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Isolation of Fenugreek Nodule Bacteria from Arid and Uncultivated Soils and Their Impact on Hostplants

(Submitted by corresponding member of NAS RA R. M. Aroutiounian 6/VI 2023)

Keywords: *fenugreek, microsymbionts, nodule bacteria, Ensifer meliloti, Rhizobium sp.*

Fenugreek is an annual plant belonging to the Papilionideae subfamily of the legume family, the Latin name is Trigonella foenum-graecum, which means Greek herb. The habitat includes the entire Mediterranean area, India, China, is found in the USA, Ethiopia and other countries. There are 130 known species of fenugreek. It is a hardy crop that can grow well in a variety of extreme conditions, withstand drought, salinity and can grow in uncultivated soils. Trigonella foenum-graecum is known for its nutritive protein content of 25-30%, a large amount of lysine, with which nodule bacteria enter into symbiotic relationships, forming root nodules and fixing atmospheric nitrogen [1 - 4]. Some authors found significant differences in plant growth parameters when inoculated with different strains, which indicates strain differences [5 - 7]. The most common nodule bacteria and other rhizosphere microorganisms associated with Trigonella foenum-graecum are known from the studies. These isolates stimulated plant growth, root and shoot length, chlorophyll content, nodule formation efficiency and nodule dry weight when co-inoculated with Ensifer meliloti rhizobia strain [8,9]. The long-term use of nitrogenous fertilizers and pesticides has led to a reduction, even the disappearance of the natural population of nodule bacteria, including alfalfa, sainfoin, fenugreek and other useful rhizobacteria in the cultivated soils of Armenia [10]. It is necessary to obtain organically pure raw materials without the use of mineral fertilizers for the use of fenugreek in pharmaceuticals and cosmetology, that is inoculation with nitrogen-fixing nodule bacteria is relevant.

The aim of the work is to isolate fenugreek nodule bacteria from soils imported from different agriculture-climatic regions of Armenia and Artsakh

and study their virulence under model conditions in order to restore the natural microbiota in soils and obtain organic primary products.

Material and methods of the research. The presence of nodule bacteria of fenugreek was studied in soil samples from Yerevan, Etchmiadzin, Vardenis, Armavir, Goris, Kapan, Meghri cities, Tegh and Lichk villages and other settlements. We investigated the virulence and efficiency of typical strains of alfalfa – *Ensifer meliloti* MDC[1] 5502 and MDC 5503 maintained in the culture collection of nodule bacteria at the Laboratory of Energy Alternative Sources, as well as newly 12 isolated strains of *Trigonella foenum-graecum* from various samples of soils.

Fenugreek seeds were sterilized with 98% sulfuric acid, washed several times with sterile water (pH 6.8-7.0), then transferred to Petri dishes with filter paper, placed in a thermostat for growing at 26-27 ^{o}C. Laboratory and vegetation experiments were carried out under sterile conditions: river sand, white sand, and perlite were taken as substrates, which were enriched with a solution of nitrogen-free mineral substances according to Vincent [11].

The investigations were carried out with 5 repetitions according to the method proposed by Somesegaran [12]. For the initial isolation of nodule bacteria from soil samples, soil extracts were prepared, placed for several hours on shakers, which were inoculated with 3-day-old sterile sprouts of the fenugreek variety and transplanted into 3-kilogram pots. After 7-8 weeks, strains of nodule bacteria were isolated from nodules that appeared on the roots of plants, their symbiotic properties were studied in a sterile slant agar mineral nutrient medium, 2 plants in each test tube. The virulence of the strains was assessed by biometric indicators.

From the isolated microorganisms' preparations were prepared, fixed with 96% alcohol for 10 min, stained with crystal violet, which made it possible to judge the purity of the isolates, as well as the size and morphological features of the strains. The purity of the strains and the study of cell morphology was performed using a Boeco light microscope with a magnification of 1800 times. The resulting pink nodules, rich in leghemoglobin, were placed on a glass slide and imprints were prepared, fixed by a similar method, and the bacteroids of nodule bacteria were studied. A large number of branched and well-developed bacteroids indicated high activity of nitrogen fixation in nodules.

Results. In Armenia, fenugreek – *Trigonella foenum-graecum* has been cultivated since ancient times, used in folk medicine for the treatment and prevention of skin, metabolic, hormonal, respiratory, gastrointestinal and genitourinary diseases. Currently, it is cultivated in some farms of Aragatsotn, Armavir, Gegharkunik, Vayots Dzor, Syunik regions, in the vicinity of Yerevan, and the wild species is found on dry stony mountain slopes, shrubs, loams, roadsides. In the course of our laboratory and vegetative-sterile experiments, it was found that nodule bacteria isolated from many soil samples of various uncultivated soils were able to form nitrogen-fixing nodules on the roots of

[1] MDC – Microbial Depository Center of the Scientific and Production Center "Armbiotechnology" NAS RA.

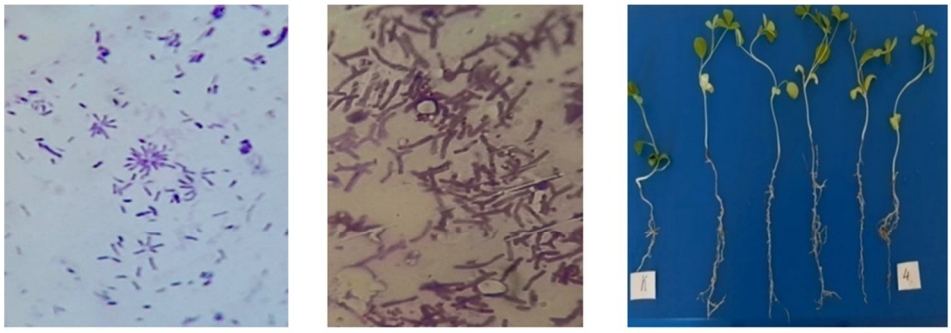
Trigonella foenum-graecum, contributing to an increase in the green mass of plants (Table 1).

Table 1

Virulence of nodule bacteria *Trigonella foenum-graecum* under sterile laboratory conditions (substrate: white sand, bud stage)

Working names of fenugreek isolates	Dry weight of plants, g	Dry weight of plants to control, %	Plant height, cm	Dry weight of nodules, g	Number of nodules, pcs
FAm-s	0.230	135.3	12.9	0.040	36
FEj-n	0.200	117.6	12.5	0.030	54
FTa-q	0.260	152.9	12.0	0.050	48
FSh-i	0.300	176.5	14.4	0.050	52
FGa-r	0.230	135.3	14.8	0.030	37
FMa-n	0.190	111.8	14.6	0.040	32
FTig-t	0.290	170.6	13.5	0.030	40
FDa-q	0.290	170.6	15.7	0.030	45
FSt-t	0.340	200.0	14.5	0.060	40
FDr-k	0.350	206.0	15.1	0.050	45
FCh-i	0.210	123.5	12.0	0.070	60
Control group	0.170	100	10.0	-	-

Table 1 shows that a significant increase in the green mass of plants grown on white sand was observed in the FDr-k strain in the amount of 0.180 g, and in the FMa-n strain it was insignificant, respectively, the mass and number of nodules also exceeded the FMa-n, FAm-s, FGa-r, FTig-t variants, in which the plant height was 12.9, 13.5, 14.6, 14.8 cm, and the weight was 0.190, 0.230, 0.290 g. FDr-k and FSt-t was 0.340-0.350 g, which is 2 times more compared to the control group.



a b C

Fig. 1. a) *Rhizobium* isolates, b) bacteroids, c) fenugreek plants with nodules of *Rhizobium* strains

In some variants, options for the nodules were more numerous, but with less weight. This pattern is explained by the dark color of the nodules. In strains FDr-k and FSt-t, the nodules differed in pink color and the presence of leghemoglobin (Figure 1).

In the case of growing plants on perlite, a significant increase in green mass was observed, in contrast to growing on white sand. The FSh-i strain showed a significant increase in green mass in the amount of 0.650 g compared to the control, and the FAm-s strain showed the smallest increase in the amount of 0.250 g. Accordingly, the mass and number of nodules were also higher in the FSh-i strain. The patterns observed in plants grown on white sand were preserved, and here the strains FTig-t, FSt-t had significantly higher values both in the mass of nodules and plants, and in height, compared with other variants and control. The effectiveness of strains expressed as a percentage ranged from 52.0 to 106.0% (Tables 1 and 2).

At the next stage, the studies were continued in an open greenhouse, at air temperature 19-24 ^{o}C, with full moisture capacity of the pots. Positive results were obtained when inoculated with all strains. Plants grown under these conditions were distinguished by splendor, increased green mass, a pronounced root system and pink, large nodules. In the variants inoculated with strains isolated from the soils of FDr-k, FLan-n, FCha-i, the number of nodules was 200 - 230 (Table 3, Fig. 2).

In the FGa-r strain, the increase in green mass was 0.570 g, in the FHa-q, FGa-r strains, the plant length was 16.2 and 16.9 cm, respectively. The mass of nodules was higher in strains FSt-t, FHa-k, FSh-i, FTig-t in comparison with other varieties from 2.6 - 7.9 g, and strains FDr-k, FLa-n, FCh-i was more distinguished by the number of nodules.

The regularities recorded in laboratory experiments were preserved, the nodules were numerous, but of small mass. The tested strains of alfalfa (*E. meliloti* 21/49, *E. meliloti* 25/49) also formed nodules on the roots of fenugreek

Table 2

Virulence of nodule bacteria *Trigonella foenum-graecum* in laboratory conditions (substrate: perlite, bud stage)

Working names of fenugreek isolates	Dry weight of plants, g	Dry weight of plants to control, %	Plant height, cm (Shoot)	Dry weight of nodules, g	Number of nodules, pcs
FAm-s	1.500	120.0	13.4	0.030	45.0
FLa- n	1.650	132.0	15.2	0.040	72.0
FQe-t	1.750	140.0	15.6	0.050	64.0
FSh-i	1.900	152.0	15.8	0.120	70.0
FVa-s	1.670	133.6	14.6	0.050	40.0
FLi-k	1.560	124.8	14.4	0.070	76.0
FTig-t	1.875	150.0	15.6	0.040	38.0
FDa-q	1.650	132.0	15.0	0.030	54.0
FSt-t	1.880	150.4	15.7	0.060	42.0
FDr-k	1.670	133.6	15.3	0.050	52.0
FCha-i	1.520	121.6	12.0	0.050	56.0
Control group	1.250	100.0	10.2	-	-

Table 3

Biometric indicators of newly isolated fenugreek strains in a vegetation test (in 3 kg pots, substrate: river sand)

Working names of fenugreek isolates	Dry weight of plants, g	Dry weight of plants to control, %	Plant height, cm	Dry weight of nodules, g	Number of nodules, pcs	Shoot / root
FAm-Yerevan	1.400	127.3	11.9	3.5	130	0.40
FLa- n	1.490	135.4	13.2	4.2	210	0.35
FHa-q	1.390	126.4	16.2	4.7	195	0.29
FSh-i	1.440	130.9	10.9	5.3	160	0.27
FGa-r	1.670	151.8	16.9	2.3	170	0.72
FMe-i	1.400	127.3	14.8	2.8	132	0.50
FTig-t	1.500	136.4	13.3	10.0	190	0.15
FDa-q	1.480	134.5	11.6	2.9	130	0.51
FSt-t	1.460	132.7	13.2	4.7	190	0.31
FDr-k	1.510	137.3	13.3	2.5	200	0.60
FCh-i	1.420	129.0	13.1	4.7	230	0.30
E.meliloti 5502	1.210	110.0	15.0	2.1	104	0.57
E.meliloti 5503	1.170	106.4	16.1	4.1	150	0.28
Control group	1.100	100.0	9.75	-	-	-

Fig. 2. Greenhouse plants of fenugreek with nodules inoculated with *Rhizobium* strain (FTig-t)

plants – 104-150 pieces per 5 plants, contributed to an increase in green mass by 6.0-10.0%, i.e., insignificantly, compared with the strains isolated from the host plant – fenugreek, in which it was 26.4-51.8%. The plant height of these variances exceeded the variance of newly ones isolated from fenugreek, but they had a thinner stem, which affected the weight index.

Conclusion. The studies were carried out for the soils of Armenia and Artsakh for the first time, *Trigonella* strains were isolated from twelve types of patterns or samples of soils and their symbiotic activity was tested under conditions of sterile microvegetation and vegetation experiments. Significant differences were found in plant growth characteristics in response to inoculation with different strains, suggesting differences at the strain level. In this study, we have isolated several strains of fenugreek with the aim of finding suitable microsymbionts that can further increase fenugreek yields in Armenia and Artsakh by inoculating them in a more sustainable agronomic condition. As a result of the research, it was found that high rates of green mass of plants were recorded in all substrates, but the mass and number of nodules in plants grown on river sand exceeded those of plants grown on white sand and perlite. These isolated strains of fenugreek nodule bacteria can be considered as PGPR species and used in biofertilizer formulations. For the use in pharmacy and cosmetology, it is necessary to obtain organically pure raw materials, therefore, it is important to inoculate with nodule bacteria with high nitrogen-fixing activity, without the use of mineral fertilizers.

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Isolation of Fenugreek Nodule Bacteria from Arid and Uncultivated Soils and Their Impact on Host-plants

From the soils of Armenia and Artsakh, 12 strains of nodule bacteria Trigonella foenum-graecum (fenugreek) were isolated and tested in laboratory and vegetation experiments. The isolated microsymbionts were characterized by high virulence and contributed to an increase in the biometric parameters of fenugreek plants. The isolates were identified as Rhizobium sp. and Ensifer.meliloti.

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Ֆենուգրեկի պալարաբակտերիաների մեկուսացումը չորային և անմշակ հողերից և դրանց ազդեցությունը տեր-բույսերի վրա

Հայաստանի և Արցախի հողերից մեկուսացվել են ֆենուգրեկի (*Trigonella foenum-graecum)*-ի պալարաբակտերիաների 12 շտամներ, որոնք փորձարկվել են լաբորատոր և վեգետացիոն փորձերում: Իզոլատներն իդենտիֆիկացվել են որպես *Ensifer meliloti* և *Rhizobium* sp.: Մեկուսացված միկրոսիմբիոնտներն առանձնացել են ֆենուգրեկի նկատմամբ բարձր վիրուլենտությամբ, նպաստել են բույսերի աճին և բիոմետրիկ ցուցանիշների բարձրացմանը:

Г. М. Хачатрян, Т. У. Степанян, С. А. Арутюнян, В. Б. Гогинян

Выделение клубеньковых бактерий пажитника из засушливых и необрабатываемых почв и их влияние на растение-хозяина

Из почв Армении и Арцаха в результате лабораторных и вегетационных экспериментов выделено и изучено 12 штаммов клубеньковых бактерий растения пажитник (*Trigonella foenum-graecum* L.). Полученные микробные симбионты идентифицированы как *Ensifer meliloti* и *Rhizobium* sp., отличаются высокой вирулентностью, способствуют росту и повышению биометрических показателей растения-хозяина.

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