INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 11, No. 5, p. 186-191, 2017

RESEARCH PAPER OPEN ACCESS

Exploring bacterial flora in rhizosphere of *Nicotiana tabbacum* at district Swabi; High tobacco production zone, Pakistan

Muhammad Idrees, Kalsoom*, Shahzeb Wahid, Sana Mateen, Amar, Faheem Ahmad Khan, Johar Jamil

Department of Microbiology, University of Swabi, Khyber-Pakhtunkhwa, Pakistan

Key words: *Nicotiana tabacum,* Rhizosphere, Bacterial population

http://dx.doi.org/10.12692/ijb/11.5.186-191 Article published on November 28, 2017

Abstract

Nicotiana tabbacum is an annually grown herbaceous plant promising great economic importance and the rhizosphere of *Nicotiana tabbacum* harbours considerable groups of microorganisms. The aim of research was to isolate and study the growth characteristics of indigenous bacterial population from the rhizosphere of *Nicotiana tabbacum* from different areas of district Swabi i.e. Marghuz, Kotha and Zaida and to maintain pure cultures for future reference. The soil samples were collected from rhizosphere with different age groups of the same species and brought into the lab for further processing. All samples were grown on different Media including Nutrient agar, MacConkey agar and Mannitol Salt Agar and a series of biochemical tests were performed for identification and characterization of the most commonly occurring rhizobacteria of this region. The result of colony morphology and biochemical tests confirmed the presence of four bacterial isolates i.e. *Micrococcus luteus, Bacillus subtilis, Bacillus cereus* and *Escherichia coli*. *Bacillus subtilis, Bacillus cereus* and *Micrococcus luteus* are gram positive while *Escherichia coli* is a gram negative bacteria. It is inferred from this study that most common isolates in rhizosphere of *Nicotiana tabbacum* was antibiotic producers which can be used as biofertilizer for enhancing growth and prevention of pathogens

* **Corresponding Author:** Kalsoom ✉ kalsoomwazir@uoswabi.edu.pk

Introduction

Nicotiana tabacum is the individual crop grown in Pakistan which yield is healthy beyond the World ordinary and equals the per hectare production in the United States and other developed countries an average production of 1900 kg/hectare. Khyber Pakhtunkhwa produces around 78% of Pakistan entire tobacco crop that generates more than 37000 full employments in the Province. Total tobacco cultivation area in KPK is 36016 hectares that produces 93080 tons of tobacco with values of 10.09 billion. In Swabi around 38% tobacco crop is produce more than the other districts of KPK. Tobacco cultivating areas here in the districts are expected to soon receive a total of Rs 256 million from tobacco cess developmental fund for the fiscal year 2016-17 (Junaid, 2011).

Rhizosphere is the narrow region of soil that is directly influenced by root secretions and associated soil microorganisms (Hiltner, 1904). These bacteria form a protective barrier to the root from infection of parasites (Shamima and Rahman, 2007) by the secrtion of poisonous metabolites and antibiotic substances (Subhashini and Padmaja, 2009a & b). The bacteria in return are nourished from the exudates of roots (Narula *et al*., 2009). In this way a cooperative association based on mutual interest is established between the roots of host plant and the rhizospheric bacteria (Starkey, 1938).

The micro flora of rhizosphere comprises nematodes, fungi, protozoa, algae and bacteria (Raaijmakers *et al*., 2001). The variety and frequency of rhizosphere microbial inhabitants is closely dependent on a number of abiotic and biotic factors prevailing in that particular natural situation but also affected by the microbial interactions (Orhan *et al*., 2006). As Tobacco is important cash crop of Pakistan and important income source in swabi district of Khyber Pakhtunkhwa. This research will provide invaluable insights of exploring the indigenous bacterial flora of healthy tobacco plants and maintenance of pure culture of isolated bacterial strains in Microbiology lab and would be used as source of biofertilizer for enhancing the growth and preventing *Nicotiana tabacum* from exogenous pathogens.

Materials and methods

Sample collection and area selection

The soil samples were collected from plants with different age groups by uprooting the plant in different fields of District Swabi. The unneeded soil particles were removed and the rhizosphere soil particles were collected into polythene bags with the help of a sterilized spatula.

Preparation of soil sample

5 grams of soil sample was added to 100 ml of distilled water and shacked so that the particles are completely dissolved into water. This suspension called master sample was carefully maintained in a beaker. Next, 9 ml of distal water was taken in 5 test tubes labeled as 1 to 5 and100ml of master sample was added to the first tube with the help of micropipette and was shaken. The same step was repeated for all 5 test tubes. One grams of soil sample was suspended in 100ml of double distilled water to make microbial suspensions (10^1 to 10^5). Dilution of 10^3, 10^4 and 10^5 were used to isolate bacteria. 100 micro liter of microbial suspension of each concentration were added to sterile Petri 12 plates (triplicate of each dilution) containing of sterile nutrient agar media. Two percent clot rim (Cotrimazole Topical Solution USP 1% w/v) was added to the media before pouring into Petri plates for preventing fungal growth. The Petri plates were then incubated at 28 + 20 ˚C in dark. The plates were visually observed for a period of three days (Warcup, 1950).

Isolation of pure culture

Considerable growth of bacterial colonies was observed on the nutrient agar media**.** After 48 hours of incubation. The Petri plates with mix microbial colonies on media were said to be the parent/master culture plates. These colonies were of different colors and morphology. The colonies were counted to be 4 in number. From the parent/master culture these different colonies were purified by inoculating the single/isolated colonies using sterilized inoculating loop on different Petri plates having nutrient agar media and subsequently incubated for 48 hours at 36°C. After 48 hours of incubation a total of13colonies were observed on different Petri plates.

The same process was repeated 3 to 4 times for all morphologically different colonies in order to get pure cultures (Naseer *et al.,* 2017).

Biochemical tests for metabolic reactions

Bacterial strains were initially characterized by using gram staining technique and detailed identification was done through eight different Biochemical tests according to standard protocols i.e. Catalase, Oxidase, triple sugar iron test, Indole, Urease, Citrate tests, Voges-Proskauer test and Methyl red test (Sharman and James, 2008).

Results

This study was performed to explore different strains of bacteria in rhizosphere of Tobacco plants. Based on Cultural, microscopic and biochemical analysis four different isolates were identified from rhizosphere of *Nicotiana tabaccum* i.e. *Micrococcus leutes* (S1), *Bacillus subtilis* (S2), *Bacillus cereus* (S3) and *Escherichia coli* (S4).

Table 1. Cultural characteristics of isolated bacteria.

S. No	Colony morphology	Color	Elevation	Margin	Texture	Pigment
S1	Filaments Colonies *M. leutes*	Beige to Yellow	Convex	Entire	Shiny, Smooth	Non-Diffusible
S2	Irregular *B. Subtilis*	White	Undulate	Undulate (Wavy)	Dry Or (Rough)	Negative
S3	Irregular *B. Cereus*	Opaque	Flattoraised	Undulate to Curled	Smooth	Negative
S4	Irregular *E. Coli*	Cream	Slightly raised	Entire	Mucoid	Negative

Cultural characteristics of isolated bacteria

Four bacterial strains were isolated from rhizosphere of *Nicotiana tabacum* on nutrient Agar. All colonies were labeled as S1, S2, S3 and S4, the color of colonies observed were beige to white, white, creamy white and brownish respectively, whereas elevation and margin of colonies showed convex and entire (S1), undulating (S2), flat to raise and undulating (S3) and slightly raised and entire (S4). The texture of colonies were shiny smooth, rough, smooth and mucoid for S1, S2, S3 and S4 respectively, Non diffusible pigment formation was observed in S1 colony, while no pigmentation occurs in other colonies.

The colony morphology recorded was filamentous for S1 and irregular for S1, S2 and S3 (Fig. 1 and Table 1).

Microscopic characteristics of isolated strains

After detailed recording of cultural characteristics all four types of colonies were subjected to Microscopy.

It was observed that S1, S2, S3 and S4 have cell sizes of 2µm, 4µm, 6.5µm and 4.5µm respectively. Three strains i.e. S2, S3 and S4 were rod shaped whereas S1 has in *Cocci* shape. The gram stain characteristics showed that S1, S2 and S3 were gram positive and S4 was gram negative (Fig 2 and Table 2).

Table 2. Microscopic Characteristics of isolated strains.

Isolates	Cellsize	Shape	Gram staining
Micrococcus letues	2mm	Cocci	Positive
Bacillus subtilis	4mm	Rods	Positive
Bacillus cereus	6.5mm	Rods	Positive
Escherichia coli	4.5mm	Rods	Negative

Metabolic characteristics of isolated strains

The metabolic characteristics of isolated strains were studied through eight different biochemical tests. S1 strain showed negative results for motility while S2, S3 and S4 were positive. All strains were Catalase positive and coagulase negative. S1 and S2 were negative for Methyl red (MR) and indole and while S3 and S4 showed positive results. Similarly S1 and S2 were Voges-Proskauer (VP) and urease Positive, and S3 and S4 showed negative results respectively (Table 3).

Table 3. Metabolic characteristics of isolated strains from rhizosphere.

S. No	Tests performed	Species Identified			
		Micrococcus letues	*B. subtilis*	*B. cereus*	*E. coli*
1	Motility	Negative	Positive	Positive	Positive
2	Catalase	Positive	Positive	Positive	Positive
3	Simon Citrate	Positive	Positive	Positive	Negative
4	MR	Negative	Negative	Positive	Positive
5	VP	Positive	Positive	Negative	Negative
6	Indole	Negative	Negative	Positive	Positive
7	Urease	Positive	Positive	Negative	Negative
8	Coagulase	Negative	Negative	Negative	Negative

Discussion

This study was performed to explore different strains of bacteria in rhizosphere of Tobacco plants. Based on Cultural, microscopic and biochemical analysis four different isolates were identified from rhizosphere of *Nicotiana tabaccum* i.e. *Micrococcus leutes* (S1), *Bacillus subtilis* (S2), *Bacillus cereus* (S3) and *Escherichia coli* (S4).

The first strain isolated in this study was *Micrococcus luteus* (S1) from tobacco rhizosphere which was non-motile, gram positive *Cocci*, non-endospore forming (Table and Fig. 1, 2 & 3).

Fig. 1. Colony morphology of isolated strains on Nutrient Agar Plate; a) *Micrococcus letueus* b)*Bacillus subtilis* c) *Bacillus cereus* d) *Escherichia coli.*

Micrococcus luteus strains are ubiquitous in the environment. In similar study it was observed that *Micrococcus luteus* cells are non-motile, non-endospore forming, Gram-positive *Cocci,* often arranged in tetrads. Creamy, yellow-pigmented colonies are typical, while cream, white or unpigmented strains have also been isolated. Biochemical analysis of similar study showed that *Micrococcus luteus* was positive for catalase, oxidase; utilization of D-glucose (Kaprelyants and Kell, 1993).

Second and third strain isolated was *Bacillus subtilis* (S2) and *Bacillus cereus* (S3) is a gram positive rods shape structure (Table & Fig. 1,2,3) the result of our study showed similar characteristics as studied in past in which they identified *Bacillus cereus* and *Bacillus subtilis* as a Gram-positive, rod-shaped, aerobic, facultative anaerobic, motile, beta hemolytic bacterium commonly found in soil and food (Ryan; Ray *et al.,* 2004) *Bacillus subtilis* is also found in association with roots of many different plants.

It exhibits many beneficial activities for the plant, *Bacillus subtilis* is widely used as a bio fertilizer.

The last strain identified in this study was *Escherichia coli* (S4) is not been identified before from rhizosphere of tobacco plant.

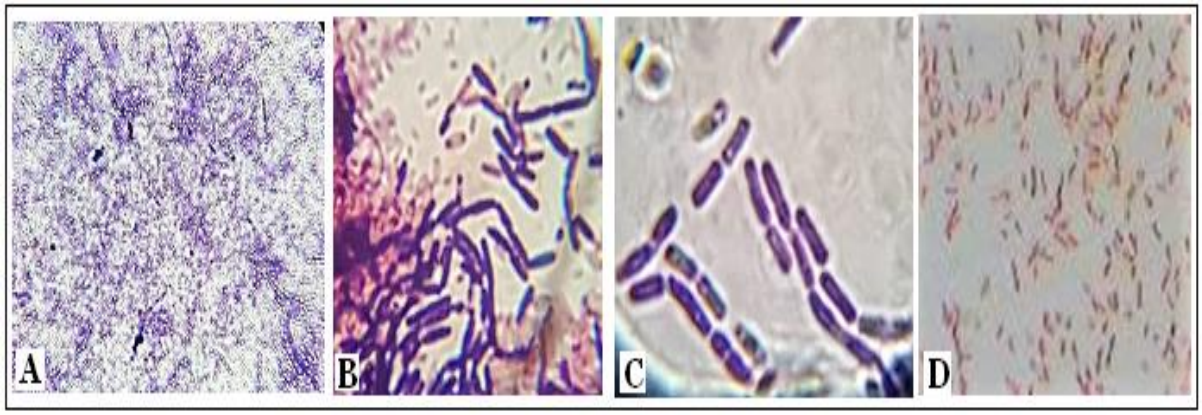

Fig. 2. Micrograph of four isolated strains; a) *Micrococcus letueus* b) *Bacillus subtilis* c) *Bacillus cereus* d) *Escherichia coli*

Further studies are recommended on this species and its effect on growth of tobacco. It is concluded from our research that the rhizosphere of *Nicotiana tabacum*is heavily populated by four species belonging to three different genera i.e. *bacillus subtilis*, *Bacillus cereus*, micrococcus and *E. coli*. These bacteria were identified through different biochemical tests and pure culture was preserved in lab of microbiology for future references. *Bacillus subtilis* and bacillus cereus can enhance plant growth. These two species would ubiquitous, saprophytic soil microscopic organisms which would thought to help supplement cycling because of its capability to process a totally assortment about proteins. These need been utilized to mechanical creation about proteases, amylases, antibiotics and chemicals. *Bacillus subtilis* has common fungicidal activity, can be utilized as bio control agent. Compound fertilizers could be decreased toward utilizing the possibility of bio inoculants which would be eco-friendly. *Micrococcus leutus*is the type genus of family Micrococcaceae are non-motile gram positive *Cocci,* non-endospore forming.

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