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Plant Growth Promoting Potential of Bacterial Endophytes in Novel Association with *Olea ferruginea* and *Withania coagulans*[1]

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Abstract—Microbially unexplored medicinal plants can have a genetically diverse microbial population with multi-functional plant growth promoting traits. In this aspect, 75 endophytic bacterial isolates with plant growth promoting traits were isolated from *Withania coagulans* Dunal and *Olea ferruginea* Royal. Many of these bacteria were able to solubilize phosphate, produce indole-3-acetic acid, ammonia as well as hydrogen cyanide, synthesize extracellular enzymes and show antagonistic activities against plant pathogenic fungi under in vitro conditions. These isolates were also characterized by morphological and biochemical analysis. Furthermore, four representative isolates with pronounced plant growth promoting activities were identified as *Enterobacter cloacae*, *Enterobacter dissolvens, Enterobacter hormaechei* and *Cronobacter sakazakii* by 16S rDNA sequencing analysis. This work for the first time, reported the isolation of endophytic bacteria, the novel association form selected plants, *Withania coagulans* and *Olea ferruginea*. The explored endophytes might have great potential in the field of biocontrol and plant growth promoting for sustainable agricultural practices.

Keywords: biocontrol agents, endophytic bacteria, PGP traits, symbiosis

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Endophytic bacteria by definition are those bacteria that colonize the internal tissue of the plants either symbiotically or in a mutualistic relationship (Holliday, 2001; Dudeja et al., 2012). Likewise, soil carries a rich pool of microbial inoculum for rhizospheric endophytes that readily colonize and exclusively invade host tissues. The microbial community isolated from rhizosphere is of special importance to the plants (Dudeja et al., 2012). Therefore, endophytes that infect plants from the soil must be competent root colonizers. The variation in the endophytic communities can be attributed to bacterial species, plant genotype, plant developmental stage, colonizing tissue type, soil type and environmental conditions (Kobayashi and Palumbo, 2000). Monocotyledonous as well as dicotyledonous plants and their compartments (i.e. roots, stem, leaves, seeds) have been explored for endophytic bacteria (Posada and Vega, 2005). Different endophytes have been identified from different plant tissues. The biodiversity of endophytes can be judged by the potential of microbes to colonize the tissues and persist in the plant (Rosenblueth and Mart nez-Romero, 2006). Molecular diversity of endophytic bacteria in a single plant is not limited to a single species, but comprises a number of different genera and

ethylene production (Glick, 1995; Tudzynski and Sharon, 2002). On the other hand, indirect mechanisms include suppression of pathogen infection via antifungal or antibacterial agents. The other indirect mechanisms include assisting plants in acquiring nutrients via phosphate solubilisation, nitrogen fixation and siderophores production. Besides these mechanisms, plant-associated microorganisms improve nutrient acquisition by supplying minerals and other micro/macro nutrients from the soil (Caldwell et al., 2000; Barrow, 2003). Therefore, isolation and characterization of endophytic bacteria with various properties from unexplored hosts will have much applications to improve plant growth promotion (Patten and Glick, 2002; Sergeeva et al., 2007).

Medicinal plants provide valuable therapeutic agents in traditional medicines which are used on a global level for a wide variety of human health issues. It has been rationalized that plants having an ethnobotanical history may harbor an endophytic population. These medicinal plants produce a plethora of microbial metabolites related closely to the plant biochemistry. In the present study, *Withania coagulans* and *Olea ferruginea* were selected for exploration of endophytes, on the basis of their wide medicinal importance. So, many interesting groups of bacterial endophytes with diverse roles in plant physiology can be expected from root, stem and leaves of both plants. Studies on isolation of bacterial endophytes from *W. coagulans* and *O. ferruginea* is not available, the study is significant and novel in its approach.

MATERIALS AND METHODS

Isolation of Bacterial Endophytes

The plants were collected in August of 2013 from Sabir abad, district Karak, KPK, Pakistan (33.14618 latitude, 71.18926 longitude) as the source material for bacterial endophytes isolation. Surface disinfection was conducted by using serial washing with 70% ethanol for 5 min, 0.1% $HgCl_2$ for 1–2 min and then double distilled water, 5–6 times for 2–5 min. A 100 μL sample of the water from the third rinse was plated on 869 medium (Mergeay et al., 1985) to verify the efficiency of sterilization. Three replicates of each sterilized plant part samples were transferred into test tubes containing 5 mL semi-solid non selective medium. These samples were incubated for 7 days at 28°C and observed for the growth of endophytic bacteria (Chaudhary et al., 2012).

SDS-PAGE of Whole-Cell Crude Protein

The methods of cell preparation, protein extraction, data analysis and gel electrophoresis was followed as described by (Kiredjian et al., 1986) and used to estimate the diversity of bacterial isolates.

Biochemical Characterization of Endophytic Bacteria

Bacterial cultures were screened for physiological and biochemical characters through molecular identification kits, QTS 24 miniaturized identification system (DESTO Laboratories Karachi, Pakistan). Bacteria were cultured for 24 h in nutrient broth medium at 35 ± 2°C to inoculate QTS kits.

Evaluation of Plant Growth Promoting (PGP) Traits

Phosphate solubilization by endophytes. Bacterial isolates were screened for their potential to solubilize insoluble calcium phosphate on Pikovskaya agar as described by Pikovskaya (1948). Bacterial colony was placed on the center of Pikovskaya agar media plates with the help of sterile loop and incubated at 30°C for 7 days. The phosphate solubilizing efficiency was measured on the basis of halo zones around the colonies by following the formulae of (Nguyen et al., 1992; Qureshi et al., 2012) and was measured by:

$$\mathrm{SE} = \frac{\textit{solubilization diameter (cm)}}{\textit{growth diameter (cm)}} \times 100.$$

Quantitative estimation of indole-3-acetic acid (IAA). Production of indole-3-acetic acid was estimated for all bacterial isolates by inoculating 500 μL of 24 h old bacterial suspension in 50 mL of broth containing 0.1% DL-tryptophan and kept in a shaker incubator at 30 ± 2°C for 5 days at 180 rpm in the dark. IAA concentration in the culture supernatant was estimated by mixing 4 mL of salkowski reagent in 1 mL of supernantant and absorbance of the resultant pink color was read after 30 min at 535 nm in UV/Visible Spectrophotometer. The IAA production was measured from the regression equation of a standard curve and the result was expressed as μg mL^{-1} (Gordon and Weber, 1951).

Ammonia production. Bacterial endophytes were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 mL peptone broth in each tube and incubated at 28 ± 2°C for 48–72 hrs. Nessler's reagent was added to each tube after incubation. The development of faint yellow to dark brown color was a positive test for ammonia production (Cappuccino and Sherman, 1992).

Bio-Control Activities of Isolated Strains

Screening of isolates for HCN. Qualitative determination of hydrogen cyanide (HCN) were carried out for all bacterial isolates by adapting the method of Lorck with few modifications (Lorck, 1948). Bacterial isolates were streaked on nutrient agar medium supplemented with 4.4 g L^{-1} of glycine. The production of cyanide was detected by placing Whatman filter paper no. 1 soaked in 0.5% picric acid on the inner side of the petri dish lids. Development of brown to red color