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Isolation and Identification of Endophytic Fungi in *Trigonella foenum-graceum* L. and their Antibacterial Activity

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Abstract*: The main aim of this work was to study the antibacterial activity endophytic fungi isolated from Trigonella foenum-graceum L. Eight endophytic fungi were isolated from leaf, stem and root and their ethyl acetate crude extract was screened for their antibacterial activity against the pathogen Pseudomonas aeruginosa. Alternaria alternata, Aspergillus flavus, A. niger, Fusarium moniliforme, F. oxysporum, F. solani, Rhizopus sp. and Verticilium dahliae, all of these endophytic fungi showed zone of inhibition. Out of these ethyl acetate crude extract of Verticilium dahliae showed a maximum of 22mm inhibition. This study shows that fenugreek harbors a good number of endophytic fungi whose crude extracts can be used for further studies in controlling the plant pathogenic bacteria P. aeruginosa in vivo.*

Key words: *Trigonella foenum-graceum L; Pseudomonas aeruginosa; endophytic fungi; crude extract.*

Introduction

Fenugreek (*Trigonella foenum-graceum* L.) belongs to the family Fabaceae. It is an aromatic annual herb (30-60 cm tall) cultivated throughout the country [23]. Fenugreek originated from the Near East and India [16]. It is cultivated in parts of Europe, Northern Africa, West and South Asia, North and South America and Australia. In India Rajasthan and Madhya Pradesh are the major states growing fenugreek [19].

Endophytic microorganisms are found in every part of the earth. These organisms reside in the living tissues of the host plant and maintain a relationship ranging from symbiotic to pathogenic [18]. Mycologists predicted that there are 1.5million species of fungi, of these 74,000 are currently known [9]. Communities of endophytic fungi can vary greatly in a single host species in different sites, climates, seasons and environments [9, 22]. Difference in endophytic communities in a single host species can increase with distance [1] or show no significant variation [2]. Endophytes have proven to be rich sources of novel natural compounds with a wide spectrum of biological activities and a high level of structural diversity. These microorganisms received considerable attention in last 20 years when their capacity to protect against insect pest pathogens was noticed [3].

The present study aims at isolation of endophytic fungi from different parts of fenugreek plant collected from different regions of Mysore district and their antibacterial activity against *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* is a gram-negative bacterium commonly isolated from soil and water [20]. As an opportunistic human pathogen, *P. aeruginosa* is also capable of causing serious infections in plants [11]. *P. aeruginosa* causes root rot in Ginseng [8]. It also affects tobacco [24].

Materials and Methods

Isolation of endophytic fungi

Endophytic bacteria were isolated from the internal tissues of healthy leaves, stem and roots of *Trigonella foenum-graceum* L. (fenugreek) plants collected randomly from different sites in the same vicinity of Mysore. Samples were thoroughly washed in running tap water to remove dust particles and then shaken in a flask containing 200ml of distilled water. After proper washing stem, leaf and root samples were cut into small pieces under aseptic conditions using sterile scalpel.

Plant material

In the present study endophytic fungal species were isolated from different parts of healthy *Trigonella foenum-graceum* L. collected from HD Kote, KR Nagar and Krishna Raja Sagar (KRS) regions of

Mysore district, Karnataka. Healthy and mature plants were chosen for isolation. The plant parts such as stem, leaf and root were brought to the laboratory in sterilized covers and processed within a few hours after sampling.

Isolation and identification of endophytic fungi

The collected plant parts were rinsed in tap water to remove dust, debris and soil. After proper rinsing stem, leaf and root samples were cut into small pieces using sterile blades under aseptic conditions. Each sample was surface sterilized by 70% ethanol for 1min. and then rinsed in sterile distilled water for 1 min. and then allowed to surface dry on the filter paper. After proper drying 4 pieces of plant parts were inoculated in petriplate containing solidified PDA supplemented with tetracycline antibiotic and incubated at room temperature (28 ± 1^0C) for 7 days [15].

Morphological identification of endophytic fungi

Individual fungal colonies were picked using sterile needle and sub cultured onto fresh antibiotic free PDA medium to obtain pure cultures. The fungi were identified in their sporulation stage by staining with cotton blue. The fungal identification was done based on colony morphology and conidial characters [4].

Calculation of colonization frequency

The relative frequency % of colonization frequency (% CF) was calculated as the number of number of segments colonized by the fungi divided by the total number of segments plated × 100 [7].

$$\%\ Colonization\ frequency = \frac{no.of\ segments\ colonized\ by\ the\ fungi}{total\ no.of\ plated} \times 100$$

Mass production of antibacterial metabolites

The isolated endophytic fungi (pure culture) were fermented in 500ml Erlenmeyer flasks containing PDB (Potato Dextrose Broth) at 28^0C for 15 days. The fermentation medium was then centrifuged at 3,600 rpm for 10 mins. The supernatant was then transferred to a separatory funnel. To this same volume of ethyl acetate was added and the funnel was strongly agitated for further separation. The process was repeated twice. The ethyl acetate extract obtained was concentrated to about 98% in a rotary evaporator at 40^0C [13].This ethyl acetate extract obtained were scrapped off by adding 2ml of Dimethyl Sulphoxide (DMSO) [12].

Evaluation of Antibacterial Activity

Test organisms

Pseudomonas aeruginosa, a pathogenic bacterium was used to assess the antibacterial activity of the fungi. The bacterium was taken from Plant Pathology Laboratory, DOS in Botany, University of Mysore, Mysore (India).

Antibacterial activity

To evaluate antibacterial activity, the Nutrient agar (NA) medium was poured into petridishes and inoculated with 100µl of the bacterial suspension [6]. Then the inoculums were spread uniformly by using a sterile cotton bud on the medium. After swabbing, 2 wells were punched on the plates. The first well was loaded with 50µl of fungal extract and the second well was loaded with 50µl of DMSO which was taken as negative control. Sterile disc already loaded with Gentamycin was placed on the plate and considered as positive control [12]. Then the plates were incubated at25±20C for 24h and measured for the inhibition zones daily.

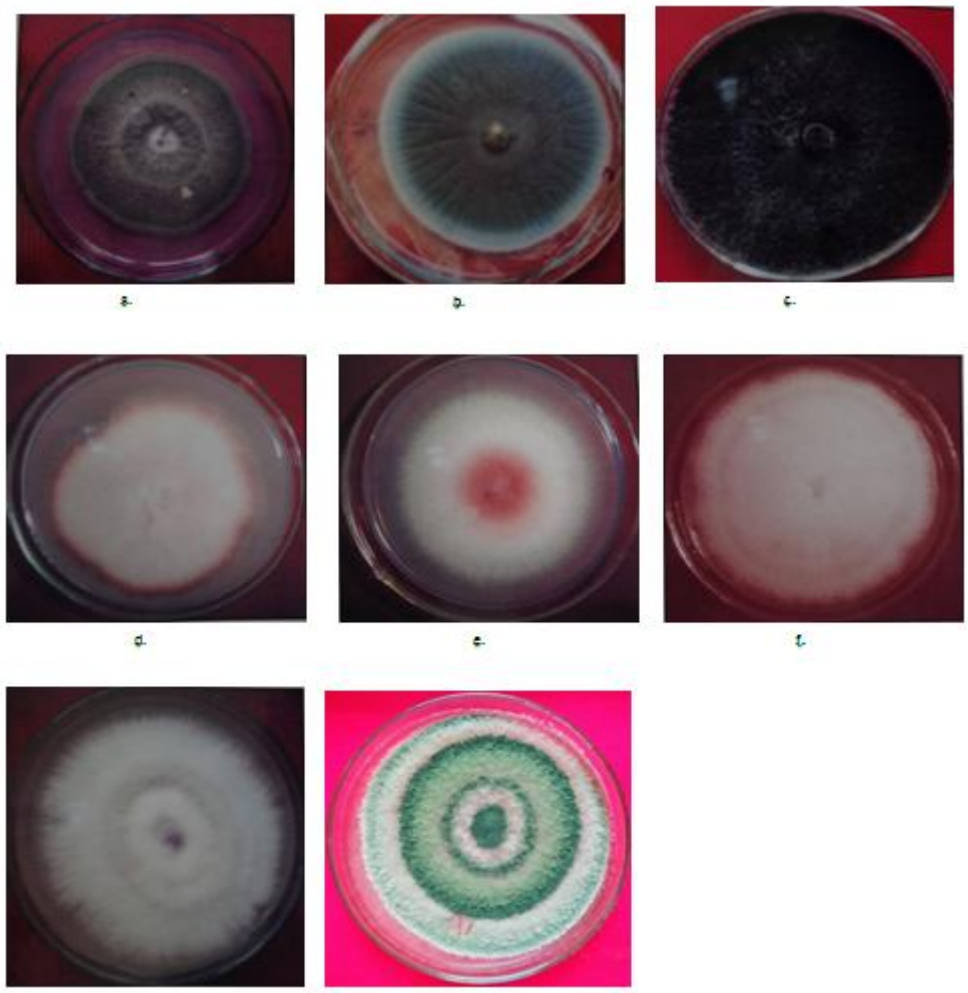
Results

Isolation of endophytic fungi

In the present study fungal sp. were isolated from different parts of *Trigonella foenum-graceum* L. Different parts of the healthy plant such as leaves, stem and roots were processed for the isolation. A total of 8 endophytic fungi were isolated.

Identification of endophytic fungi

Identification of the isolated endophytic fungi was done by using manual of Barnett and Hunter (1998) on the basis of morphological characters of the colony and microscopic studies. These fungi were identified as a) *Alternaria alternata*, b) *Aspergillus flavus*, c) *A. niger*, d) *Fusarium moniliforme*, e) *F. oxysporum*, f) *F. solani*, g) *Rhizopus sp*. and h) *Verticilium dahliae*. **(fig.1.)**



Colonization frequency

The colonization frequency on leaf, stem and roots varied. The results are shown in **table 1.** *Alternaria alternate* (33.3%) *and Fusarium moniliforme* (66.7%) showed maximum colonization in stem, *Aspergillus falvus* (16.7%), *Aspergillus niger* (16.7%) and *Rhizopus sp*. (16.7%) showed colonization only on leaf. *Fusarium oxysporum showed maximum colonization in leaf (*50.0%). *Fusarium solani* showed colonization only on stem (16.7%) and *Verticillium dahliae* showed colonization only on root (33.3%)

Table 1. Colonization frequency of endophytic fungi isolated from different parts of *Trigonella foenum-graceum* L.

Plant parts	Leaf	Stem	Root
Endophytes	Colonizing Frequency (%)		
Alternaria alternate	-	33.3	16.7
Aspergillus falvus	16.7	-	-
Aspergillus niger	16.7	-	-
Fusarium moniliforme	-	66.7	50.0
Fusarium oxysporum	50.0	33.3	-
Fusarium solani	-	16.7	-
Rhizopus sp.	16.7	-	-
Verticillium dahliae	-	-	33.3

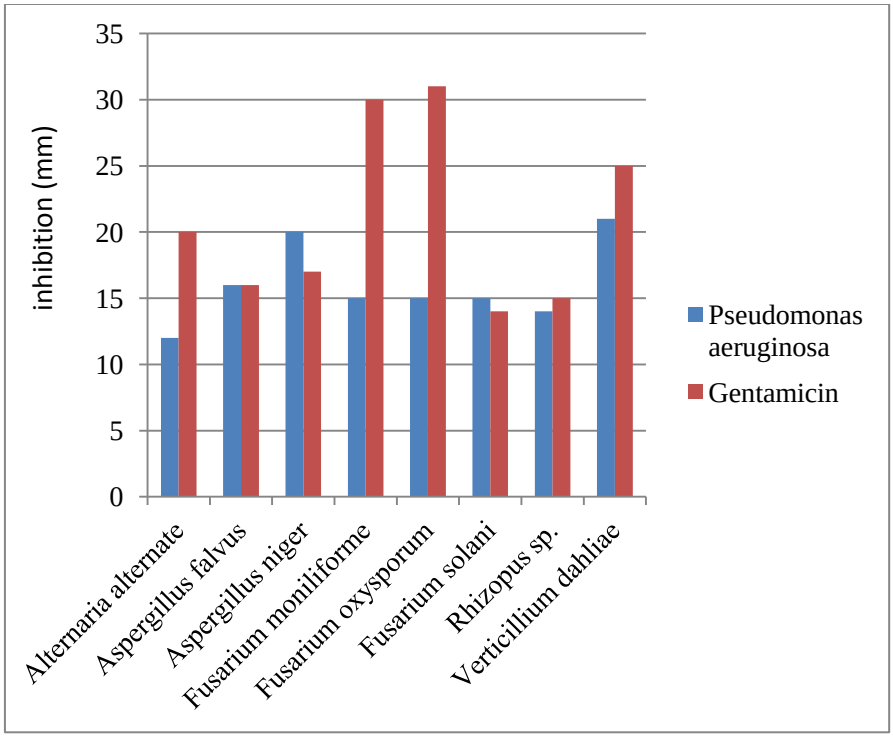
Screening of endophytic fungi for antibacterial activity

Agar well method was used to determine the antibacterial activity against *P. aeruginosa*. The results obtained are shown in **table 2.** The extent of antibacterial activity is expressed in diameter of inhibition zone (mm). Standard 15 days old metabolites of fungi were used for screening. *V. dahliae* showed maximum zone of inhibition against *P. aeruginosa* (21mm) and *Alternaria alternata* showed least zone of inhibition (12mm). **(fig.2.)**

Table 2. Antibacterial activity shown by endophytic fungi isolated from Fenugreek

Endophytes	*Pseudomonas aeruginosa*	Inhibition Zone (mm)	
		Pseudomonas aeruginosa	Gentamicin
Alternaria alternate	+	12.66±1.15	20.66±1.15
Aspergillus falvus	+	16.00±1.00	16.66±1.52
Aspergillus niger	++	20.00±0.00	17.33±1.15
Fusarium moniliforme	+	15.00±1.50	30.00±1.63
Fusarium oxysporum	+	15.00±1.00	31.00±1.00
Fusarium solani	+	15.00±0.00	14.00±1.00
Rhizopus sp.	+	14.33±1.55	15.00±1.00
Verticillium dahliae	++	21.33±1.00	25.33±1.15
Positive control	++		
Negative control	-		

+++=inhibition zone <30mm, ++=20mm, +=<10mm and - = no inhibition zone



Antibacterial screening of endophytic fungi on *Pseudomonas aeruginosa*

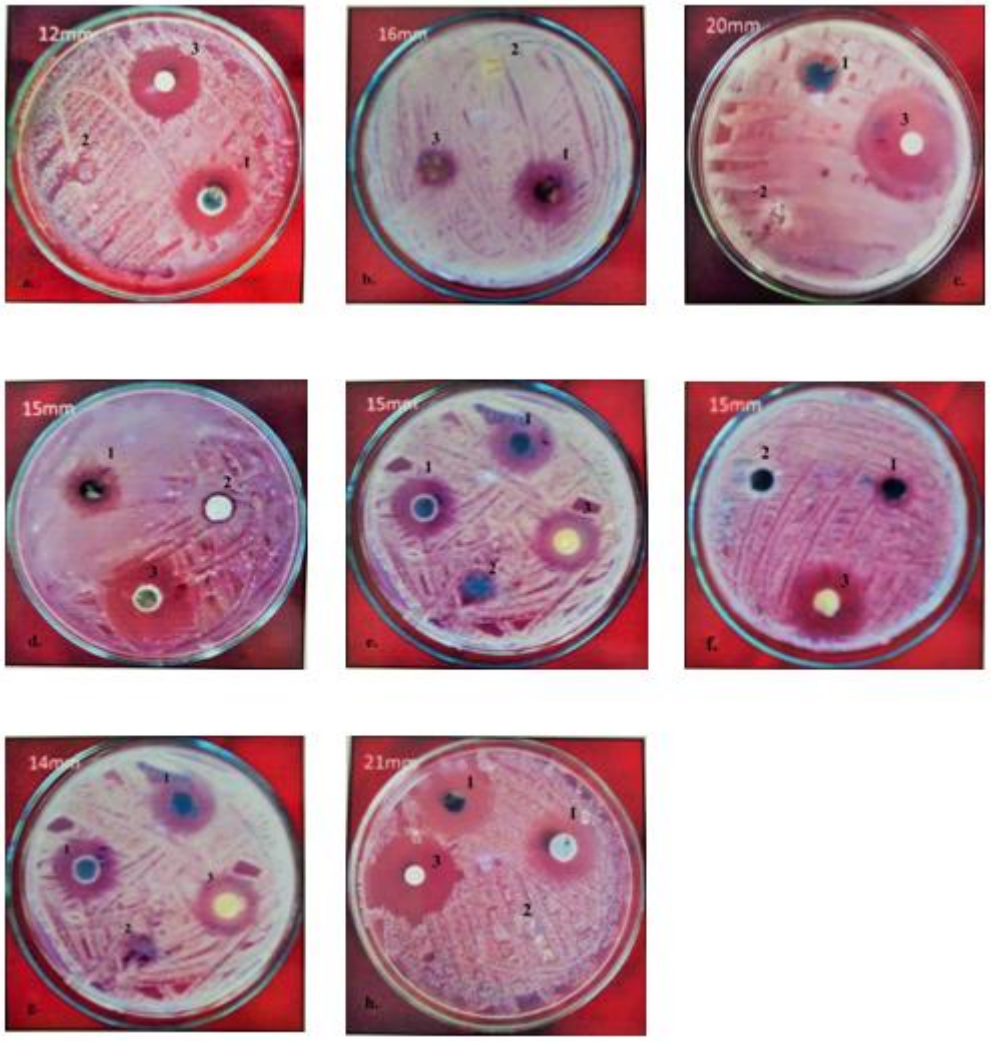


Fig.2. a) ***Alternaria alternate*** **b)** ***Aspergillus flavus*** **c)** ***A. niger*** **d)** ***Fusarium moniliform*** **e)** ***F. oxysporum*** **f)** ***F. solani*** **g)** ***Rhizopus sp.*** **and h)** ***Verticilium dahliae*** **showing zone of inhibition**

Discussion

Endophytes are a rich source of functional metabolites which benefits host plants by preventing pathogenic organisms from colonizing them; they also stimulate the production of secondary metabolites.

Trigonella foenum-graceum L. is a plant having a broad spectrum of medicinal value. In India countable number of report showed on diversity of endophytic bacteria or fungi in medicinal plants [17]. Medicinal plants are good source for isolation of endophytic fungi that colonize the tissue without causing apparent symptoms [14]. Endophytic organisms have received considerable attention as they are found to protect their hosts against pests, pathogens and even domestic herbivores [21]. Isolation of endophytic fungi from medicinal plants results to produce bioactive compounds which have greater activity against various pathogenic microbes. Endophytic fungi from *Ocinum sp*. (Tulsi) showed 21mm zone of inhibition against *Pseudomonas aeruginosa* [10].

Conclusion

In the present study a total of 8 endophytic fungi were isolated from stem, leaf and roots of healthy *Trigonella foenum-graceum* L. against *Pseudomonas aeruginosa*. Of these the crude extract of *Verticillium dahliae* showed maximum inhibition. Hence the crude extract of *Verticillium dahliae* can be used for further studies on the plant pathogenic bacteria *Pseudomonas aeruginosa.*

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