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Isolation and Characterization of *Rhizobium meliloti* Isolated from Rhizosphere Soil and Roots of Fenugreek from Different Locations

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A B S T R A C T

The nine isolates of *Rhizobium meliloti* were isolated on CR-YEMA medium from root nodules and rhizosphere soil of fenugreek. Four isolates showed absorption of Congo red and rest five isolates did not absorbed Congo red. All the isolates showed similar cultural characteristic for colony colour and shape except for colony size. Colonies of all isolates were whitish, translucent, sticky nature, rounded and had a diameter in the range of 3.2 to 5.2 mm. All the isolates showed positive reaction for biochemical test viz., catalase test, potassium hydroxide and starch hydrolysis test and showed negative reaction for Gram's staining.

Introduction

The fenugreek (*Trigonella foenum graecum* Linn.) commonly called methi belongs to family Leguminoceae. There are two species of *Trigonella* genus which are of economic importance i.e. *Trigonella foenum graecum* (common methi) and *Trigonella corniculata* (Kasuri methi).Fenugreek is cultivated in India and other parts of world for leafy vegetables, condiments, medicinal as well as fodder purpose. Seeds of fenugreek are used as spice for preparation of different tasty dishes. (Rai and Yadav, 2005). According to Willems (2006), Beijerinck was the first to isolate the root nodule bacteria and named it as *Bacillus radicicola*. Later Frank changed the name to *Rhizobium leguminosarum*. Fenugreek has a potential to fix a substantial amount of atmospheric nitrogen. Inoculation of fenugreek with suitable strains of *Rhizobium* is expected to improve the quantity and quality of the produced seeds and consequently, the nutritional and economic status of the population will be improved. *Rhizobium* inoculation of fenugreek has been reported to increase the biomass of plant and seed production. Fenugreek was reported to fix 48% of its total N_2 during the growing season (Singh *et al.,* 2008). The *Rhizobium* isolates

were rod shaped, Gram negative, acid and mucous producing. They were found to be temperature and pH sensitive, with optimum values of 29.4°C and 7.0, respectively. The bacteria was sensitive to the antibiotics; chloramphenicol, kanamycin and streptomycin. It utilizes glucose, sucrose and starch as sole carbon source. The *Rhizobium* species isolated from fenugreek roots have the potential to produce industrially important enzymes; amylase and cellulose (Pawar *et al.*, 2014).

Materials and Methods

Collection of root nodules and soil samples

The fresh and plump root nodules from the roots of fenugreek plants and rhizosphere soil samples from fenugreek were collected from the adjoining farmer's fields. These nodules and soil samples were brought to the laboratory and subjected for isolation from soil by serial dilution method and from root nodules by streak plate method on Congo-red Yeast Extract Mannitol Agar (CR-YEMA) medium.

Isolation and purification of *Rhizobium meliloti*

The soil samples and root nodules of fenugreek plant samples collected were subjected to isolations on selective medium. Isolation of rhizobia from root nodules of *Trigonella foenum-graecum* was done by the method suggested by Somasegaran and Hoben (1994). From each sample, two-three nodules were picked up and washed thoroughly with sterile distilled water. After washing, nodules were surface sterilized in 75% ethanol for 30–40 second to remove wax coating if any and subsequently immersed in 3% sodium hypochlorite for 3–4 min. Then nodules were immediately washed 5–6 times with sterile distilled water to remove traces of sodium hypochlorite. The surface-sterilized nodules were transferred to sterile tubes containing100 µl sterile distilled water. Nodules were crushed with the help of sterile glass rod and then one loopful of each nodule suspension was streaked on to sterilized plates of Congo red-yeast extract mannitol agar (CR-YEMA) medium. Then, the streaked plates were incubated at 28 ± 2^{O}C in an inverted position for 2 to 4 days until colonies appeared along the lines of spreading. The soil samples were serially diluted in distilled water up to 10^{7} - 10^{8} cfu/ml and isolated the *Rhizobium* on Congo red-yeast extract mannitol agar (CR-YEMA) medium and incubated at 28 ± 2^{O}C. After completion of incubation period of 2 to 4 days, the plates were observed for development of the *Rhizobium* colonies.

Colonies of isolates were picked with sterile inoculating loop, streaked on sterile YEMA plates and incubated at 28 ± 2^{0}C. The purity and uniformity of colony type was carefully examined through repeated re-streaking and a single well isolated colony was picked to YEMA medium in a petri plate and in conical flask containing YEMA broth for mass multiplication and incubated at 28 ± 2^{0}C. (Somesagaran and Hoben, 1994 and Aneja, 2003).

Characterization of*Rhizobium meliloti* isolates

Visual characterization of colony morphology was done for isolation of pure cultures of *Rhizobium*. Nine such pure isolates were used for further studies and all tests were performed.

Cultural characters

The different isolates of *Rhizobium* collected from the fenugreek plants grown in pots and farmer's field in the nearby vicinity were grown on YEMA medium and incubated at

28±20C for 48 hours and observed for colony colour, shape and size (Aneja, 2003).

Biochemical characters

Biochemical characters of *Rhizobium* were studied by subjecting the bacterial isolates to various biochemical tests, *viz.* Gram's staining, catalase oxidation test, potassium hydroxide (KOH) solubility test and starch hydrolysis test (Aneja,2003 and Vishunavat and Kolte, 2005).

Gram's staining

A loop full of the 24- 28 hrs. old bacterium suspension was smeared on clean glass slide, air dried and fixed by gentle heating on flame of the spirit lamp.

Aqueous Crystal violet solution (0.5%) was spread over this smear for 30 to 60 second and then washed with running tap water for a minute; this stained smear was later flooded with Grams iodine solution for one minute and rinsed in tap water. Later decolorized with 95% of ethanol until color runoff, washed with water and treated with Safranin as counter stain for about 10 seconds, washed with water, air/blot dried and observed under research microscope (make:- Olympus) at 40X.

Catalase oxidation test

A loop full of 24-28 hrs. old culture of test bacterium was placed on the clean glass slide, and to this a drop of 3% hydrogen peroxide (H_2O_2) was mixed and allowed to react for few minutes and observed for the production of gas bubbles.

KOH test (Potassium hydroxide)

A drop of 3% potassium hydroxide was placed on clean glass slide and to this 48 hrs. old test bacterial culture was mixed with clean inoculation loop and stirred for 10 second and observed for slime threads.

Starch hydrolysis

The autoclaved and cooled starch agar medium was poured in sterile glass Petri plates and on solidification of the medium, pure culture of the test bacterium was streaked and incubated for 96 hrs. at 28 ± 2^0C.

Then these plates were flooded with lugol's iodine and allowed to react for few minutes.

Results and Discussion

Collection of root nodules and soil sample

Fenugreek root samples having nodules with pinkish coloured, present on tap root or lateral roots as a single or in clustered form and fenugreek rhizosphere soil samples were collected from the adjoining farmer's fields.

Isolation and purification of *Rhizobium meliloti*

The nine test isolates were isolated from rhizosphere soil and root nodules of fenugreek on basal culture medium YEMA with Congo-red, by applying serial dilution and streak plate method. Out of nine, five isolates were isolated from root nodules of fenugreek and nomenclatured as, LFRR-1, LFRR-2, LFRR-3, LFRR-4 and LFRR-5 and another four were isolated from fenugreek rhizosphere, nomenclature as LFRS-1, LFRS-2, LFRS-3 and LFRS-4 (Table 1). Colonies with whitish translucent appearance of *Rhizobium* were obtained on CR-YEMA medium after incubation at 28 ± 2^OC for two days. Generally the Isolates did not absorbed Congo red (Fig. 2), but isolates LFRS-1, LFRR-3, LFRS-3 and LFRR-5 slightly absorbed Congo red (Table 1, Fig. 1), showing the variation in absorption capacities of Congo red among the isolates.

Fig.1 Showing Congo-red absorption

Fig.2 Showing no Congo-red absorption

Fig.3 Whitish-translucent, rounded colonies of *R. meliloti*

Fig.4 Gram Staining reaction

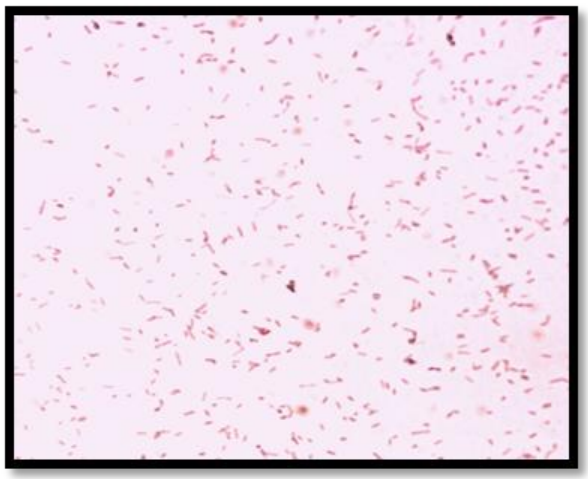

Table.1 Growth of *Rhizobium meliloti* isolates on CR-YEMA medium

Sr. No.	Isolates	Growth on CR-YEMA medium
1	LFRR-1	Colorless
2	LFRR-2	Colorless
3	LFRR-3	light red
4	LFRR-4	Colorless
5	LFRR-5	light red
6	LFRS-1	light red
7	LFRS-2	Colorless
8	LFRS-3	light red
9	LFRS-4	Colorless

LFRR = Latur fenugreek *Rhizobium* from root
LFRS = Latur fenugreek *Rhizobium* from soil
CR-YEMA = Congo-red Yeast Extract Mannitol Agar

Table.2 Cultural characters of *Rhizobiummeliloti* isolates on YEMA

Sr. No.	Isolates	Colony colour	Colony shape	Colony size (mm)
1	LFRR-1	White	Round	4.6
2	LFRR-2	White	Round	3.2
3	LFRR-3	White	Round	3.5
4	LFRR-4	White	Round	4.2
5	LFRR-5	White	Round	3.2
6	LFRS-1	White	Round	4.3
7	LFRS-2	White	Round	4.5
8	LFRS-3	White	Round	5.2
9	LFRS-4	White	Round	3.2

LFRR = Latur fenugreek *Rhizobium* from root
LFRS = Latur fenugreek *Rhizobium* from soil
YEMA = Yeast Extract Mannitol Agar

Table.3 Biochemical characters of *Rhizobium meliloti* isolates

Sr. No.	Isolates	Characteristics			
		Gram Reaction	Catalase Oxidation	Potassium hydroxide	Starch hydrolysis
1	LFRR-1	-ve	+ ve	+ ve	+ ve
2	LFRR-2	-ve	+ ve	+ ve	+ ve
3	LFRR-3	-ve	+ ve	+ ve	+ ve
4	LFRR-4	-ve	+ ve	+ ve	+ ve
5	LFRR-5	-ve	+ ve	+ ve	+ ve
6	LFRS-1	-ve	+ ve	+ ve	+ ve
7	LFRS-2	-ve	+ ve	+ ve	+ ve
8	LFRS-3	-ve	+ ve	+ ve	+ ve
9	LFRS-4	-ve	+ ve	+ ve	+ ve

-= Negative, + = Positive
LFRR = Latur fenugreek *Rhizobium* from root

LFRS = Latur fenugreek *Rhizobium* from soil

The isolates were purified and mass multiplication on YEMA medium and YEMA broth for further study. Similar results were earlier reported by Kneen and Larue (1983), Yousef and Abdul Karim (2012) who reported that *Rhizobium meliloti* absorbed Congo red strongly and Tsegaye *et al.*, (2015) reported that some isolates of *Rhizobium* isolated from fenugreek, slightly absorbed Congo red.

Characterization of *Rhizobium meliloti*

Cultural characters

Results (Table 2) indicated that the variation does not seen with the characters like, colony colour and shape except colony size which showed slight variation among the isolates of *Rhizobium meliloti*. The colonies of the isolates appeared to have a sticky nature, indicating the production of mucous substances. After two days of incubation, colonies were observed as whitish, rounded (Fig.3) and had a diameter in the range of 3.2 to 5.2 mm. The isolate LFRS-3 recorded maximum colony diameter of 5.2mm, followed by the isolates *viz*., LFRR-1 (4.6mm), LFRS-2 (4.5mm), LFRS-1 (4.3mm) LFRR-4 (4.2mm) and LFRR-3 (3.5mm). Rest of the isolates, LFRR-2, LFRS-4 and LFRR-5 recorded the colony growth 3.2mm.

Result of present study revealed that all the isolates of *Rhizobium meliloti* showed whitish and rounded colonies with slight difference in colony size, Singh *et al.,* 2008 reported that colonies of *Rhizobium* isolates, isolated from fenugreek were white, rounded, had a diameter from 5 to 7 mm and production of mucous substances which is indeed one of the characteristics Rhizobia, similar results were earlier reported by many workers like, Tamas *et al.,* 2010, Jain *et al.,* 2012, Prerna Rajpoot and Panwar 2013 and from different host like Berseem (Gauri *et al.,* 2011), Faba bean (Anteneh Argaw, 2012), Mungbean (Shraddha Bhatt *et al.,* 2013 and Amin, 2014), Pea (Deshwal and Chaubey, 2014), Soybean (Pawar *et al.,* 2014), Black gram (Satyanandam *et al.,* 2014) Groundnut (Benson *et al.,* 2015) and fenugreek (Tsegaye *et al.*, 2015).

Biochemical characters

Different biochemical tests *viz.*, Grams staining, Catalase oxidation test, KOH test (Potassium hydroxide), Starch hydrolysis, etc. were attempted in respect of *R. meliloti* and results (Table 3) revealed that all test isolates showed positive reaction with the catalase oxidation, potassium hydroxide and starch hydrolysis test and also gave negative reaction to Gram's staining (Fig. 4).

Similar results of biochemical tests *viz.*, Gram staining, catalase test, potassium hydroxide (KOH) test and starch hydrolysis test, in respect of *R. meliloti* observed under these investigations were earlier reported by several workers in different crops like, Singh *et al.*, (2008), Gauri *et al.*,. (2011), Shahzad *et al.*, (2012), Prerna Rajpoot and Panwar (2013), Singh *et al.*, (2013).

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