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Article

Nitrate Uptakes by Microorganisms Isolated from the Soils of Greenhouse

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Salinity of soils in greenhouse has been increased by massive application of fertilizers. Nitrogen fertilizer was most popular, and thus nitrate became the majority of soil salinity. Accumulation of nitrate led to deleterious effects on the growth and development of crops and vegetables. Microbial strains able to utilize nitrate and thus remove excess nitrate from farm land soils were isolated from 15 different soils of greenhouses and plastic film houses. Four strains able to grow in medium containing 50 mM KNO_3 were isolated, among which only E0461 showed high capacity of nitrate uptake. Nitrate uptake by E0461 was dependent on culture medium and was increased by addition of tryptone and peptone. Although E0461 was able to grow without tryptone and peptone, growth was slow, and no nitrate uptake was observed. Nitrate appeared to facilitate E0461 growth in the presence of tryptone and peptone. Through kinetic analysis, nitrate uptake was measured at various concentrations of nitrate, and half-life was calculated. Nitrate concentration decreased with increasing incubation period, and plot between half-lives and initial concentrations of nitrate fitted to single exponential function. These results suggest one major factor plays an important role in microbial nitrate uptake.

Key words: *Greenhouse soil, nitrate uptake, salt stress, soil bacteria*

Nitrate is an essential nutrient for plant growth and reproduction. However, excess nitrate is harmful to the plant as well as the biological environment.[1,2] Nitrogen fertilizers have been used more than necessary for crop and vegetable cultivations, because the productivity and quality are apparently improved proportional to the amount of N-fertilizer used.[1] As a result, the accumulation of nitrate salt occurred in vast area of the domestic farm lands and reached levels that affect the plant growth negatively, known as 'salt stress'.[3,4] Plants use much energy to reduce cytosolic salt concentration via active transport systems;[5,6] thus, the growth and development of plant severely deteriorate under the salt-stressed condition. Currently, salt stress is a nationwide phenomenon. It is more serious in the soils of greenhouse and plastic film house, because rainfalls are physically blocked and water supplies by irrigation are not enough to wash out excess salts.[7] Reducing the amount of fertilizer used will alleviate this problem. Field researches to calculate the amount of fertilizer needed for the cultivation of crops and vegetables have helped farmers to use adequate amount of fertilizers.[8] However, as the greenhouse and plastic film house cultivations increase, the agricultural problems caused by salt stress could become more serious.[9]

Many salt management protocols to improve plant growth have been developed. Seasonal rainfalls were used for natural leaching.[10,11] Leachings with rainfalls and irrigation waters can move salts downward beyond the root zone. These methods are useful in soils with good internal drainage, because the salts are removed by the groundwater. However, some difficulties are encountered using natural leaching to reduce the salt contents in soil. Firstly, drainage by rainfalls is not applicable to the soils of greenhouse and plastic film house due to physical barriers, and, secondly, the salts removed by leaching can be moved back to the surface by high temperature-induced evapotranspiration during a dry season.[12] Furthermore, natural leaching of fertilizer can contribute to the contamination of groundwater resources,[13] and the resulting accumulation of nitrate in groundwater can cause health problems to humans and animals.[14]

Because microbial treatments have been successful for the removal of nutrients and heavy metals from wastewater resources,[15,16] similar strategies could be applied to reduce the nitrate content in the soils of greenhouse and plastic film house.[17] In this study four microorganism strains able to grow in the presence of high nitrate concentration were isolated from the soils of various farm lands, and their characteristics and capabilities of nitrate uptake were examined.

Materials and Methods

Soil analysis. Soils of greenhouse and plastic film house were collected from 15 different places located at southern counties of Chungbuk province. Chemical characteristics of soils were analyzed by the methods developed by the National Institute of Agricultural Science and Technology of the Rural Development Administration in Korea.[18] Briefly, pH and electrical conductivity (EC) of soils were determined using an extract of a 1 to 5 dilutions of soil with distilled water.[19] EC

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and pH were measured using a conductivity meter (YSI-32, Yellow Springs Inc., Yellow Springs, OH, USA) and a pH meter (Radiometer M-92, MeterLab Co., Lyon, France), respectively. Concentrations of organic matters and available phosphate were determined by Tyurin's and Lancaster's methods, respectively. NO_3-N concentration was determined by the Kjeldahl method.

Isolation of soil microorganisms. One gram of soil sample was extracted with 5 m*l* of sterilized double distilled water, and the extract was centrifuged at 1,200 rpm for 2 min. The supernatant was diluted up to 1,000 times, and 50 μ*l* of the diluted aliquot was spread onto a potato dextrose agar (PDA) plate. To isolate nitrate-utilizing microbial strains, 50 mM KNO_3 was added to PDA medium. EC of the medium was 5.35 dS · m^{-1}, concentration high enough to give salt stress to plants. PDA plates were incubated for 24 h at 28°C. Colonies were identified and chosen based on their color, shape, and size. Microbial strains were isolated from the colonies and subjected to growth measurements.

Growth analysis. Isolated microbial strains were cultured in the broth of *Pseudomonas* agar factor (PAF) or potato dextrose (PDB) containing various amounts of nitrate. Cultures were incubated at 30°C with continuous agitation at 135 rpm. Cell density was monitored as the optical density at 600 nm.

Nitrate uptake measurement. Nitrate uptake was measured by analyzing the amount of remaining nitrate in the culture media. The concentration of nitrate was determined potentiometrically using a nitrate ion-selective electrode (ISE) in conjunction with a double-junction reference electrode connected to an Orion 960 ISE meter.[8] Standards and samples were mixed 50 : 1 (v/v) with an ionic strength adjustment solution. The electrodes were equilibrated for 2 h in a 50 mM nitrate standard solution before use. A two-point calibration using 5.0 and 50.0 mM nitrate standard solutions was performed prior to analysis.

Results and Discussion

Isolation of soil microorganisms. Twenty-five strains of soil microorganism were isolated based on the color and shape of colonies from full nutrient media, four of which were able to grow in the medium containing 50 mM KNO_3. Table 1 shows the strain numbers and the physicochemical properties of their corresponding soils. Some differences were found in the physicochemical properties, such as the content of organic matter, electrical conductivity, and the content of soil nitrate.

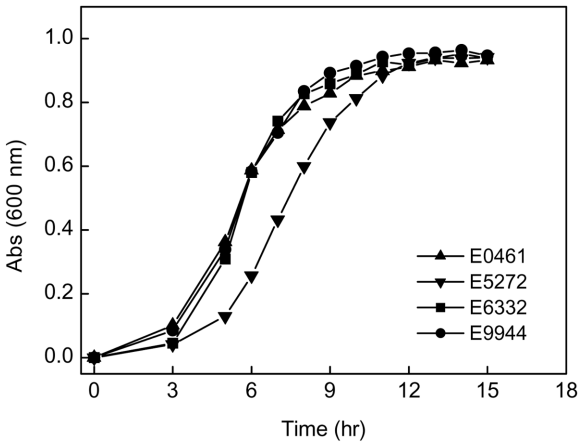

Fig. 1. Growth curves of the isolated soil microorganisms. Isolated microorganisms were incubated in a PAF broth containing 50 mM KNO_3. The inoculated broth was agitated at 135 rpm and incubated for 15 h at 30°C. Absorbance was measured at 600 nm.

When the isolated microorganisms were cultured in PAF broth containing 50 mM KNO_3, their growth curves were very similar except for that of E5272 (Fig. 1). Stationary phases were observed after 12 h incubation. In the growth of E0461, the absorbance increased to 0.91 and the number of microorganism was 3.7×10^6 ml^{-1} after 12 h. The growth of E5272 was relatively slow, apparently late by 2 h. However, it also reached stationary phase with a similar increase in absorbance. Among the rest of the isolated microorganisms, some grew after long incubation period; however, their growth rates were very poor (data not shown).

Nitrate uptake. When the isolated microorganisms were incubated in the modified PAF media containing 50 mM KNO_3, the concentration of nitrate did not change even after the growth reached stationary phase, indicating that the isolated microorganisms are able to grow in the presence of high nitrate concentration, and they do not need nitrate for growth. However, in the presence of other nitrogen sources, such as tryptone and peptone, E0461 decreased the concentration of nitrate in the media (Fig. 2). Although the

Table 1. Isolated microorganisms and physicochemical properties of the corresponding soils

Microbial Strain No.	pH	OM[a] (%)	P_2O_5 (mg · kg^{-1})	CEC[b] (cmol · kg^{-1})	EC[c] (dSvm^{-1})	NH_4-N (mg · kg^{-1})	NO_3-N (mg · kg^{-1})
E0461	6.1	1.5	940	12.3	0.40	3.31	6.62
E5272	6.8	3.0	1042	19.7	5.20	8.31	151.31
E6332	6.9	3.4	1418	25.1	6.30	7.81	94.53
E9944	5.2	3.1	921	27.1	11.65	9.13	141.46

[a]OM: organic matter.
[b]CEC: cation exchange capacity.
[c]EC: electrical conductivity.

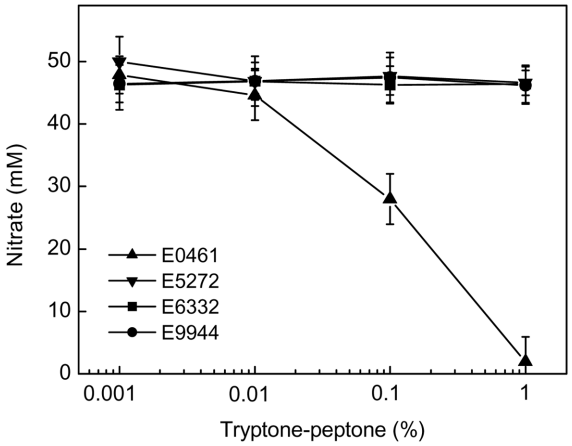

Fig. 2. Nitrate uptakes at various concentrations of tryptone and peptone. Microbial strains were cultured in the modified PAF broth containing tryptone and peptone (0.001 to 1%), and 50 mM KNO_3. Tryptone and peptone were added to the media at 1 : 1 ratio, and 1% of tryptone and peptone in the figure represents a medium containing 1% of each. The amount of nitrate remaining in the medium was analyzed at the stationary phase of growth using a nitrate ion-selective electrode (n=5).

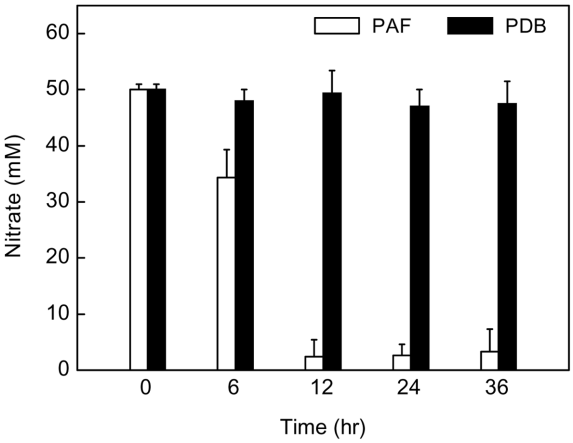

Fig. 3. Effect of culture medium on the nitrate uptake. E0461 was cultured in both PAF and PDB media containing 50 mM KNO_3. The same concentrations of mineral nutrients were added in both media. The amounts of nitrate remaining in the media were analyzed after the indicated incubation period (n=3).

growth rates of all four strains were similar, the nitrate uptake was observed only in the culture of E0461; thus, E0461 was chosen for subsequent experiments.

The microbial uptake of nitrate could be reduced or suppressed by additional supplies of other nitrogen sources in the media.[20-22] However, the nitrate uptake by E0461 was initiated and increased by the addition of tryptone and peptone. Furthermore, the amount of uptake was increased by high concentrations of tryptone and peptone. Nitrate uptake was completed in the media containing 1% tryptone and 1% peptone, whereas only 40% uptake was observed in the media containing 0.1% each of these. Although the mechanism involved has not yet been elucidated, the decrease in the

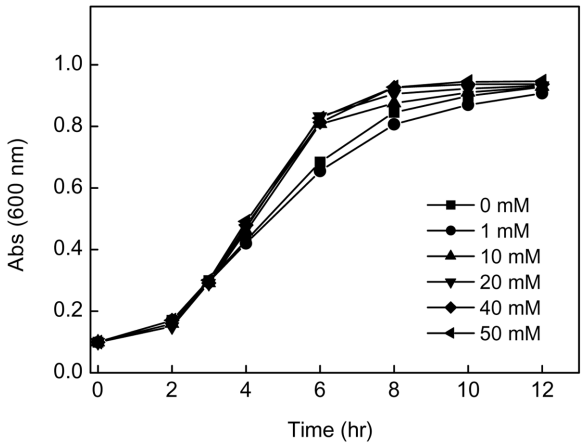

Fig. 4. Effect of nitrate on the growth of E0461. E0461 was cultured in the PAF broth containing various concentrations of nitrate. The amount of initial inoculation was 1%, and the growth was measured by the absorbance at 600 nm.

amount of nitrate in the culture of E0461 was probably mediated by microbial uptake rather than by any other physical or chemical processes, because no decreases in the nitrate concentration were observed in the cultures of other microorganisms under the same condition.

Nitrate uptake by E0461 was observed in PAF broth, but not in PDB (Fig. 3). In the PAF medium, stationary phase was observed after 12 h incubation (Fig. 1). However, cell density in PDB increased slowly, and the growth reached stationary phase after 25-30 h incubation. These results showed that E0461 is able to grow in both media, with faster growth in PAF broth than in PDB. A big difference was observed in the nitrate uptake. Nitrate uptake by E0461 was completed after 12 h incubation in the PAF media, but no significant uptake was observed in the PDB even after 30 h incubation. The difference in uptake is probably due to the presence of tryptone and peptone in PAF media. It is also possible that a component of PAF media is able to facilitate the nitrate uptake. If the factor is identified, it will be very useful in understanding the mechanism of microbial nitrate uptake. For further support, the effect of nitrate concentration on the growth of E0461 was measured (Fig. 4). E0461 was cultured in the presence of nitrate. The growth was relatively slow in the media containing low concentrations of nitrate, 0 and 1 mM, and the logarithmic phase was also delayed at these conditions. These results indicate that nitrate is not necessary for the growth of E0461. Nevertheless, it is possible that nitrate may increase the microbial growth, because the growth was fast at high concentrations of nitrate.

Kinetics of nitrate uptake. The rates of nitrate uptake by E0461 were measured at various concentrations of nitrate (Fig. 5). When 1 to 50 mM nitrate was added, it was depleted and virtually undetectable in the media after 8-10 h incubation. The patterns of decrease in the nitrate concentration were similar under all conditions, and single exponential functions were fitted to the data. A half-life of nitrate uptake

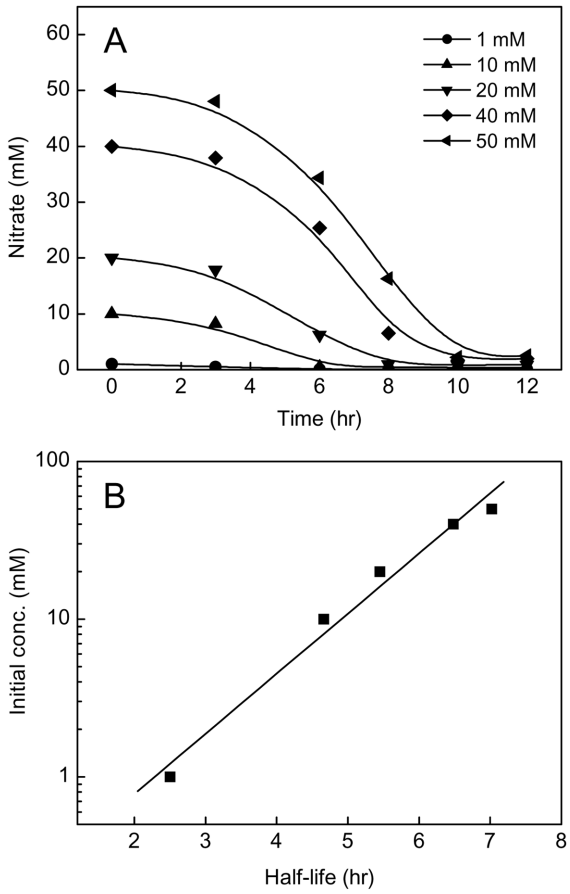

Fig. 5. Time course of nitrate uptake at various initial concentrations of nitrate. (A) Nitrate uptake was measured at various concentrations of nitrate (1 to 50 mM). Initial inoculation of E0461 was 1%. Data were fitted to single exponential functions in each experiment. An average value was obtained from two independent experiments. (B) Relation between the initial concentration of nitrate and half-life, time required for 50% nitrate uptake. A linear function was fitted to the data.

was measured when the nitrate was reduced to 50% of the initial concentration. Relationship between initial concentration of nitrate and half-life was fitted to a linear line (Fig. 5B). These results imply that only one major factor is involved in the nitrate uptake. Although detailed molecular mechanism is not yet known, the factor could be a nitrate-transporting enzyme in the plasma membrane of E0461.[23)]

In this study, four strains among the isolated microbial strains from various soils of greenhouse and plastic film house were able to grow in the presence of 50 mM KNO_3, with one of them showing a high capability of nitrate uptake. E0461 could be a candidate strain for use in the microbial removal of nitrate from the soils of greenhouse as well as from wastewaters polluted with nitrate. However, detailed mechanisms of microbial nitrate uptake should be investigated further to increase the uptake capacity and versatility of application, because nitrate does not appear to be a necessary factor for the growth of this strain. In a diagnostic examination, we found that the strain E0461 is a rod-shaped Gram-negative bacterium. Biochemical and genetical investigations are under way to identify this strain. These characteristics will be very valuable for the successful applications of E0461 in agriculture.

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