Effects of different forms of nitrogen on rhizosphere microbial community structure of *Eichhornia crassipes* (Pontederiaceae)

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Abstract: Rhizosphere microbial communities are important for phytoremediation, plant nutrition, health and metabolism. Many factors, including plant species, pH and nutritional factors influence rhizosphere microbiology. In this study, we analysed the effects of different forms of nitrogen on the structures of rhizosphere microbial communities of *E. crassipes*. Using a conventional culture method with special media, bacteria, actinobacteria and molds were cultured. We found that the numbers of bacteria were largely similar across the three culture conditions, while the numbers of actinobacteria and molds from the rhizosphere of *E. crassipes* cultured in NH_4Cl solution were two orders of magnitude higher than those from the rhizospheres of plants cultured in distilled water and KNO_3 solution. Using a culture-independent method of polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) of 16S rDNA, we found that the form of nitrogen could influence the components of the rhizosphere microbial community. *Pseudoxanthomonas*, *Enterobacter* and *Citrobacter* were present in all of the samples cultured under the three different experimental conditions. The genus *Reyranella* was found only in samples cultured in KNO_3 solution; *Acinetobacter* and *Streptomyces* were unique to samples cultured in NH_4Cl solution, and *Pseudomonas*, *Pseudacidovorax* and *Methylosinus* were found only in samples cultured in distilled water. *Pseudoxanthomonas* and *Acidovorax* were the dominant genera in the rhizosphere microbial community of *E. crassipes* cultured in KNO_3 solution, while *Novosphingobium* was the dominant genus in the sample cultured in a nitrogen-deficient medium. Our results provide a theoretical foundation for using *E. crassipes* as a phytoremediation plant and controlling the widespread distribution of *E. crassipes* around the world using principles of nutrient metabolism. Rev. Biol. Trop. 64 (1): 213-220. Epub 2016 March 01.

Key words: *Eichhornia crassipes*, nitrogen, rhizosphere microbial community, PCR-DGGE.

Eutrophication of water bodies is becoming an increasingly severe problem with current levels of industrial development and the increasing population worldwide. Phytoremediation is an effective solution to this problem. In phytoremediation of water, aquatic plants remediate eutrophic water, a process that relies not only on the absorption and utilisation of nutrients by plants (Fu et al., 2014) but also on their relationship with rhizosphere microbiology (Lu, Hu, Liang, & Zhu, 2010; Tanaka et al., 2012). Rhizosphere microbial communities can decompose organic substances and denitrify nitrogenous compounds by the processes of ammoxidation, nitrification and denitrification and transform phosphorus compounds (Stottmeister et al., 2003; Lu et al., 2010). In this way, they play an important role in plant nutrition, plant metabolism, phytoremediation and plant health (Elsgaard, Petersen, & Debosz, 2001; Filip, 2002). Many environmental factors can influence the structure of the rhizosphere microbial community, thereby affecting phytoremediation and the growth of aquatic plants (Chen & Shu, 2008; Ma, Xiong, He, & Ma, 2008). Thus, the extent to which these

communities vary with various environmental factors is of considerable interest to plant-microbial ecologists (Marschner, Crowley, & Yang, 2004).

Eichhornia crassipes is an aquatic invasive plant, originated in South America and represents one of the most productive plants in the world (Aboul-Enein et al., 2011). It has become a serious weed in freshwater habitats in rivers, lakes and reservoirs in both tropical and warm temperate areas worldwide. Owing to its strong ability to assimilate nitrogen and phosphorus (Reddy, Agami, & Tucker, 1989; 1990) and accumulate heavy metals (Deng, Ye, & Wong, 2004; Caldelas, Iglesia-Turino, Araus, Bort, & Febrero, 2009), it is useful in wastewater phytoremediation (Casabianca, Laugier, & Posada, 1995; Lu, Fu, & Yin, 2008; Chen, Chen, Wan, Weng, & Huang, 2010). In this study, we analysed the structure of the rhizosphere microbial communities associated with *E. crassipes* cultured under different forms of nitrogen (1.8 mM ammonium, 1.8 mM nitrate and nitrogen deficiency). Our results provide a theoretical foundation for using *E. crassipes* as a phytoremediation plant and controlling the widespread distribution of *E. crassipes* around the world using principles of nutrient metabolism.

MATERIALS AND METHODS

Culture: *E. crassipes* plants were collected from a lake from the Western part of Guangdong University of Technology, Guangzhou, China in fall (September). The plants were cultured in nutrient solution (0.25 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.25 mM KNO_3, 0.1 mM $MgSO_4 \cdot 7H_2O$ and 0.05 mM KH_2PO_4) for 20 days at 25 °C with 16 h of light (2000 Lx) and 8 h of darkness indoors. The solution was replaced completely every two days. After preculture, the *E. crassipes* plants were cultured in either 1.8 mM NH_4Cl solution, 1.8 mM KNO_3 solution, or distilled water for two weeks under the same conditions as the preculture.

Sample preparation: The first 1-2 cm of root tips (90 g) of *E. crassipes* mixed with 90 mL sterile water and some glass beads were vortexed (2 000 rpm) for 20 min and then allowed to rest for 20-30 s. The supernatant solution was collected. Some of it was stored in -80 °C for DNA extraction and some was used as a sample solution which was diluted in 10-fold steps with sterile water.

Determination of the number of culturable rhizosphere microbial communities: The densities of microbial communities in the plant rhizosphere were determined by a conventional culture method using the following media: salt beef extract tryptone medium for bacterial culture; Gao 1 synthetic medium for actinobacteria, with the addition of potassium dichromate (100 µg/mL) to inhibit bacteria and molds and salt Czapek Dox agar for mold culture. Diluted sample (100 µL) was spread onto plates and cultured at 37 °C for one or two days (for bacteria) or 28 ºC for a week (for actinobacteria and molds). The number of clones in each type of culture medium was determined. Each solution was assayed in triplicate to obtain the average (Matsuzawai, Tanaka, Tamaki, Kamagata, & Mori, 2010).

Extraction of DNA and PCR amplification: DNA was extracted from rhizosphere samples prepared as described above using a soil DNA extraction kit (Hangzhou Haoji Biotechnology Ltd.) according to the manufacturer's instructions. Two rounds of PCR amplification were performed with primers listed in Table 1. The primers for this procedure were designed from the V3 region of bacterial 16s rRNA. The conditions for the first amplification were 94 °C for 2 min, 35 cycles (94 °C for 30 s, 58 °C for 30 s and 68 °C for 1.5 min). The conditions for the second amplification were 94 °C for 2 min, 30 cycles (94 °C for 30 s, 55 °C for 45 s and 68 °C for 30 s). PCR products were separated on 1 % agarose/EtBr gel. Amplification experiments were performed in triplicate on every sample (Mota et al., 2008).

Primer name	Primer sequences
Bac-V3-F1	5-GAGTTTGATCCTGGCTCAG-3
Bac-V3-R1	5-AGAAAGGAGGTGATCCAGCC-3
Bac-V3-F2	5-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG CCTACGGGAGGCAGCAG-3
Bac-V3-R2	5-ATTACCGCGGCTGCTGG-3

TABLE 2
The number of rhizosphere microbiology from *Eichnornia crassipes* cultured in three conditions (cfu/mL)

Kind of microbiology	Distilled water	KNO$_3$ solution	NH$_4$Cl solution
Bacteria	4.70×10^7	4.40×10^7	3.51×10^7
Actinobacteria	7.90×10^5	7.00×10^4	3.50×10^7
Moulds	7.00×10^3	4.00×10^3	1.29×10^6

Denaturing gradient gel electrophoresis (DGGE) analysis of microbial rRNA genes: PCR products were purified with a purification kit (Hangzhou Haoji Biotechnology Ltd.) and then separated by DGGE using the Decode Universal Mutation Detection System (Bio-Rad, USA) with a run time of 12 h in Tris-acetate-EDTA buffer heated to 60 °C. Bacterial 16s rRNA gene PCR products were electrophoresed in an acrylamide gel with a gradient of 35-60 % denaturant. After electrophoresis, the gel was stained with SYBR Green I for 30 min and the bands were examined using the Gel Doc 2000 gel imaging system (Bio-Rad, USA).

Statistical analysis of DGGE image: The DGGE image was scanned with the Quantity One program and the intensity of every band was reported. The evenness and diversity indexes were compared by one-way analysis of variance and Tukey HSD test for post-hoc analysis using SPSS (13.0). Diversity was calculated as the Shannon index of diversity (H) to compare changes in diversity of microbial communities between the three treatments using the following function: $H = -\sum(P_i) \log_2 P_i$, where $P_i = n_i/N$, n_i is the band intensity and N is the sum of all band intensities in the curve. Simpson's diversity index (D) for infinite populations is equal to $1 - \text{sum}(P_i * P_i)$. The DGGE evenness (E), a measure of how evenly DGGE bands are distributed in a given sample, is calculated as $E = H/\log_2(S)$ where richness (S) refers to the number of bands detected in a given sample.

Sequencing and analysis of bright bands: Clear differential display or common display bands were cut out and redissolved in Tris buffer (pH 8.0). The supernatant was subjected to PCR (94 °C for 2 min, 30 cycles; each cycle included 94 °C for 30 s, 55 °C for 45 s and 68 °C for 30 s) with the primers Bac-V3-F2 and Bac-V3-R2 (listed in Table 1). PCR products were recycled, cloned and sequenced. All sequences were blasted against the nonredundant nucleotide database in NCBI to identify the most similar sequences in the database (http://www.ncbi.nlm.nih.gov). These findings were used to classify of our sequences.
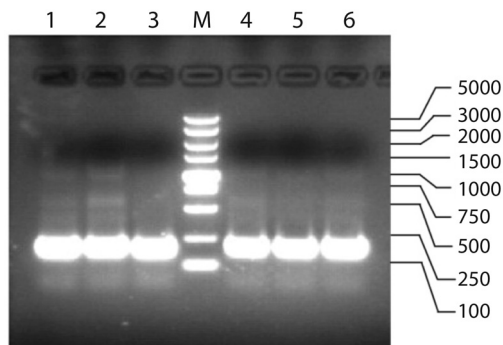
RESULTS

Determination of the total numbers of culturable bacteria, actinobacteria and molds: The numbers of rhizosphere bacteria, actinobacteria and molds are listed in Table 2. Under each culture condition, the numbers of bacteria were the highest, followed by actinobacteria, and less molds. The numbers of

bacteria were largely similar across the three culture conditions, whereas the numbers of actinobacteria and molds from the rhizosphere of *E. crassipes* cultured in NH_4Cl solution were two orders of magnitude higher than those from the rhizospheres of plants cultured in distilled water and KNO_3 solution.
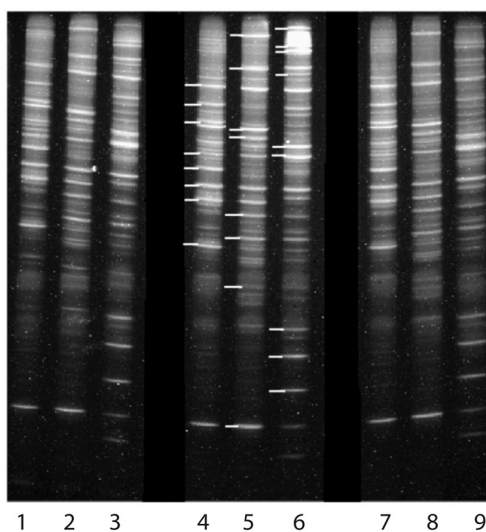
PCR amplification of extracted DNA and DGGE analysis: The results of electrophoresis of the second round of PCR products are shown in Fig. 1. There was only one 230 bp band in every lane (with each sample run in two lanes). All three samples from the DGGE results showed many clear bands (Fig. 2). The Shannon index of diversity (H), Simpson's diversity index (D) and the evenness (E) of every sample are listed in Table 3. They indicate that all three samples had an abundance of diversity in the microbial community of the rhizosphere. But there was no significant difference (all P > 0.05) among the Shannon index of diversity (H), among Simpson's

diversity index (D), or among the evenness (E) of three samples.

Sequence analysis: Clear differential display bands or clear common display bands were selected to be sequenced. The selected sequences were used for BLAST against the non-redundant nucleotide sequence database to search for the most similar sequences whose classifications were known. Among the eight sequences from the sample cultured in KNO_3 solution, two were similar to sequences of *Pseudoxanthomonas* and two were similar to those of *Acidovorax*. The other four sequences were similar to those of *Devosia*, *Novosphingobium*, *Enterobacter* and *Reyranella*. Among

Fig. 1. Electrophoresis of the PCR products (lanes 1 and 2, samples cultured in KNO_3 solution; M, marker; lanes 3 and 4, samples cultured in NH_4Cl solution; lanes 5 and 6, samples cultured in distilled water).

Fig. 2. Denaturing gradient gel electrophoresis result (lanes 1, 4, and 7, samples cultured in KNO_3 solution; lanes 2, 5, and 8, samples cultured in NH_4Cl solution; lanes 3, 6, and 9, samples cultured in distilled water). The short segment beside the lane indicates the bands that were sequenced.

TABLE 3

The Shannon index of diversity (H), Simpson's diversity index (D) and the evenness (E) of every sample

Diversity indices	Distilled water	KNO_3 solution	NH_4Cl solution
H	5.201±0.072	5.293±0.016	5.294±0.077
D	0.968±0.003	0.971±0.001	0.972±0.001
E	0.953±0.013	0.954±0.010	0.968±0.006

Mean±standard deviation (SD).

the eight sequences from the sample cultured in NH_4Cl solution, there were sequences similar to those of *Acinetobacter*, *Flectobacillus*, *Cytophaga*, *Streptomyces*, *Devosia*, *Enterobacter*, *Rhizobium* and *Citrobacter*, whereas among the nine sequences cultured in distilled water, there were three sequences similar to that of *Novosphingobium* and six sequences similar to those of *Pseudomonas*, *Pseudacidovorax*, *Acidovorax*, *Brevundimonas*, *Rhodospirillum* and *Methylosinus*. *Pseudoxanthomonas*, *Enterobacter* and *Citrobacter* were found in bands from all three samples. The genus *Reyranella* was found only in samples cultured in KNO_3 solution; *Acinetobacter* and *Streptomyces* were unique to samples cultured in NH_4Cl solution, and *Pseudomonas*, *Pseudacidovorax* and *Methylosinus* were found only in samples cultured in distilled water.

DISCUSSION

It has been previously reported that the diversity and abundance of different components of rhizosphere microbial communities were influenced by plant nutrition and other environmental factors. Hamonts et al. (2013) reported that nitrogen limitation significantly influenced the structure and function of denitrifier communities in the rhizosphere of wheat. An investigation by Marschner et al. (2004) showed that bacterial community structure was influenced by soil pH and type of phosphorus fertilisation. Söderberg and Bååth (2004) found that bacterial activity in the barley rhizosphere decreased when ammonium was added.

Our study also found that not only the numbers of culturable microbial communities but also the compositions of the microbial communities of *E. crassipes* were influenced by different forms of nitrogen. Although *Pseudoxanthomonas*, *Enterobacter* and *Citrobacter* were found in all three samples, different treatment samples had unique genera and dominant genera. *Pseudoxanthomonas* and *Acidovorax* were the dominant genera in the rhizosphere microbial community of *E. crassipes* cultured in KNO_3 solution, whereas *Novosphingobium*

was the dominant genus in the sample cultured in distilled water. The differences among the rhizosphere microbial communities were a response to environmental factors. Because individual components of the rhizosphere microbial community had different functions that allowed them to exploit specific environmental conditions. In the environment with plenty of ammonium, the special microbial communities of *E. crassipes* rhizosphere can effectively absorb or transform the ammonium; while in the environment with much nitrate, the microbial communities of rhizosphere will change into adapted compositions which can take in or transform the nitrate. Through all these microbial communities of rhizosphere, the nitrogen can be removed from water or transformed into other forms which can be utilized by other organisms. The study of the effects of various forms of nitrogen on these communities can provide a theoretical foundation for rhizosphere microbiology in phytoremediation, plant growth, plant protection and other areas. In the rhizosphere microbial community of *E. crassipes*, several bacterial species are common in soil and water. Some of them, including *Acidovorax*, *Acinetobacter*, *Streptomyces*, *Enterobacter*, *Citrobacter*, *Novosphingobium* and *Pseudomonas*, can decompose organic or macromolecular compounds. Some are nitrogen fixers, such as *Rhizobium*, *Devosia*, *Pseudacidovorax* and *Rhodospirillum*. *Methylosinus* is an example of a methanotroph. Many bacteria are very important in phytoremediation (Lu et al., 2010; Bai, Liang, Liu, Hu, & Qu, 2014). But from the results of our study, we could not identify specific nitrifying or denitrifying bacteria in the NH_4Cl or KNO_3 treatment samples, probably because the removal of nitrogen in water with NH_4Cl or KNO_3 is a complex process that comprises not only nitrification and denitrification, but also absorption and use by bacteria and plants. Thus, nitrogen removal represents the integrated action of plants and bacteria in phytoremediation.

There are several likely reasons for the effects of different forms of nitrogen on

rhizosphere microbial communities. First, the form of nitrogen present influences the pH of culture media (O'Donnell, Seasman, Macrae, Waite, & Davies, 2001; Kemmitt, Wright, Goulding, & Jones, 2006). Nitrogen in the form of ammonium decreases the pH of the soil, while nitrogen in the form of nitrate increases the pH. Ma et al. (2008) reported that ammonium increased the numbers of actinobacteria and fungi in the rhizosphere of Yumai 49 (a medium-gluten wheat), and our study confirmed this result. The numbers of culturable actinobacteria and molds from the rhizosphere of *E. crassipes* cultured in NH_4Cl solution were more than two orders of magnitude higher than those cultured from plants in distilled water or KNO_3 solution. DGGE analysis also confirmed that NH_4Cl can promote the growth of actinobacteria such as *Streptomyces*. We are aware that NH_4Cl can reduce the pH of solutions. Molds prefer slight acidity and survive better in acidic solutions. In contrast, actinobacteria prefer slight alkalinity or neutrality (Su et al., 2013), so the mechanism whereby NH_4Cl improved the growth of actinobacteria was not pH-dependent.

Another reason why different forms of nitrogen influenced the rhizosphere microbial community is that the form of nitrogen present can directly affect the growth of the community. Because of various preferences for the type of nitrogen, the presence of different forms of nitrogen alters the components of the rhizosphere microbial community. The experiments of Zhu, Yao, Long, and Yang (2004) indicated that ammonium ion could inhibit the hyphal growth of AM fungus. Liu, Huang, and Kong (2005) also confirmed that *Aspergillus niger* ML2 and ML4 exhibited high efficiency of phosphate solubilisation when cultured in a medium with ammonium as the only nitrogen source.

The final reason why different forms of nitrogen can influence the rhizosphere microbial community is that different forms of nitrogen can differently influence the growth and development of plants, particularly roots (Gao & Pan, 1998; Ma, Wang, Wang, & Wang,

2004). The rhizosphere of plants provides a microenvironment for the growth of rhizosphere microbes. Respiration and other physiological processes of plant roots can affect this microenvironment. Rhizosphere microbiology is associated with the secretion and abscission of plant roots (Liljeroth, Bååth, Mathiasson, & Lundborg, 1990), and different forms of nitrogen can influence these processes, thereby influencing the microbiology of the rhizosphere. Amino acids secreted from the roots of *E. crassipes* induced chemotaxis in its rhizosphere bacteria (Zhao & Zheng, 1996). The root exudates from *E. crassipes* can stimulate the growth of phenol-degrading *Enterobacter* sp. nov (Wu & Zheng, 1993). In our experiment, ammonium was found to stimulate the growth of actinobacteria; however, whether any secretion or abscission from the roots of *E. crassipes* can stimulate the growth of actinobacteria remains to be investigated.

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RESUMEN

Comunidades microbianas de la rizósfera son importantes para la fitorremediación, nutrición vegetal, salud y metabolismo. Muchos factores, incluyendo la especie de planta, el pH y los factores nutricionales influyen en la microbiología de la rizósfera. En este estudio, se analizaron los efectos de las diferentes formas del nitrógeno en la estructura de las comunidades microbianas de la rizósfera de *E. crassipes*. Mediante métodos de cultivo convencional con medios especiales se cultivaron: bacterias, actinobacterias y mohos. Se encontró que el número de bacterias era en gran parte similar a través de las tres condiciones de cultivo, mientras que el número de actinobacterias y mohos de la rizósfera de *E. crassipes* cultivadas en solución de NH4Cl era dos órdenes de magnitud superior a los de las rizósferas de plantas cultivadas en agua destilada y solución de KNO3. Utilizando un método de cultivo independiente de electroforesis en gel con gradiente de desnaturalización (PCR-DGGE) del ADNr 16S, se encontró que la forma de nitrógeno podría influir en los componentes de

la comunidad microbiana de la rizósfera. *Pseudoxanthomonas*, *Enterobacter* y *Citrobacter* estaban presentes en todas las muestras cultivadas en las tres condiciones experimentales. El género *Reyranella* se encontró sólo en muestras cultivadas en solución de KNO3; *Acinetobacter* y *Streptomyces* eran las únicas muestras cultivadas en solución de NH4Cl, y *Pseudomonas*, *Pseudacidovorax* y *Methylosinus* se encontraron sólo en muestras cultivadas en agua destilada. *Pseudoxanthomonas* y *Acidovorax* eran los géneros dominantes en la comunidad microbiana de la rizósfera de *E. crassipes* cultivadas en solución de KNO3, mientras que *Novos phingobium* fue el género dominante en la muestra cultivada en un medio deficiente de nitrógeno. Nuestros resultados proporcionan una base teórica para el uso de *E. crassipes* como planta fitorremediadora y para controlar la distribución generalizada de *E. crassipes* en todo el mundo a través de los principios del metabolismo de nutrientes.

Palabras clave: *Eichhornia crassipes*, nitrógeno, comunidades microbianas de la rizósfera, PCR-DGGE.

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