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Isolation, characterisation and identification of native 
*Azotobacter* spp. strains

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Abstract

In this research, 50 soil samples were collected from ecologically different provinces in 9 country in Turkey, for isolation of the indigenous *Azotobacter chroococcum* strains. Fifty-five like *A. chroococcum* strains were isolated on Ashby agar after incubated for 3 days at 30 °C. The isolated strains were maintained on Ashby agar slopes and mycelial fragment in glycerol (25% v/v) at -25 °C. The isolated 55 indigenous *A.chroococcum* strains were tested for nitrogen fixation capability into Ashby broth after 72 h at 30 °C and three soil samples with different texture after 8 weeks. The highest N fixed 10 isolates and the type strain of the *A. chroococcum* Beijerinck 1901 were examined on the field and greenhouse conditions for determine effect of the plantal productivies. The selected indigenous *A. chroococcum* and the related type strains were also tested for ability to use sole carbon sources for energy and growth. The isolate strains identified based on 16S rRNA gene sequencing. Phylogenetic dendograms of 16S rRNA sequence analysis were made using the least-squares, maximum-parsimony and neighbour-joining algorithms.

**Key words:** *A.chroococcum*, N fixation, identification, soil

INTRODUCTION

*Azotobacter* sp. is free-living aerobic bacteria dominantly found in soils. A large number of experiments have been performed to investigate the effects of inoculation of cereals with *Azotobacter* sp. Results of these studies showed that in many cases grain, yield and N concentration in plants increased by inoculation with *Azotobacter* sp. (De Freitas, 2000; Kumar et al., 2001; Emtiazì et al., 2004). *A. chroococcum* is the most prevalent species found but other species described including *A. agilis, A. vinelandii, A. beijerinckii, A. insignis, A. macrocytogenes* and *A. paspali* (FAO, 1982). Cereal plants inoculated with *A. chroococcum* increased number of root hairs, tillering ratio, dry matter concentration, N uptake or yields

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of wheat (Haathela et al., 1988; Ishac et al., 1986; Rai and Gaur, 1988). Several studies have shown that A. chroococcum as soil inoculant is not only effective in N fixation but also has other properties such as production of growth hormones (Remus et al., 2000), production of fungicidal substances (Lakshminarayana, 1993), siderophore production (Suneja et al., 1994) and the property to solubilize phosphate (Kumar and Narula, 1999; Narula et al., 2000).

MATERIALS AND METHODS

Test strains

Eight strains which were found to have the potential to be used as N fixing rhizobacteria, and these were designated as RK33, RK34, RK38, RK39, RK40, RK41, RK46, RK48, RK49, RK50 (Kizilkaya, 2008) were subjected to molecular taxonomy.

DNA preparation, amplification and Sequencing of 16S rRNA genes

Extraction of genomic DNA and PCR-amplification of 16S rRNA genes from the strains were carried out as described by Pitcher et al. (1989), using the modifications of Sembiring (2000). The amplified fragments were purified with QIAquick purification kits (Qiagen, Valencia, USA) and sequenced directly using ABI PRISM BigDye Terminator Cycle Sequencing kits (Applied Biosystems) and previously described oligonucleotide primers (Lane 1991; Chun and Goodfellow 1995). Sequencing gel electrophoresis was carried out and the nucleotid sequences automatically obtained by using an Applied Biosystem DNA sequencer (model 377) and software provided by the manufacturer.

The identification of phylogenetic neighbours and the calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (http://www.eztaxon.org; Chun et al., 2007). The CLUSTAL W version 1.8 (Thompson et al., 1994) were used to align the sequences of strains with related taxa retrieved from public databases. Phylogenetic analysis was performed using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 3.1 (Kumar et al., 2004), using the neighbour-joining (Saitou & Nei, 1987) and maximum parsimony (Fitch, 1971) methods with bootstrap (Felsenstein, 1985) values based on 1000 replications.

RESULTS AND DISCUSSION

Comparison of the almost complete 16S rRNA nucleotide gene sequences obtained for strains with corresponding sequences of N fixing bacteria, Azotobacter and Pseudomonas species showed that 8 isolates assigned for morphological properties to the Azotobacter chroococcum, formed a closely related, but also distinct group with the species A. chroococcum DSM 2286\(^T\). It can be seen from phylogenetic tree that isolates RK39, RK40, RK46, RK48 and RK50 belong to or is most closely associated with the type strain A. chroococcum DSM 2286\(^T\). The strains RK48, presented 100% 16S rRNA gene nucleotide (nt) similarities, while RK46 and RK50 presented 99.94% (1 nt differences), RK40 presented 99.87% (2 nt differences) and RK39 presented 99.80 16S rRNA gene nucleotide similarities (3 nt differences). The two of the remaining 3 isolates, RK38 and RK41 is also closely related to A. chroococcum DSM 2286\(^T\). These organisms share a 16S rRNA gene similarity of 99.5%, a value which corresponds to 7 nt differences. Whereas, the strain RK49 presented 99.3% 16S rRNA gene nucleotide (nt) similarities (9 nt differences) with A. chroococcum DSM 2286\(^T\), were found to the higher grain and straw yield and N concentration responses in the previous study (Kizilkaya, 2008).
Figure 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the relationships among the new isolates and the phylogenetically closest *Azotobacter* and *Pseudomonas* species. Bootstrap percentages (from 1000 replicates) above 50% are shown at nodes. Bar, 0.005 substitutions per nucleotide position.

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