of salinity on the development of five-week old *Astragalus vilosissimus* plants.

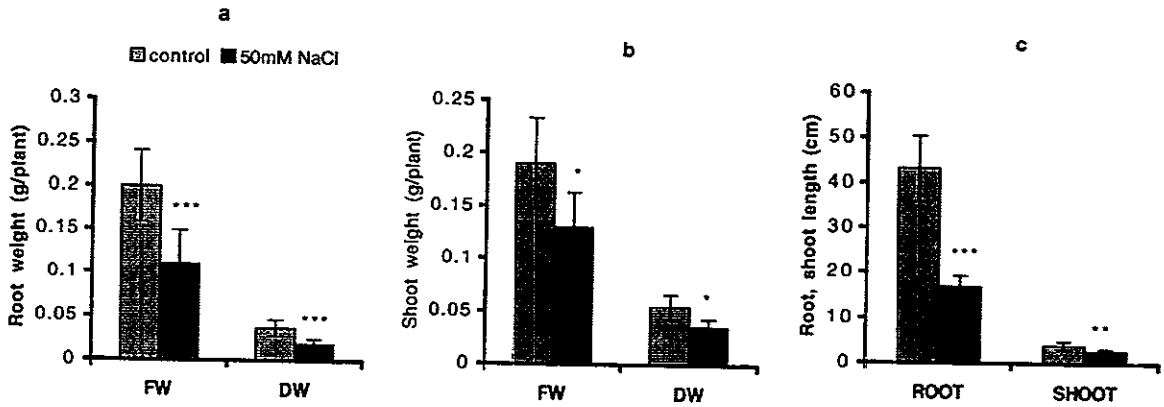

Figure 10: Effect of salinity on the development of five-week old *Amodendron connollyi* plants.

Experiments were then set up with the objective of testing the effect of inoculation and nodulation on the development of the three plant species. During the first week, the seedlings received 3 mM NH_4NO_3, then they were inoculated with one of the 15 bacterial strains by dispensing, close to the root, 2 ml of a suspension with approximately 10*9 cells/ml. From the time of inoculation, the concentration of nitrogen in the nutrient solution was lowered to 1 mM NH_4NO_3. Two weeks later, NaCl was added at the concentrations of 50, 100 and 300 mM for *A. unifoliatus*, of 100 and 300 mM for *A. vilosissimus*, and of 50 and 100 mM for *A. connollyi*. For each plant/bacterium combination there were controls with or without nitrogen in the nutrient solution, and controls without added NaCl. Un-inoculated controls were also set up for each plant species.

The highest level of NaCl tested was fatal. Plant development at all other concentrations was similar to that shown in Figs 8-10. Root nodules were not found in any of the plants, and the experiments were repeated twice with the identical results.

II. *Onobrychis transcaucasica*

II. 1. Effect of salinity on plant development

Two ranges of NaCl concentrations were tested: up to 200 mM NaCl and from 300 to 800 mM. Seeds of *O. transcaucasica* (Bge) were germinated on wet filter paper and the seedlings were planted in 15 l pots filled with a mixture of equal volumes of vermiculite and local loess soil. The plants grew for eight weeks under irrigation frequencies suitable to temperature and plant size, using 10% complete Hoagland nutrient solution containing one of the following nitrogen sources: 0.5 mM KNO_3, 2 mM NH_4Cl or 1 mM NH_4NO_3. The plants were then grown for further 8 weeks in the presence of 0, 50, 100 or 200 mM NaCl. The experiments were carried out in a greenhouse, and each treatment consisted of four replicate pots with two plants each. In the experiment at the higher salinity range, plants were grown in 1.5 l black-plastic bags, and the NaCl concentrations applied to the eight-week plants were 0, 300, 400, 600 or 800 mM, for four weeks. Each treatment consisted of two replicate bags containing five plants each. Harvesting, processing of plant material, and data analysis were performed as described above.

Plants of *O. transcaucasica* showed considerable tolerance to salinity, and although the plants dry weight increased at a lower rate with increased salinity, all plants survived at 800 mM NaCl (Figs 11 and 12). Plants receiving ammonia as sole nitrogen source were the most susceptible to salinity; compared to their controls, at 800 mM NaCl the root and shoot dry weight were reduced by approximately 85% and 96% respectively.

Increasing the concentration of NaCl from 50 to 200 mM had little effect on the number of nodules formed on the roots of plants growing under all regimes of nitrogen nutrition (Fig. 11). High variability was observed in the number of nodules in the plants treated with 300-800 mM NaCl (Fig. 12). However, it is interesting to note that nodulation occurred at 800 mM NaCl in plants receiving KNO_3 as sole nitrogen source.

II. 2. Effect of drought on plant development and nodulation

Plants were pre-grown for 40 days in 6 l pots (6 plants/pot) filled with local soil and irrigated every two days with 10% complete Hoagland nutrient solution containing one of four concentrations of NH_4NO_3: 0 mM (control), 0.25 mM, 1 mM and 3 mM. The plants were then grown for further 32 days during which the interval between irrigations was 2 days (control), 4 days, 8 days or 16 days. Irrigation consisted of adding, all at once, 500 ml of nutrient solution per pot. Each treatment included six replicate pots.

Irrigation frequency (Fig. 13) had a marked effect on the biomass production, and the formation of nodules improved with irrigation frequency and low levels of nitrogen.

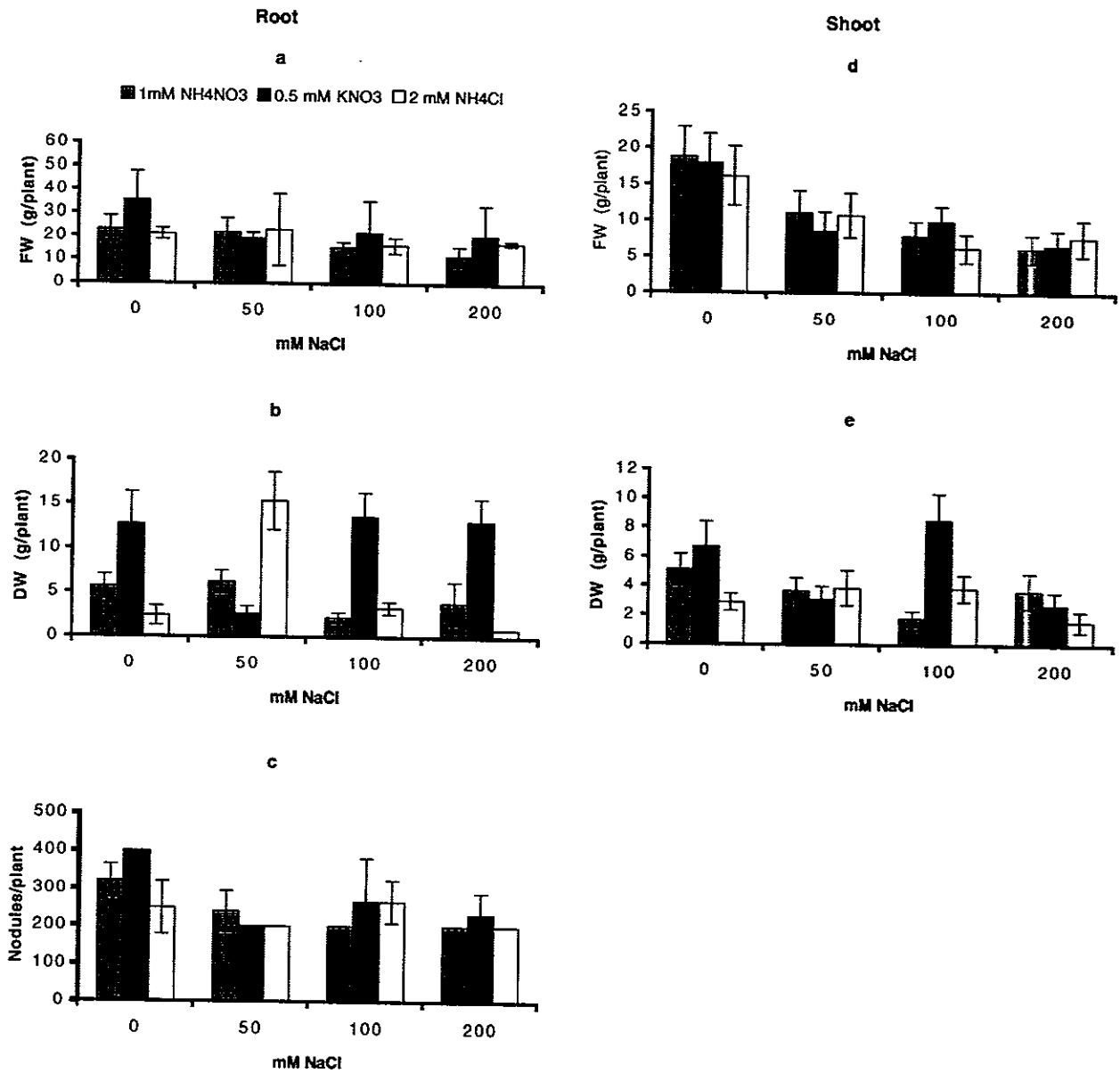

Figure 11: Effect of NaCl concentration on the development of 15 weeks old *O. transcaucasica* plants. The salinity treatment lasted 7 weeks.

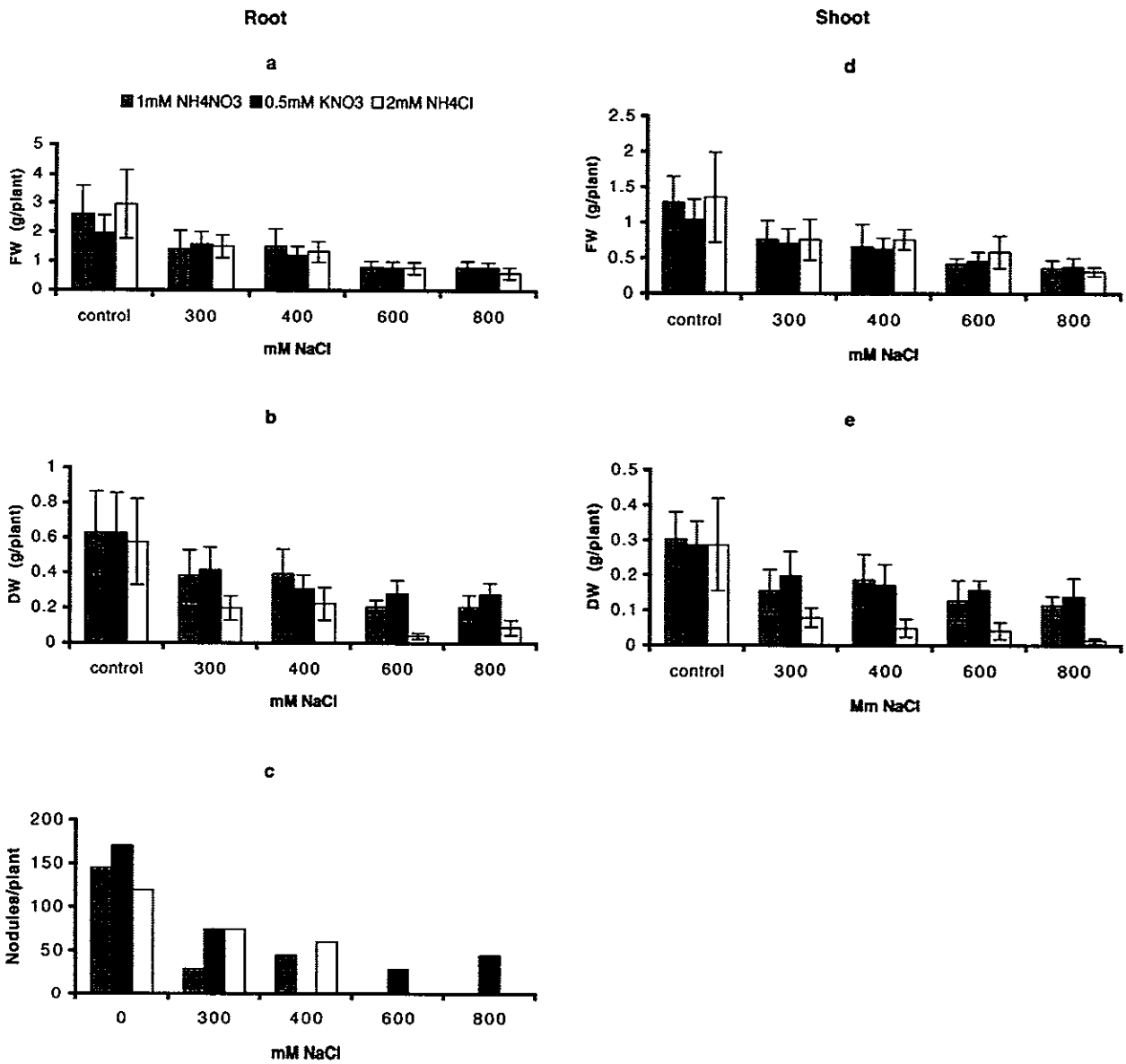

Figure 12: Effect of NaCl concentration on the development of 10 weeks old *O. transcaucasica* plants. The salinity treatment lasted 6 weeks.

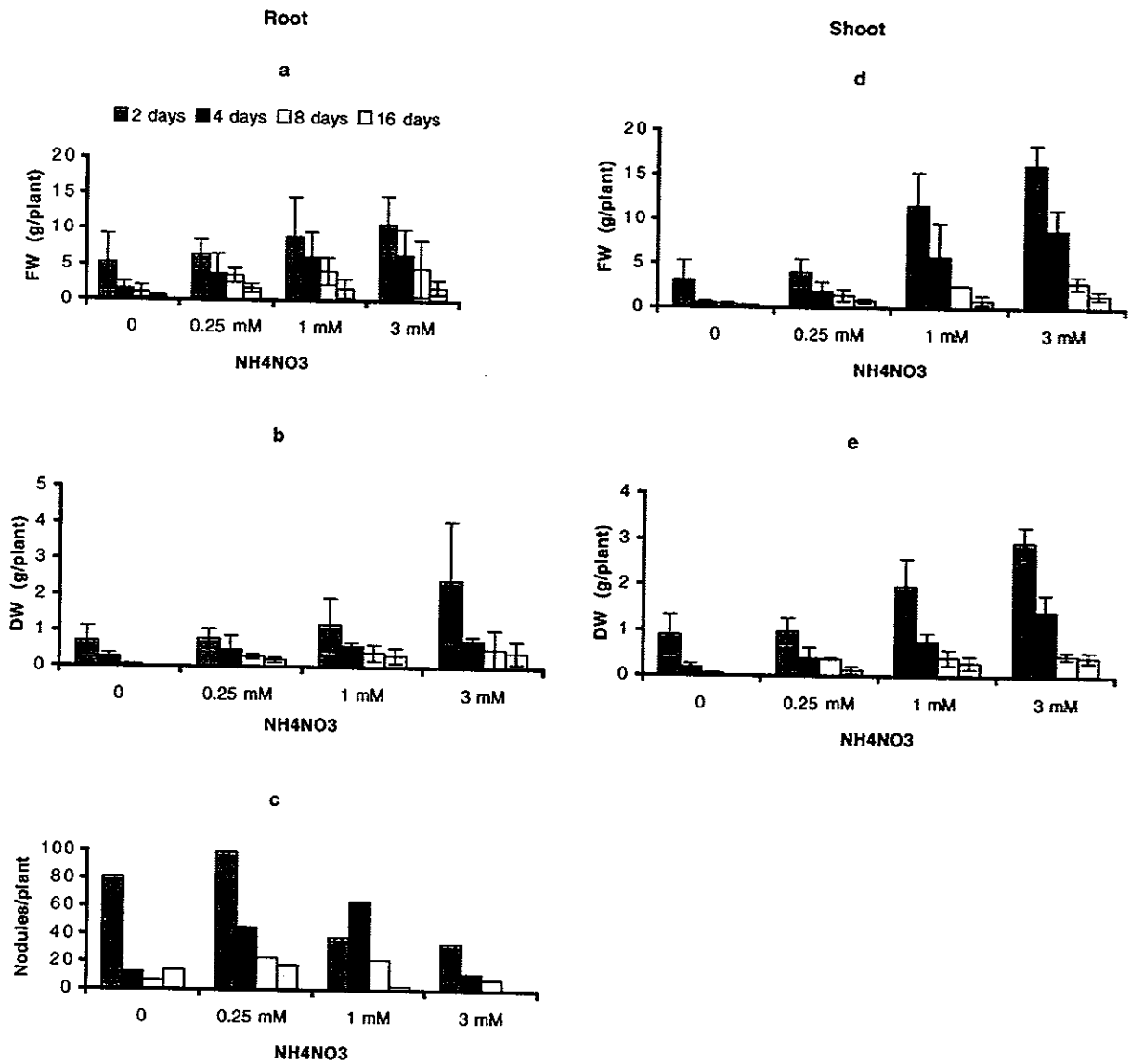

Figure 13: Effect of irrigation frequency on the delopment of 9 weeks old *O. transcaucasica* plants grown at various concentrations of ammonium nitrate.

Discussion

Astragalus unifoliatus (Fig. 8) and *Astragalus vilosissimus* (Fig. 9) developed NaCl concentrations up to 200 mM. It was interesting to observe that even when grown in the presence of 200 mM NaCl and with a drastic loss of dry matter production, plant length was affected to a much lower extent; this was possible by a more focused allocation of dry matter to the central shoot and by cutting down on side growth. This effect was evident both on root and shoot development. Also notable is the increase in dry matter production of the shoot of plants kept under 50 mM NaCl, **indicating a reverse of the traditional trend shown by stressed plants in which shoot growth is inhibited more than root growth**. All these changes indicate that these plants have the capacity to change the patterns of dry mass distribution under increasing saline conditions in order to survive and produce seeds.

Amodendron connollyi (Fig. 10), when compared to the *Astragalus* plants, was considerably more sensitive to 50 mM NaCl in all growth parameters measured: root and shoot fresh and dry mass, as well as root and shoot length. The potential biomass production capacity of these plants would require optimization of nutritional and irrigation management, as well as selection of specimens with higher dry-mass productivity.

Onobrychis transcaucasica (Fig. 11), exposed to NaCl between 0 and 200 mM for 7 weeks, showed remarkable salt tolerance as expressed by fresh and dry mass production. This plant species has the potential of an excellent salt tolerant forage crop. The capacity of 15 weeks old plants to develop high numbers of nodules within the entire range of salinity tested was quite surprising and also points out to the great potential of this species as a desert and arid zone forage crop. At higher NaCl concentrations, between 300 to 800 mM, *O. transcaucasica* showed increased sensitivity to salinity (Fig. 12), although the plant survived even at the highest salinity (800 mM NaCl) and kept forming nodules when provided with low levels of KNO_3 (0.5 mM). At 800 mM NaCl, plants developed better in the presence of nitrate and of ammonium-nitrate than in the presence of ammonium nitrogen sources. This effect may have to do with the hormonal balance in plant roots as affected by different inorganic nitrogen ions (**Lips, S. H., M. Sagi and R. Omarov. 2000**. Mo-enzymes at the crossroads of signal transmission from root to shoot. In: Nitrogen in a sustainable ecosystem from the cell to the plant. M. A. Martins-Louçao and S. H. Lips, Eds. ISBN 90-5782-063-3 Buckhuys Publishers, Leiden, Netherlands. Pp. 239-250).

Irrigation frequency (Fig. 13) had, as expected, a large effect on the biomass production of plants of *O. transcaucasica*. The formation of N_2-fixing nodules improved with irrigation frequency and low levels of N (0.5 mM N in 0.25 mM NH_4NO_3). The results of these experiments point out the enhanced capacity of these plants to produce biomass in response to water and NH_4NO_3. Under conditions in which these two inputs are available, **O. *transcaucasica* would constitute a very valuable forage crop when taking into account the high nutritional** value of its shoots.

C) Scientific Impact of Collaboration

The Uzbek PIs have learned new and advanced molecular biology techniques in the Sede Boqer laboratories in close cooperation with Israeli investigators. The possibility to fund their travel and stay in Israel has permited remarkable advances by them in the more sophisticated aspects of the joint study undertaken, with an excellent integration of basic and applied aspects of the project. They have also been acquainted with a framework of planning and carrying out long range research in a methodical way. They have been exposed to the use of modern laboratory instrumentation, both in molecular biology and on the use of modern computers, including literature searches through electronic libraries and the Internet. Their cooperation with Israeli colleagues has been developing extremely well.

D) Description of Project Impact

By now it is obvious that some of the experimental results are an important basis for a more ambitious program to introduce the use of salt tolerant N_2-fixers into native and foreign plant

species which can either increase their stress tolerance in ecosystems threatened by worsening drought and saline conditions, or maintain sustainable rates of biomass production in aride areas of Central Asia. The impact of this project in practical terms may be boosting the capacity to produce leguminous crops in desert areas affected by drought and salinity, if local government and funding agencies will lend their support for more extensive projects with a more active participation of local farmers. The isolation of highly stress tolerant strains of N_2-fixers and their ongoing characterization are very valuable factors which could be put to use valuable salt tolerant N_2-fixing strains within a relatively short time in Uzbekistan and neighboring countries.

E) Strengthening of Developing Country Institutions

New equipment and training are the two major elements that this project has been providing to the Uzbek laboratory, including long periods of cooperative work in the same laboratory. Our collaborators have greatly developed their capacity to prepare and present grants to obtain independent funding for their valuable work and are now the recipients of two grants with a third one selected for presentation in full version.

F) Future Work

Work will now proceed along two parallel lines of work, one with the isolated strains of N_2-fixers and the second with the leguminous plants collected from the Kyzil-Kum desert area.

- To study the capacity of the isolates to form nodules with their traditional host plants from which they where originally isolated.
- To test the capacity of the selected N_2-fixers to make nodules with alternative wild and cultured leguminous plant species.
- To attempt the transfer of *nod* genes from peanut Rhizobium to our stress tolerant N_2-fixers and test their effect on peanuts growing in field conditions
- To study the stress tolerance of native plant species such as *Ammodendron connollyi* with and without association with N_2-fixers.

Section II

A) Managerial Issues

None

B) Budget

There are problems with the payments of fellowships of the Uzbek investigators in Kazakhstan. We hope that the valuable capacity building these fellowships allowed will be considered above purely technical legalities.

C) Special Concerns

None

D) Collaboration, Travel, Training and Publications

Collaboration has been developing nicely. In June 1999 the Israeli PIs visited the Uzebek laboratory and took part in a collecting expedition to the Kyzil-Kum desert. Drs. Khakimov and Shakirov are spending the period between May and October 2000 in Sede Boqer for work in the laboratories of Drs. Soares (Microbiology) and Lips (Plant Sciences). Another visit of the Uzbek scientists took place during 2002. Publication of some of the results is at a preliminary stage of preparation while additional experimental work will be completed. The Uzbek scientists have presented the results of the cooperative research at the International Microbiology Conference in Paris (August 2002) were it elicited great interest.

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

None