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Research Article

Isolation and Biochemical Characterization of Endophytic Bacteria Associated with Roots of *Salvadora Oleoides*

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Abstract

The reduction of plant diseases by the use of plant associated microbes is being increased nowadays. Microorganisms which are isolated from surface sterilized plant tissues and which have no negative impact on plant growth are called as endophytic microbes. In the present investigation, sampling of the *Salvadora Oleoides* was done from 6 different sites of Rajasthan state and endophytes associated with these plants were isolated and cultured. Then those were identified by biochemical and culture techniques. Results of our investigations confirmed the presence of *Pseudomonas syringae* in all the isolates.

Keywords: Biochemical, Culture technique, Endophytes, *Salvadora Oleoides* etc.

INTRODUCTION

Loss of a crop due to plant diseases is common problem in agricultural sector which can be controlled by using optimum agrochemicals but their excessive use may be harmful to our environment. Nowadays, applications of biological materials for plant protection are increasing which are also safe to environment (Pandya & Shelat, 2017; Urooj et al., 2018).

The reduction of plant diseases by the use of plant associated microbes is being topic of interest for many researchers (Afzal et al., 2013; Costa et al., 2013; Habiba et al., 2016; Noreen et al., 2015). Some bacteria directly supress growth of phytopathogens whiles other develop resistance into host plants against these pathogens (De Meyer & Hofte, 1997; De Meyer et al., 1999; Rahman et al., 2016).

Microorganisms which are isolated from surface sterilized plant tissues and which have no negative impact on plant growth are called as endophytic microbes (Schulz & Boyle, 2006). Nowadays they are achieving scientific and commercial interest because of their positive impacts on plant growth, reduction of diseases and induction of systemic resistance against biotic and abiotic stresses (Afzal et al., 2013; Boddey et al., 2003; Khan & Lee, 2013; Rahman et al., 2016; Ryan et al., 2008).

Among the bacterial antagonists, fluorescent *Pseudomonas* are gaining attention as biocontrol agents like rhizo bacteria (Afzal et al., 2013; Habiba et al., 2016; Tariq et al., 2009; Shafique et al., 2015). Besides siderophore production, antifungal metabolites and siderophores produced by the plant growth promoting bacteria and induction of systemic resistance in plants against pathogens are considered as mechanisms involved in biocontrol of plant diseases (DeMeyer & Hofte 1997; DeMeyer et al., 1999; Ramamoorthy et al., 2001; Shafique et al., 2015; Siddiqui et al., 2001).

In Rajasthan, *Salvadora* plants are known as miswak tree and its roots are used as a tooth brush (Chelli-Chentouf et al., 2012). They are able to tolerate a wide range of soil pH, salinity, water logging and drought (Korejo et al., 2014). It was hypothesized that plants from unique environments may harbour unique endophytes with biocontrol potential against a wide range of plant pathogens (Ehteshamul-Haque et al., 2013; Korejo et al., 2014). In the present study we tried an

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attempt to isolate and characterize endophytic fluorescent *Pseudomonas* by biochemical tests which were isolated from roots of *Salvadora Oleoides*.

MATERIALS AND METHODS

Field Survey and Collection of Seeds

Sampling of the *Salvadora Oleoides* was done from 6 different sites of Rajasthan state (Jaipur, Jhunjhunu, Khetri, Jatoowas, Jaisalmer, and Jodhpur) in India. Approximately, 250 g roots were collected from each sampling site. Collected roots were subjected for isolation of associated pathogenic bacterial strains.

Isolation and Purification of the Pathogen

Collected healthy roots of Salvadora were surface sterilized by immersion in 90% (v/v) ethanol for one minute followed by 1% (v/v) NaOCl for 10 min and then washed six times with sterile distilled water. Sterilized roots were pleated on Nutrient Agar (NA) medium and incubated for 24-48 h at 30°C. The colonies appeared under the roots were picked up and diluted by dilution series and subsequently streaked on TZC agar plates (Kelman, 1954).

Colonies showing similar characteristics were confirmed by plating on various semi selective media, and by other biochemical and molecular studies described below:

A. Identification by Morphological Characteristics

(i) Gram's staining: To study the morphological features of bacterial isolates, a smear from its suspension was prepared on glass slide; air dried and stained by Hucker's modified Gram's staining method (Lelliot and Stead, 1987). The airdried stained slides were examined under compound microscope under high power and oil immersion objectives.

(ii) Gram's KOH solubility test: From well grown colonies of pathogen, a loopful was taken on a glass slide and a drop of 3% aqueous solution of KOH was mixed gently using a toothpick to avoid any chemical reaction. A viscous thread was formed when this mixture was stretched over a few centimetres with toothpick.

B. Identification by Cultural Characteristics

a. Identification using differential media: Bacterial colonies were taken from NA, transferred, and incubated on different kind of differential media for detection and purification of isolates. The bacterial colonies were examined by naked eyes as well as under stereo-binocular microscope for the external features such as margins, elevation and colony shape**.**

b. Identification on semi-selective media: For identification of the isolates MSP agar medium (Mohan & Schaad, 1987) was used as semi-selective medium. MT medium enables identification of species of the isolates**.**

C. Identification using physiological/ biochemical methods

On the basis of morphological and cultural characteristics of colonies, the isolates were differentiated and subjected to other biochemical tests:

(i) Lopat test

a. Levan information test: Sucrose nutrient agar (5%) containing petri plates were inoculated by making dots at 4 corners and centre. These plates were inoculated for 3 days at 25±2°C in darkness for development of smooth, mucoid and domed colonies (Kovac's, 1956; Hinderbrand and Schroth, 1972; Lelliot and Stead, 1987). Observation of Levan positive or non-Levan forming colonies were taken after 24 hours regularly up to 3 days.

b. Kovac's oxidase test: In a petri plate Whatman's filter paper no. 1 was placed and 3-4 drops of freshly prepared 1% aqueous solution of tetramethyl Para phenylene diamine dihydro chloride (Kovac's solution) was added in the centre, a loopful from well grown bacterial isolate from the NA by using a platinum loop was streaked on the moistened filter paper. Colour change into pink red due to the reaction within 10 s was recorded as a secretion of oxidase enzyme by the pathogen (Kovac's, 1956; Hilderbrand and Schroth, 1972).

c. Potato soft rot: Healthy potato tubers were washed and disinfected by dipping in 95% alcohol followed by briefly flaming and cut aseptically into slices of about 7-8 mm thickness (Goszczynska T., 2000). These slices were placed on moistened sterile filter papers in the petri plates, inoculated with the test bacterial isolates individually and incubated in darkness for 3 days at 25 ± 2°C. Any rotting in the slices was taken as positive responses.

d. Arginine dihydrolase: In the test tubes containing 3 ml of Thorley's medium 2A (Lelliot and Stead, 1987). Pathogens were inoculated by stabbing with fresh bacterial culture. After inoculation the test tubes were covered with molten Vaseline and incubated for 3 days at 25 ± 2°C. Observation for change in colour from pale-yellow to pink-red was taken as positive reaction.

e. Tobacco hypersensitivity test: From 24 hours old cultures (10.8-10.9 cfu/ml, OD 0.3-0.4 at 600nm) of the test bacterium aqueous suspension was prepared add in-filtered into intercellular spaces of the tobacco (*Nicotiana tabacum* L.) leaves by an infiltration method (Klement, 1963).

f. Gelatine liquefaction: In test tubes containing semi-solid gelatine agar medium, bacterial isolates were stab inoculated. The inoculated tubes were observed for gelatine liquefaction after 3 days specifying gelatine utilization by the test bacterium (Lelliot and Stead, 1987).

g. **Aesculin hydrolysis**: Petri plates containing aesculin medium were inoculates and incubated for 2-5 days (Goszczynska T., 2000). Development of dark colour showed the presence β-galactosidase activity.

h. **Tyrosinase activity test**: On petri plates containing L-tyrosine, isolates were inoculated and these were incubated for 2-5 days (Goszczynska T., 2000). Appearance of red to reddish-brown, diffusible pigment indicated tyrosinase activity.

i. **Tartrate reduction test**: On petri plates containing tartrate, isolates were inoculated and these were incubated for 2-5 days (Goszczynska T., 2000)

j. **Starch hydrolysis**: Starch hydrolysis agar medium containing petri dishes were inoculated by streaking the test bacterium (Schaad and Kendrick, 1975) and were incubated at 30°C in darkness for 4 days. Then plates were sprayed with Lugol's iodine solution and observed for change in colour of medium from translucent to blue showing a positive indication of starch hydrolysis by the test bacterium (Sharma et al., 2002).

k. **Nitrate reduction test**: A semi-solid medium containing nitrate was prepared and used to study the nitrate reducing capability of the isolates. The inoculated sterilized tubed were incubated at 25 ± 2°C for 3-7 days. a few drops of solution 1 (consisting starch 0.4 g, $ZnCl_2$ 2g, distilled water 100 ml and 0.2% KI in equal amounts), and solution 2 (consisting HCl and distilled water 56 ml) were added to incubated test tubes and change of colour of the medium from light to blue was recorded as the positive test by the bacteria by (Fahy & Pershey, 1983).

l. **Catalase test**: On 24 hours old colony, few drops of 3% H_2O_2 were added. Production of gas bubbles showed positive reaction (Goszczynska T., 2000).

m. **Sorbitol utilization test**: On petri plates containing sorbitol, isolates were inoculated and incubated for 3-5 days (Goszczynska T., 2000).

RESULTS

Isolation and cultural condition of isolates: A total 6 pathogenic bacterial strains were isolated and purified from roots of *Salvadora* plants growing at 6 sampling sites in Rajasthan (one strain from each sampling site). All bacterial isolates showed pink boundaries in Gram's staining showing all isolates to be Gram negative. Formation of viscid thread by Gram's KOH solubility test confirmed all isolates to be gram's negative.

All the bacterial colonies showing whitish, smooth and mucoid on NA medium and fluorescent on KB were taken as *Pseudomonas spp*. (differential medium). Colonies of all isolates were characterized as creamy white, flat and circular with a diameter of 4.5-5 mm on MT medium and no zone of hydrolysis was produced around colonies which showed the presence of *pseudomonas spp*. (Mohan & Schaad, 1987).

Results of biochemical test: Results for all biochemical tests are shown in (Table 1). All the isolates showed positive reaction for Levan formation test, Tobacco HR test, catalase test, gelatine test, aesculin hydrolysis, and sorbitol utilization test while all isolates showed negative reaction for Kovac's oxidase test, potato soft rot test, arginine dihydrolase test, tyrosinase activity, metabolism of tartrate, nitrate reductase test, and starch hydrolysis test. Results of LOPAT (+---+) test, and GATTa (++--) test confirmed the presence of *Pseudomonas syringae* in all the isolates. Positive results of other biochemical tests also confirmed the presence of *Pseudomonas syringae* (Figure 1, 2 & 3).

Table 1. Biochemical Test and their results for the isolates.

Name of Test	Result
Levan formation	+
Kovac's oxidase	-
Potato soft rot test	-
Arginine Di hydrolases test	-
Tobacco HR test	+
Catalase test	+
Gelatine liquefaction	+
Aesculin hydrolysis	+
Tyrosinase Activity	-
Metabolism of Tartrate	-
Nitrate reduction test	-
Starch hydrolysis	-
Sorbitol utilization test	-

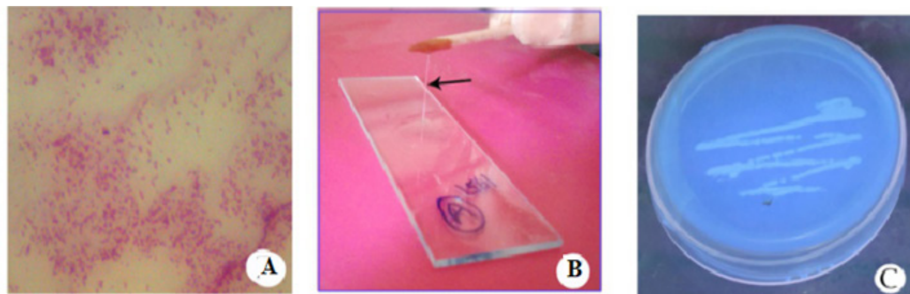

Figure 1. Cultural characterization of the isolate (A- Gram staining, B- KOH solubility test, C- Fluorescence on modified King's B medium).

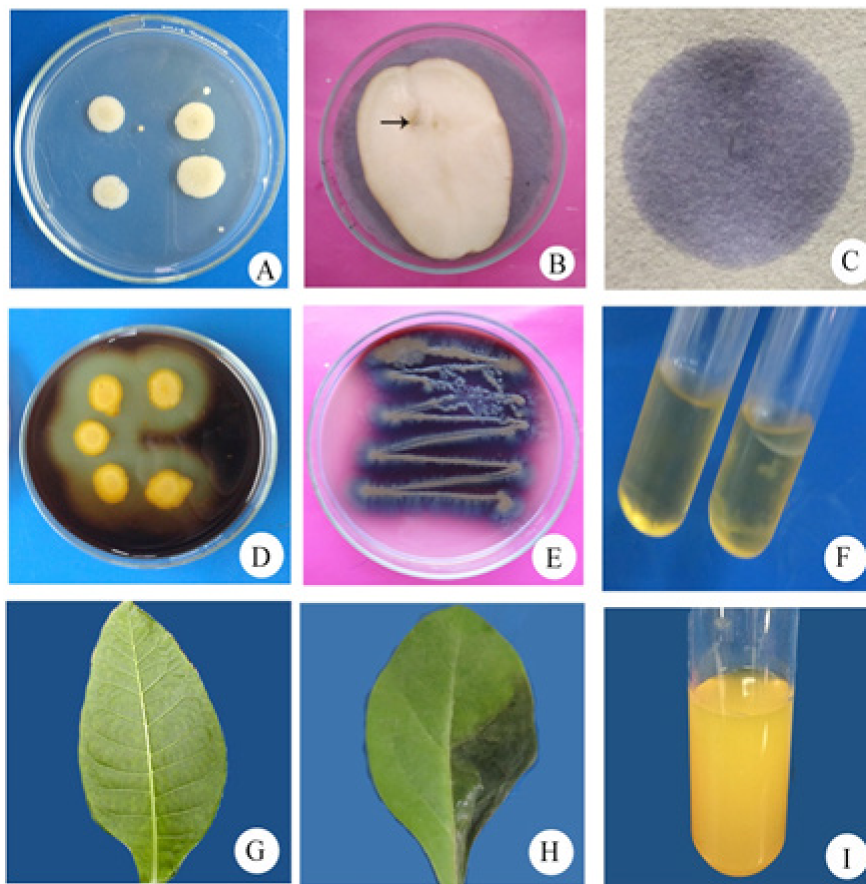

Figure 2. Biochemical characterization of the isolate. A- Levan production (+ve), B-Potato soft rot (-ve), Oxidase (-ve), Starch hydrolysis (+ve), Aesculin hydrolysis (+ve), gelatine liquefaction (+ve), G- Tobacco hypersensitivity test of sample, H- Tobacco hypersensitivity test of control, I- Arginine dihydrolase (-ve).

DISCUSSION

Many bacteria are present in soil which are generally found on surface of roots to obtain nutrients but sometimes these bacteria enter inside plant tissues (Prieto et al., 2011; Rosenblueth & Mattinz-Romero., 2006) or these may enter aerial parts of plants (Romero et al., 2014). Among different soil bacteria, *Pseudomonas* is a common bacterium which is found around the plant roots or inside the roots (Tariq et al., 2009).

In this study, fluorescent *Pseudomonas* was isolated from roots of *Salvadora Oleoides* collected from different locations. By cultural or biochemical characterization, these were confirmed as *Pseudomonas syringae*.

By many studies, it has been reported that endophytic bacteria also have positive effects on host plant as these may supress pathogens and are useful for growth promotion of the host plant (Afzal et al., 2013; Hallmann et al., 1997; Tariq et al., 2009). Bacteria found within roots are useful because they

can enhance plant growth by promoting production of plant growth hormones (Inam-ul-Haq et al., 2012; Weller, 2007).

In various studies, it was reported that application of endophytic fluorescent *Pseudomonas* suppressed infection of root rotting fungi and the root knot nematode. They also improved plant growth (Kloepper, 1993; Lazarovits & Nowak, 1997). Growth of host plant can be promoted by various mechanisms like production of stimulatory phytohormones, lowering of the ethylene level, liberation of phosphates and micronutrients etc. (Anton & Prevost, 2005). These bacteria suppress spread of pathogens by production of certain antibiotics (Raaijmakers et al., 2002) and siderophores (De Meyer & Hofte, 1997).

Raaijmakers & Weller (1998) reported the role of 2, 4-diacetyl-phloroglucinol, an antifungal metabolite from species of fluorescent *Pseudomonas* in plant root disease suppression. Like P. fluorescens and P. putida, P. aeruginosa has also been reported as endophytic bacteria showing activity against plant pathogenic fungi and parasitic nematodes (Afzal et al., 2013; Kumar et al., 2013 Tariq et al., 2009). Due to their ability to colonize plant tissues internally, endophytic bacteria are now gaining importance (Prieto et al., 2011), an ecological niche similar to that of phytopathogens (Berg et al., 2005)

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