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First report of Isolation and Characterization of *Bacillus amyloliquefaciens* from *Artemisia dracunculus in* Iran

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Abstract

Endophytice bacteria promotes plant growth of different herbals by various process such as nitrogen fixation, solubilization of phosphate, production of phytohormones, sidrophore, posing antagonistic activity and induce high biotic and abiotic resistance for plants. Sustainable agriculture emphasizes yield improvement without negative affect on environment, Through the current study, the presence as *Bacillus amyloliquefaciens* endophytic bacteria was identified from *Artemisia dracunculus* in Iran for the first time, By further research about plant growth promoting feature of them, medicinal plant-based industries improved to achieve their commercial success and plant maintenance facilitate in sustainable ecosystems.

Keywords: endophytic bacteria**,** *Atrimisia dracunculus***,** *Bacillus amyloliquefaciens* Medicinal plants

Introduction

The genus of *Artemisia dracunculus belongs* to Asteraceae family. This genus is one of the most economically important herbal in many areas. *Artemisia dracunculus* is cultivated as culinary and medicinal purposes (Swanston-Flatt et al, 1991). This genus is industrially important and it is known for its antifungal, antibacterial and allellopathic properties (Ved and Goraya, 2008). Endophytic bacteria can be defined as those bacteria that can be isolated from healthy, superficially disinfected plant tissues and do not cause any damage to the host plant and these endophytes use many mechanisms to gradually adapt to their living environments (Compant *et al*, 2005). They are ubiquitous; colonize more plants and different plant part structures. Their association with their host plant is complicated. There are some reports which indicate they can be obligate or facultative. The population density of endophytic bacteria can vary from 10^2 to 10^9(Misaghi and Donndelinger, 1990) depends on age, plant genotype, structure of host plant and also environmental factor such as Carbon dioxide, Oxygen concentration and etc. (Compant *et al*, 2005). It seems to have beneficial effects on their hosts(Ulrich *et al*, 2008). Endophytic bacteria can produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which decrease plant ethylene levels. This microorganism also able to produce Auxin and Gibberellins that promotes plant growth. As it mentioned endophytic bacteria are more important, therefore in this research, we have tried to find endophytic bacteria in *Artemisia dracunculus* and then in supplementary research, we are going to test plant growth promoting characteristics in order to save this valuable medicinal herb from extinction.

Plant material

Samples were collected from different part of *Artemisia dracunculus* during spring of 2015. The plants which collected were free symptom. The collected samples were washed in running water totally, and then it was disinfected superficially through the following protocol: sodium hypochlorite (2.5%) for 4 min, ethanol for 30 s, Tween 80% for 1 minute and finally 3 rinses in sterile, distilled water. then the final wash was spread plated on to nutrient agar plate (g/L; peptone 5, beef extract 2, yeast extract 3, sodium chloride 5 and agar 18, pH 7.0) and King B plate (g/L; protease peptone 10, anhydrous K_2HPO_4 1.5, glycerol15, $MgSO_4$ 1.5) as control, then at 28 ºC for 15 days and the plates are examined for the presence or absence of microorganism growth colony. The samples were ground with 50 micro liter sterile water using a sterile mortar and pestle. The tissue extract was maintained to release of endophytic bacteria then with sterile loop spread on nutrient agar and King B plate. All plates including the control were incubated at room temperature for 14 days and observed daily for bacterial growth. Morphologically definite colonies were determinated, purified and used for further studies.

Identification and phylogenetic analysis of endophytic bacteria

DNA from each isolate was extracted using the following protocol: 1.5 ml of a 24-hour bacterial culture was centrifuged for 5 minutes at 14000 *g* and resuspended in 1 ml of TE buffer (mM Tris-HCl, 1 mM EDTA, pH 8.0), centrifuged, resuspended in 500 µl of TE buffer and ultimately adding 0.5 g of glass pearls (0.1 mm in diameter) (Sigma-Aldrich, USA) and 15 µl of 20% SDS. The cells were then homogenized for 30 s in a vortex mixer (AP56 – Phoenix), 500 µl of buffered phenol was added, and the solution was mixed and centrifuged for 5 min at 14000 *g*. The aqueous phase was extracted once with phenol-chloroform (1:1) and once more with chloroform. Following the extraction of the aqueous phase, 20 µl of 5M NaCl was added; the DNA was precipitated with isopropanol and collected by centrifugation for 10 min at 13000 *g*. The DNA pellet was washed with 70% ethanol, air dried and resuspended in 30 µl of autoclaved, ultrapure water. The amplification of 16S rDNA was accomplished in a reaction with a final volume of 25 µl containing 1 µl of total DNA, 0.5 µl of the RpF(AGAGTTTGATCATGGCTCAG) 0.5 µl of the Fd2(AGAGTTTGATCATGGCTCAG), 1.6 µl (200 µM) of each dNTP,12.5 µl of Master mix and 10.5 µl of dH_2o. A negative control (PCR mix without DNA) was included in all PCR experiments. The PCR reaction conditions were as follows: 95ºC for 4 min one cycle, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 51ºC for 1 min and extension at 72ºC for 2 min, before a final extension at 72ºC for 7 min. The PCR products were purified and sequenced by Macrogen Inc. (Seoul, South Korea). The identification of the isolates was performed using the Ribosomal Database Project Then with Bioedit we checked forward and Reverse sequences and made consensus one. The consensus sequence blasted in NCBI

(http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi). (Zhang et al, 2000). The phylogenetic analysis of the 16SrDNA sequences of the isolates obtained in the study was conducted with MEGA 6 using neighbor-joining method with 1,000 bootstrap replicates (Tamura et al, 2011).

Results and Discussion:

Fresh and cleaned plant tissue of *Artemisia dracunculus were* used for the isolation of endophytic bacteria. The Leaf and stems were surface sterilized to remove the epiphytic microorganisms. The surface sterilization procedure for the isolation of endophytic bacteria as standardized in the experiment was quite satisfactory as no growth appeared on the control plate. Molecular identification of the isolates was done by sequencing part of the 16S rDNA. The amplification of the 16S rDNA was confirmed by agarose gel electrophoresis which was shown in figure 1.

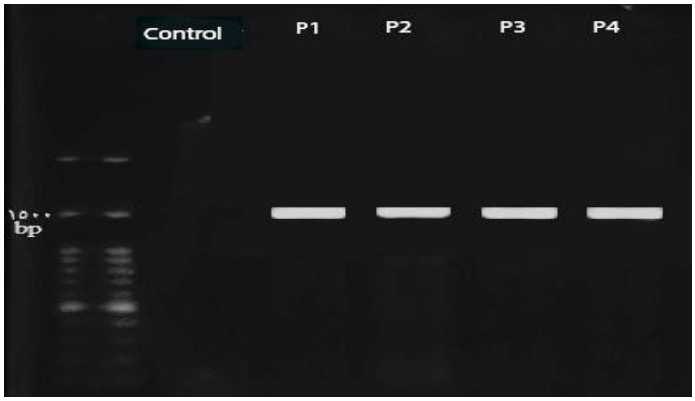

Fig 1. PCR amplification with 16 srDNA

In this experiment diverse community of endophytic bacteria associated with *Artemisia dracunculus* isolated and identified by 16SrDNA PCR and sequence analysis. The 1500 bp fragment confirmed and several time replicated till sharp bands were obtained. Based on nucleotide sequence, as endophytic bacteria characterized from the leaf and stem. The phylogenetic analysis of 16S rDNA sequence of the isolates along with the sequences retrieved from the NCBI was carried out with MEGA 6 using the neighbor-joining method with 1,000 bootstrap replicates. The result of phylogenetic analysis indicaeted in Fig2.

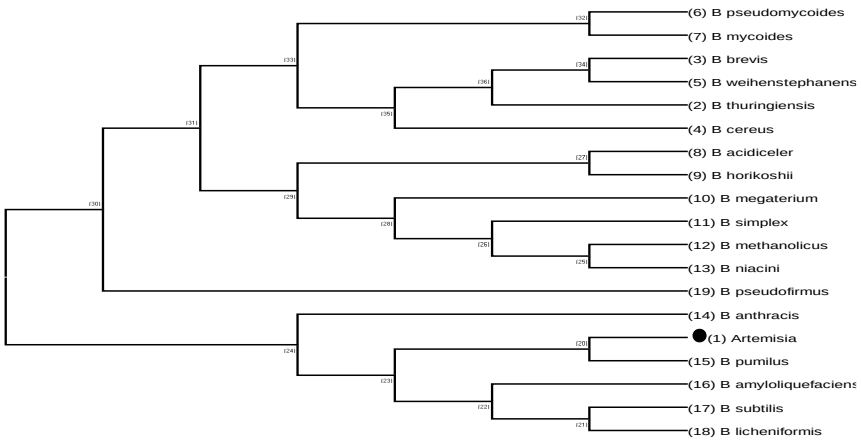

Fig2-Phylogenetic tree showing the relationship between the 16S rDNA gene sequences from representative isolates of endophytic bacteria (Bacillus strain type) with isolate of this research(indicate in bold circle).

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