Isolation and characterization of an endophytic bacterium related to
Rhizobium/Agrobacterium from wheat (Triticum aestivum L.) roots

Endophytic bacteria live in plant tissues without causing substantive harm to the host or gaining any benefit other than a non-competitive environment inside the host. Bacterial endophytes have been isolated from surface-sterilized plant tissue or extracted from internal plant tissue. A number of facultative endophytes have been reported from rice, maize, wheat, sorghum, cotton, potato, and Arabidopsis. Endophytes enter the plant tissue primarily through the root zone; however, aerial portions of plants, such as flowers, stems and cotyledons may also be used for entry. Endophytes either become localized at the point of entry or are able to spread throughout the plant and such isolates can live within cells, in the intercellular spaces, or in the vascular system.

The potential of endophytic bacteria to fix nitrogen and promote plant growth has renewed the interest in such associations. The presence of diazotrophic endophytic bacteria has been identified from kallar grass, sugarcane, rice, cotton, potato, and maize. A number of facultative endophytes have been reported from wheat roots. Bacterial endophytes have been isolated from internal plant tissue extracted from surface-sterilized plant tissue or ex-tractive water to remove adhering soil and root debris. Attempts to isolate diazotrophic endophytic bacteria from wheat have not been successful, although a few actinobacteria, including Streptomyces, Microbiobiospora and Micromonospora have been identified as endophytes of wheat roots.

In the present study a new endophytic bacterium belonging to the group Rhizobium/Agrobacterium has been identified from surface-sterilized wheat roots.

Wheat plants of different cultivars (WH711, WH736, WH755, PBW343, Raj 3765, and Sonalika) were uprooted 75-90 days after sowing from Agronomy area of CCS Haryana Agricultural University, Hisar (India). Roots were washed in running water to remove adhering soil and detritus. Roots were cut into 1 cm pieces; surface sterilized and endophytic bacteria were isolated using Luria Bertani (LB) and diazotrophic medium (DM), as described elsewhere. Bacterial isolates were picked from the DM plates and purified by streaking 2-3 times. In this manner, a total of 150 isolates were recovered, which were further characterized for the production of indoleacetic acid (IAA) and stimulation of wheat root and shoot elongation, as described earlier.

For genotypic characterization, isolate 24 was grown in TY medium for 48 h and total DNA was isolated using the CTAB method. The 16S rDNA was amplified using the primers 41F and 1488R (ref. 19) under the following conditions: one cycle of denaturation at 94°C for 3 min; 30 cycles at 94°C for 30 s, annealing at 48°C for 30 s, elongation at 72°C for 1 min and final elongation at 72°C for 10 min. The amplified DNA was partially sequenced using forward primer 41f on an ABI Prism 3700 (Applied Biosystems, USA) DNA sequencer. The 16S rDNA sequence of isolate 24 was compared to the NCBI data bank using the Blast X 2.2.9.

To study colonization of wheat root, isolate 24 was tagged with constitutively expressed gusA/gfp genes. For this, plasmid pHRGFPGUS containing the gfp and gusA genes expressed under the control of a gentamycin promoter was introduced by biparental mating using E. coli S17-1, as described previously. Plasmid pHGFPGUS (kindly provided by F. O. Pedrosa, UFPR, Curbita, Brazil) is a derivative of plasmid pBBR1, which was a small (2.6 kb), broad-host range plasmid and stably maintained in a number of Gram-positive and Gram-negative bacteria. After transfer of the plasmid, derivatives showing green fluorescence on a UV transilluminator and forming blue colonies on TY medium containing X-GlucA substrate were selected for colonization. Wheat variety (Feldkronen) was surface-sterilized using 2.5% sodium hypochlorite for 3 min and washed 4-5 times with sterilized distilled water. Seeds were germinated for 24 h on 1% agar. Isolate 24 tagged with gfp and gusA was grown in TY broth with kanamycin for 48 h. One ml (about 10⁶ cells) was centrifuged at 8000 rpm for 1 min and washed with sterile distilled water three times. The pellet was resuspended in 1 ml

![Figure 1](image-url)
sterile water. Ten germinated seeds were soaked in 1 ml culture and transferred to 100 ml tubes (25 x 150 mm) containing 25 ml YEMA salts with 1% agar, but without mannitol and yeast extract. After 10 days of growth in an environmental chamber (22°C, 10 h daylight), plants were removed from the tubes and sections were cut. Small root segments (0.5 cm) were fixed in 5% paraformaldehyde and 10% sucrose for 24 h, dehydrated in a graded ethanol series, and embedded in 1% agarose and cut in ultra thin sections (50 µm) on a vibratome Leica VT 1000S. The sections were observed and documented on a confocal laser microscope.

Endophytic bacteria were isolated from all six wheat cultivars; however, extent of colonization varied. Bacterial counts ranged between 3.5 x 10^6–5.1 x 10^7 and 1.2 x 10^7–3.5 x 10^8 CFU g^-1 roots on LB and DM medium, respectively (Table 1). Such variations may be due to plant source, rooting pattern and soil conditions. Similar variations in the population of endophytic bacteria have been observed in alfalfa, sweet corn, sugar beet, cotton, potato and sorghum 8,24. Strain no 24, isolated from cultivar PWB 343, produced maximum level of IAA (4.6 µg ml^-1) and was selected for further studies. Re-inoculation of wheat cultivar PWB 343 with isolate 24 increased the seedling shoot and root length by 63.1 and 26.6% compared to uninoculated control (data not shown). Inoculations of endophytic bacteria on different hosts have been described to improve seedling vigour 10,11,28. The count of endophytic bacteria from inoculated wheat roots was 4.88 log CFU g^-1 roots after 14 days.

A large and diverse range of endophytic bacteria like Herbaspirillum seropedicae, Serratia marcescens IRBG500 (Gyaneshwar et al.30), Pantoea sp., Ochrobactrum sp.30, Azorarcus sp.31, R. leguminosarum bv trifolii, R. etli, Rhizobium sp., A. caulinodans, photosynthetic Bradyrhizobium, Bacillus licheniformis, B. endophyticus, B. pumilus, B. subtilis and B. amyloliquefaciens have been identified as endophytes 10,11,28. Recently, filamentous actinobacteria and some fungi have been identified as endophytes of wheat roots 12,13. This is a report of bacteria belonging to the Rhizobium/Agrobacterium group as wheat root endophytes.

![Figure 2](image-url)

**Figure 2.** Wheat roots showing the presence of bacterial cells in the intercellular spaces (a) and epidermal cells (b), and vascular tissue (c). The infection is spreading to the intercellular spaces and vascular tissues (d).

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>LB CFU g^-1</th>
<th>DM CFU g^-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raj 3765</td>
<td>1.1 x 10^6</td>
<td>5 x 10^2</td>
</tr>
<tr>
<td>PBW 343</td>
<td>1 x 10^6</td>
<td>5.1 x 10^4</td>
</tr>
<tr>
<td>WH 711</td>
<td>5.1 x 10^5</td>
<td>3.5 x 10^3</td>
</tr>
<tr>
<td>Sonalika</td>
<td>2 x 10^5</td>
<td>1.2 x 10^3</td>
</tr>
<tr>
<td>WH 736</td>
<td>4 x 10^2</td>
<td>1.6 x 10^3</td>
</tr>
<tr>
<td>WH 755</td>
<td>3.5 x 10^3</td>
<td>1.2 x 10^2</td>
</tr>
</tbody>
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Dactylorhiza hatagirea (D. Don) Soo – a west Himalayan orchid in peril

Dactylorhiza hatagirea (D. Don) Soo (family Orchidaceae), a high-value medicinal plant, is reported to occur in temperate to alpine regions (2500–5000 m asl) in India, Pakistan and Nepal. It is categorized as critically endangered (CAMP status), critically rare (IUCN status) and is listed under appendix II of CITES. Besides these, being an orchid, D. hatagirea can be considered as an inherently slow-growing and poorly regenerating species because of pollinator specificity and requirement for mycorrhizal association. Thus it becomes more important from conservation point of view. Further the species is categorized as near endemic. All these attributes call for conservation of the target species.

To formulate the conservation plan for a particular area and to understand the ecology of the species, studies on quantitative information play a vital role. Since studies on extent of availability of high-value medicinal plants in wild are essential to develop appropriate strategies for their sustainable use, the present study focuses on assessment of quantum of availability of D. hatagirea in its natural habitats.

Six populations, namely Valley of Flowers (VoF), Nagtal, Pindari, Lata, Donidhar and Kedarnath in Uttarakhand Himalaya were selected for the study. Site characteristics of the species are presented in Table 1. Three belt transects (200 m long and 20 m wide) were laid in each population. Transects were divided into three strata (i.e., base, middle and top) and three plots (20 × 20 m) were marked in each strata. Fifteen (1 × 1 m) quadrats were laid randomly in every plot. Number of individuals of all the species was recorded in each quadrat. The target species was localized and not distributed uniformly. Hence the calculated density represents the density of the species in its habitats. Quadrat data were analysed for frequency, density, abundance, relative density (RD) and abundance/frequency (A/F) ratio. Data were pooled for plots in each site. To assess the difference among density of the species at different sites,