Screening of endophytic diazotrophic bacteria in wheat under saline conditions from central regions of Uttar Pradesh

SUMMARY OF

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Soil salinity and sodicity are some of the major constraints to global cereal production that affect the expression of potential for development, growth and reproduction of the wheat plants. The presence of excess salts also interferes with nitrogen nutrition in a direct and indirect way, usually simultaneously. Nitrogen serves as a major component of amino acids (the building blocks of proteins) and chlorophyll utilized by plants for photosynthesis. The nutrient is essential to life and plays a very important role in plant growth, development, and reproduction. Microorganisms are the most natural inhabitants of diverse environments possessing enormous metabolic capabilities to mitigate various abiotic stresses such as drought, salinity, low or high temperatures. The ability to biologically fix N is limited to certain microbes which involve the representatives of various bacterial phylogenetic groups, which are collectively called as diazotrophs. Common wheat *(T. aestivum)* is a widely cultivated and significant cereal crop occupying the prime position among the 'big three' food crops which in India is usually cultivated in the months of October-November and harvested around April. It is typically milled into flour which is known to be used to make a wide range of foods including bread, noodles, pasta, biscuits, cakes, pastries, cereal bars, sweet and savoury snack foods, crackers, crisp-breads, sauces and confectionery. Enriched wheat flour is known to be a good source of iron, calcium, and vitamin B6 and also a decent source of several vitamins and minerals, including selenium, manganese, phosphorus, copper, and folate. In order to obviate the complexities of abiotic stress and increase the growth and yield of wheat plant, we need to recline towards sustainable agricultural alternatives such as use of PGPE in respect to plant growth promotion and mitigation of abiotic stresses. Application of endophytes in the form of bioinoculants are

considered to be an eco-friendly, biotic, and safe substitute to chemical fertilizers that will maximize the productivity of *T. aestivum* without causing ecological disturbances in the soil.

In the present study, a total of 42 bacterial endophytes were isolated from the root tissues of wheat plant collected from Lucknow and adjoining districts. Plant roots exudes a lot of organic compounds which stimulates microbial growth and can have a major impact on the composition of the microbial communities. Bacterial endophytes colonizing the plants are diverse in nature and help in proper functioning of their host under abiotic as well as biotic stresses. All the isolates were characterized on the basis of morphological, physiological, biochemical and molecular basis and showed multifariousness in their size, shape, color, and growth pattern. 31 bacterial endophytic isolates were found to be Gram negative in nature and 47.61% isolates showed positive results for motility. The endophytic isolates were also found to be positive for the utilization of various carbon sources (glucose, dextrose, lactose, galactose, sucrose and maltose) and nitrogen sources (yeast extract, potassium nitrate, sodium nitrate, ammonium chloride, ammonium sulphate, and tryptophan). Further, isolates were also checked for their growth over different pH, temperatures, and salt stress conditions. It was observed that the optimum temperature of isolated endophytes was 28ºC and maximum number of isolates showed growth at ranges of pH (6 and 8). In the case of salt stress, most of the bacterial isolates were able to grow upto 2%, 4%, and 6% salt concentration whereas very few (26.1% and 9.5%) were able to tolerate 8% and 10% NaCl. Biochemical assays showed endophytic isolates to be also positive for catalase (all), amylase (50%), protease, lipase (30.9%), citrate utilization (35.7%), MR test

(30.9%), VP test (21.4%), and ammonia production (all). On the basis of the biochemical properties. it was observed that the results were relatively diverse in nature and were displayed by significantly different bacterial endophytic communities. Next, the endophytic bacterial isolates were characterized for their plant growth promoting attributes and most of them were demonstrated to be positive for siderophore production (69%), IAA production (52.3%), phosphate solubilization (45.2%), zinc solubilization (30.95%), and 19% have been found positive for potassium solubilization. Whereas production of HCN was observed by none of the endophytic isolates. Qualitative ACC deaminase assay of the selected isolates was also performed and it was found that eight isolates (19%) were able to grow on the DF minimal salt medium indicating the positive test result.

All the isolates were screened for their potential to fix N by checking their growth on Jensen's medium, detecting the presence of *nifH* gene, and nitrogenase activity by acetylene reduction assay (ARA). 59.5% endophytic isolates were able to efficiently grow on N free medium and twenty-three of the total isolates (54.7%) were demonstrated to be positive for the amplification of *nifH* by yielding and end product of ~390 bp length. On the basis of the screening, seven endophytic isolates i.e., AU15, CP18, PD22, PD25, KA28, KA31, OC36 were chosen for further experimental work as the isolates showed best results for nitrogen fixation, PGP traits as well as salt tolerance.

Colonization efficiency of these selected isolates were investigated further and it was noted that the isolates were able to reinfect the roots of host plant i.e., wheat which was confirmed by re-isolation from the root tissues of treated seedlings and scanning

electron microscopy. Electron micrographs of plant roots colonized by inoculated endophytes under gnotobiotic conditions were observed. No evidence of rhizospheric or endophytic colonization was seen in test control and it was interpreted that this ruled out the possibility of cross contamination.

The isolates AU15, CP18, PD22, PD25, KA28, KA31, OC36 were subjected to 16S rRNA gene sequence analysis for the identification and genomic analysis confirmed them as *Bacillus firmus, Alcaligenes faecalis, Bacillus aryabhattai, Proteus mirabilis, Pseudomonas marginalis, Alcaligenes faecalis,* and *Brevibacterium antiquum* respectively. The isolates were then tested to determine biosafety issues on human health prior to the application of endophytes to the plants in pot experiments. Enzymatic activities including protease and gelatinase, hemolytic activity on hemoglobin supplemented medium, and presence of catalase, was assayed in order to determine the pathogenicity. These initial level tests proved that the isolates were non-pathogenic and avirulent; however, clinical trials will also be done to completely ensure the safety before using them at large scale.

To assess the plant growth promoting effects of the selected endophytic isolates, pot experiments were conducted for two consecutive years (2019 and 2020) using *T. aestivum* as test crop. For this wheat seeds of variety Annapurna-PBW 343 were selected and bioformulations were developed at lab scale. Sterilized seeds of wheat were treated with prepared bioformulation and sown according to different set of treatments under saline conditions (EC 9.72 dS/m). The treatment for pot study were designed as: i) Untreated control ii) Seeds + AU15 iii) Seeds + CP18 iv) Seeds + PD22 v) Seeds + PD25 vi) Seeds + KA28 vii) Seeds + KA31 viii) Seeds + OC36. Various

plant growth parameters such as root length, shoot length, plant dry weight and fresh weight, spike length, number of grains per spike and the tiller numbers were analyzed and it was noticed that seed bio priming with the endophytic isolates significantly improved growth parameters of their host plant i.e., wheat in respect to other treatments and experimental control.

Out of all the isolates applied, maximum increment was observed in the case of plant treated with two isolates namely AU15 and OC36 which were considered to be the best plant growth promoters. *C. firmus* AU15 increased the germination rate by 78.5% in comparison to experimental control while *B. antiquum* resulted into 64.2% increment. The incoulation of seeds with AU15 was the most effective treatment that caused increase in the root length by 156.1% and two-fold increment in shoot length as compared to untreated plants. Similarly, other parameters including fresh weight, dry weight, tiller numbers, spike length, and number of grains per spike were also significantly escalated by 84.5%, 293.5%, 119.7%, 88.9%, and 62.6%, respectively when AU15 was applied. The two potential isolates were deposited in National Agriculturally Important Microbial Culture Collection (NAIMCC), an international culture collection centre approved by International Depository Authority (IDA) and assigned with accession number *B. firmus* NAIMCC-B-03040 (AU15) and *B. antiquum* NAIMCC-B-03041 (OC36).

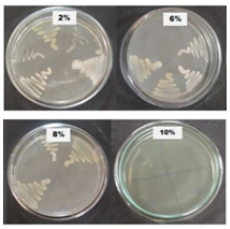
The present work elucidates the mutualistic associations of potent diazotrophic salt tolerant endophytes such as *C. firmus* AU15 and *B. antiquum* OC36 which not only played an important role in plant growth promotion of wheat plants but also mitigated the detrimental effects of salinity. Though *C. firmus* and *P. aryabhattai* have been

recognized as endophytes from a variety of plants but their role in nitrogen fixation and salt tolerance in wheat plants has not been studied yet. The procured endophytic isolates are of significant interest for bacterial taxonomy and thereby development of versatile bio-formulations using these PGPE could remove the deleterious constraints associated with the utilization of chemical formulations and be a sustainable solution for improving crop productivity of wheat in saline agroecosystems in the near future.

Isolation of bacterial endophytes

Morphological, biochemical, and plant growth promoting characterization of the isolates

SCREENING OF NITROGEN FIXERS
-Growth on nitrogen free medium
-Acetylene reduction assay
-Detection of *nif*H gene



SALT TOLERANCE ASSAY

AU15, CP18, PD22, PD25, KA28, KA31, OC36 WERE SELECTED AS BEST ENDOPHYTIC ISOLATES

Pot culture experiments were performed under saline conditions

Endophytes (AU15 and OC36) showed maximum growth enhancement in comparison to non-inoculated ones i.e., control.

Summary of the work done