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The Impact of Mineral Nitrogen Fertilization on the Occurrence of Native Diazotrophic Bacteria in Kohlrabi (*Brassica oleracea)* Shoots and Roots

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Abstract

Biological Nitrogen Fixation (BNF) is a process of great importance in crop production systems, as it provides additional natural sources of mineral nitrogen. BNF is catalyzed by diazotrophs that are identified by the *nif* operon presence comprising the *nif*H gene that encodes for enzyme nitrogenase synthesis. Thoroughly understanding of factors that influence diazotrophic abundance is crucial for their utilization to enhance sustainability and prevent land degradation in modern agriculture. In this study the impacts of nitrogen fertilization on diazotrophic abundance in *Brassica oleracea* roots and leaves was investigated in greenhouse experiments by real-time qPCR. One way ANOVA was used to compare means and bivariate Pearson correlation tested for relationships between variables. Increased nitrogen fertilization significantly increased the nitrogen content in leaves but not in roots. No significant changes in *nif*H gene copy numbers nor in proportion of *nif*H gene copy numbers were detectable. This indicates no effect of mineral N fertilization on the abundance of total native diazotrophic bacterial numbers in *Brassica oleracea* plants.

Keywords: diazotrophs, NH_4NO_3 fertilization, nitrogen fixation, *nif*H gene

1. Introduction

Biological nitrogen fixation (BNF) is a process through which the atmospheric inert gas N_2 is converted to nutrient ammonia (NH_3). The process is carried out by bacteria. In both symbiotic and free-living bacteria, BNF is genetically controlled by a group of *nif*, *anf* or *vnf* genes (Joerger et al., 1991; Waugh et al., 1995; Coelho et al., 2007a; Sugitha & Kumar, 2009). The *nif*H gene codes for dinitrogenase reductase and is part of the *nif* cluster that produces the nitrogenase catalytic enzyme complex and its regulatory proteins that are responsible for reducing atmospheric N_2 to ammonia (Sugitha & Kumar, 2009; Rosado et al., 1998; Poly et al., 2001). This functional gene has been a center of focus for researchers studying detection, dynamics and diversity of bacterial nitrogen fixing communities (Coelho et al., 2007b) and thus a potential indicator of microorganisms' ability to carry out BNF. The *nifH* gene is often used to quantify diazotrophs using real-time qPCR (Juraeva et al., 2006) and for their molecular identification. Among all the nitrogenase genes, *nifH* is the most extensively studied, with well-documented sequences from both cultured and uncultured diazotrophs (Coelho et al., 2007a; Tan et al., 2003; Deslippe & Egger, 2006). Consequently, *nifH* is utilized in phylogenetic, diversity, and abundance studies of diazotrophic microorganisms (Gaby & Buckley, 2012; Sugitha & Kumar, 2009).

The N_2 fixing enzyme nitrogenase is highly influenced by the availability of the mineral form (such as ammonium nitrate) in which nitrogen is present (Lovell, 2000; Fritzsche & Niemann, 1990; You et al., 2005). The existence of adequate availability of mineral nitrogen in any form in the plant growth substrate and eventually in plant tissues should inhibit N_2 fixation by bacteria, as the process is energetically expensive than mineral nitrogen uptake (Fritzsche & Niemann, 1990; Hartmann, 1988). The impact of nitrogen fertilization on plant growth, photosynthetic capacities, *nif*H expression and isolated diazotrophs has been extensively studied (Vos & van der Putten, 1998; Zhao et al., 2005; Cechin & Fumiss, 2004; Pati & Chandra, 1992), but surprisingly no study has yet focused on the quantification of diazotrophs or diazotrophs abundance dynamics in the

phyllosphere and in rhizosphere in relation to nitrogen fertilization and the relationship between shoots and roots. More knowledge is still needed on the relationships between mineral nitrogen availability, N_2 fixing activity, and the colonization and abundance of diazotrophic organisms around the plant. In order to characterize the interconnections between plant mineral N availability and native diazotrophs abundance we quantified diazotrophs (*nif*H-gene real-time qPCR) in leaves and roots of a vegetable plant kohlrabi (*Brassica oleracea*) grown at increasing nitrogen fertilization. *Brassica oleracea* is one of the non-leguminous traditional German vegetable of the cabbage family, with edible tuber and leaves and it can be eaten raw or cooked. Kohlrabi have high content of amino acids and glucosinolates which of nutritional importance to humans (Coi et al., 2010). We hypothesized that rising nitrogen content within the plant tissues by NH_4NO_3 fertilization should negatively affect the abundance of diazotrophic bacteria in both roots and leaves. This was based on the assumption that provision of plant species' optimum or above-optimum mineral nitrogen nutrients decreases the need to get additional mineral nitrogen via BNF and thereafter down-regulate the growth and settlement of diazotrophic bacteria around and within plant tissues.

2. Materials and Methods

2.1 Greenhouse Experiment and Harvesting

The study was conducted and repeated at the Leibniz-Institute of Vegetable and Ornamental Crops in Großbeeren (Brandenburg, Germany). Kohlrabi (*Brassica oleracea*) plants were grown at 5 different nitrogen fertilization levels including a negative control. Each nitrogen level consisted of 24 plants. All were grown for 8 weeks in the greenhouse in pots (33 cm diameter) containing 1kg of non-sterilized quartz sand (0.5-1 mm particle size). Three times a week essential nutrients were provided as 100 ml half strength nitrogen-free Hoagland solution (Hoagland & Arnon, 1950). Plants were provided with reverse-osmosis purified water according to demand (about 150 ml per day); the water that leaked through the plant pots was collected in plates and reused. The different N fertilization treatments were applied by injecting 10 ml NH_4NO_3 solution around the root zone with a pipette once a week. The total nitrogen administered per plant was applied during the growth period and administered at weekly intervals to prevent any negative impact of high salinity due to high nitrogen ion concentrations. The N levels applied as total amount of NH_4NO_3 per plant for the entire experiment were: 0g for N level 0 (N0 negative control), 0.5 g for N level 1 (N1), 1 g for N level 2 (N2); 1.5 g for N level 3 (N3) and 2 g for N level 4 (N4). Other essential macronutrients were provided at a uniform rate at all nitrogen levels in this experiment (Table 1). After 8 weeks plants were harvested and separate samples of shoots and roots collected (three plants pooled per replicate). For DNA extraction and molecular analyses, samples were stored at -20 °C overnight before lyophilization. The remaining roots and leaves were dried at 60 °C for one week, weighed and milled with a high speed plant grinder (Fritsch Pulverisette; Oberstein, Germany) for nitrogen content analysis. This experiment was repeated once.

Table 1. NPK fertilization rates (grams per plant of nitrogen (N), phosphorus (P) and potassium (K))

N level	N (g)	P (g)	K (g)
N0	0	2.8	5
N1	10	2.8	5
N2	20	2.8	5
N3	30	2.8	5
N4	40	2.8	5

2.2 Nucleic Acid Extraction and qPCR

DNA was extracted from 50 mg lyophilized plant material using DNeasy plant mini kits (Qiagen, Hilden GmbH, Germany) according to the manufacturer's instructions. Lysis of bacterial cells was ensured by adding five 5 mm sterile metal beads and using a Retsch MM200 mechanical disrupter (Haan, Germany) at 30 rpm for 5 minutes. Quantitative real-time PCR (qPCR) was run using an Advanced™ Universal SYBR® green I dye Supermix system (Bio-Rad Laboratories, Hercules, CA, USA) as an indicator of resultant PCR amplicons. Diazotroph amplification was done with *nif*H specific primer pair 19F (5′-GCIWTYTAY GCIAARGGIGG-3′) and 388R (5′AAICCRCCRCAIACI ACRTC-3′) (Juraeva et al., 2006) by the following protocol: Cycle 1, 95 °C/5 min; cycle 2, 94 °C/30 sec, 50 °C/60 sec, 72 °C 75 sec (recording fluorescence data) 40 repeats; cycle 3, 72 °C/10 min;

cycle 4, melt curve 55 °C increasing temperature every 10 sec by 0.5 °C (recording fluorescence data), 85 repeats; and cycle 5, 4 °C hold (Juraeva et al. 2006). Total bacterial copy numbers were quantified using the primers 519f (5′-CAGCMGCCGCGGTAANWC-3′) and 907r (5′-CCGTCAATTCMTTTRAGTT-3′) specific for the bacterial domain (Lane, 1991) with the PCR protocol: Cycle 1, 95 °C/5 min; cycle 2, 95 °C/15 sec, 53 °C/30sec, 72 °C/42 sec (recording fluorescence data) 40 repeats; cycle 3, 72 °C/10 min; cycle 4, melt curve 55 °C increasing temperature every 10 sec by 0.5 °C (recording fluorescence data), 85 repeats; and cycle 5, 4 °C hold (Ruppel et al., 2006). Fluorescence was recorded at 520 nm after excitation at 490 nm with a Bio-Rad CFX96 real-time qPCR detection system (BIO-RAD, Foster City, California, United States of America). Total bacterial and *nif*H gene copy numbers were quantified according to a standard curve generated from PCR products specific for bacteria (*Escherichia coli*) and *nif*H (*Kosakonia radicincitans*) genes of known copy numbers. The results were log10 transformed and calculated as gene copy numbers per µg DNA.

2.3 Nitrogen Content Analysis

Dried root and leaf samples were milled to a fine powder with a high speed plant grinder (Fritsch Pulverisette, Oberstein, Germany) and total nitrogen content was analyzed using a CN-Vario EL analyzer (Elementar, Hanau, Germany) (Berger et al., 2013; Chintala et al., 2013).

2.4 Statistical Analysis

Comparison of mean values of 8 replicates was performed using one-way ANOVA analysis and Bonferroni-test at a P-level of ≤ 5% (IBM® SPSS® 20). Statistical differences between means are indicated by uppercase letters in roots and lowercase letters in leaves. Bars indicate the standard errors. Pearson-type correlations (r) were calculated at a P-level of ≤ 5% and at ≤ 1% (IBM® SPSS® 20).

3. Results

3.1 NH_4NO_3 Fertilization Impact on Dry Weight and Plant Nitrogen Content

To demonstrate the N-fertilization impact on plant growth and nitrogen content, leaves and roots were sampled and analyzed separately after a growth period of 8 weeks. For both leaves and roots, provision of nitrogen fertilization resulted in a substantially increased plant dry matter between N0-N1, however further increase in fertilization did not result in continuous increase in plant dry matter (Figure 1). That is to say, increased shoot to root growth ratio with increasing N fertilization could only be seen from N0 to N1. As a result, there were significant differences ($p < 0.05$) in dry matter between N0 and fertilized N levels in both roots and leaves. The mean nitrogen content was significantly higher in leaves than in roots at all levels ($p < 0.05$). There was a continuous and significant increase ($p < 0.05$) in leaves' mean nitrogen content with the lowest mean recorded at N0 (0.94 ± 0.09 g, $n = 8$) and the highest recorded at N4 (7.18 ± 0.61 g, $n = 8$) (Figure 2). Roots did not exhibit the same trend; the lowest mean was also recorded at N0 (0.62 ± 0.79 g, $n = 8$) but the highest mean was at N1 (1.69 ± 0.22 g, $n = 8$) and values remained essentially constant from N1 to N4 (Figure 2). However, plant root dry weight was significantly low ($p < 0.05$) at N0 than at fertilized N levels.

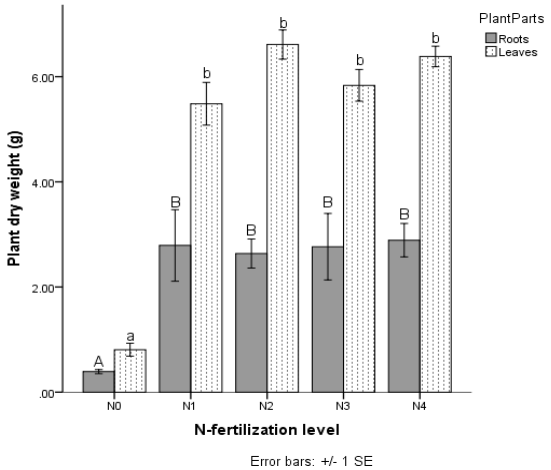

Figure 1. Dry weight (g $plant^{-1}$) of kohlrabi leaves and roots (both $n = 8$) at increasing levels of NH_4NO_3 fertilization. Statistical differences between means are indicated by different uppercase letters in roots and different lowercase letters in leaves

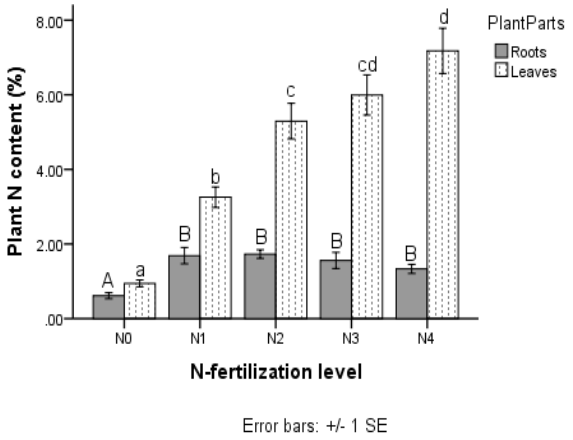

Figure 2. Plant nitrogen content (%, dry weight basis) of kohlrabi leaves and roots (both n = 8) at increasing levels of NH_4NO_3 fertilization. Statistical differences between means are indicated by different uppercase letters in roots and different lowercase letters in leaves

3.2 Diazotrophic Bacterial (nifH) Quantification

Quantification of total diazotrophic organisms using functional *nif*H gene copy numbers in real-time qPCR analysis revealed significantly ($p < 0.05$) higher *nif*H gene copy numbers in leaves than in roots (Figure 3). Nonetheless, there was no detectable influence ($p > 0.05$) of increased N-fertilization on mean *nif*H gene in either roots or leaves (Figure 3). To further evaluate the N-fertilization impact on the plant microbiome community structure at each N-fertilization level, *nif*H gene was calculated as a percentage of the total bacteria (16S rDNA gene copy numbers) to reveal the extent to which the total bacteria population within and around the plant was accounted for by diazotrophs. This additional calculation was done to validate the *nif*H quantity results in Figure 3. Similar to *nif*H copy numbers results in Figure 3 diazotrophs proportion (*nif*H gene copy numbers of total bacterial copy numbers) also did not reveal a decreasing or an increasing trend with increasing N-fertilization on the plants (Figure 4). In addition these results also indicated a high variation in *nif*H proportion at different N-fertilization levels. Thus there were no significant difference ($p > 0.05$) in the mean *nif*H proportion in leaves and roots. Similar to *nif*H quantification, diazotrophs proportion at the total bacterial counts was higher in leaves than in roots when molecular methods were applied.

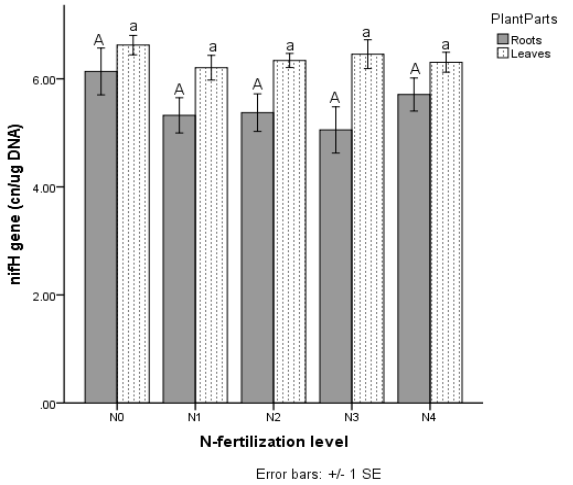

Figure 3. Total *nif*H gene copy numbers (cn) (*nif*H gene cn μg^{-1} DNA, n = 8) of kohlrabi leaves and roots (both n = 8) at increasing levels of NH_4NO_3 fertilization. Statistical differences between means are indicated by different uppercase letters in roots and different lowercase letters in leaves

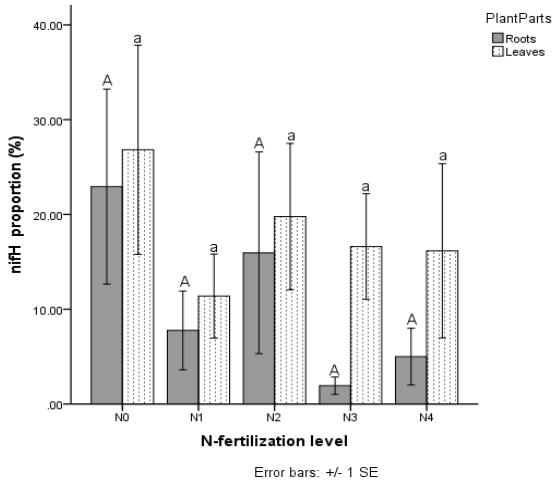

Figure 4. Proportion of *nif*H gene copy numbers in total bacterial copy numbers (%) of kohlrabi leaves and roots (both n = 8) at different levels of NH_4NO_3 fertilization. Statistical differences between means are indicated by uppercase letters in roots and lowercase letters in leaves

3.3 Correlation

As expected, increasing NH_4NO_3 fertilization was significantly positively correlated to kohlrabi leaf dry matter (r = 0.72**) and leaf nitrogen content (r = 0.86**) (Table 2). Root dry matter also showed significant correlation to NH_4NO_3 fertilization (r = 0.46**) while, N-content showed no significant correlation to NH_4NO_3 fertilization (r = 0.31). Nevertheless, increased NH_4NO_3 fertilization did not significantly impact *nif*H gene copy numbers in leaves (r = 0.10) or in roots (r = -0.15). Likewise the *nif*H gene copy number proportion of total bacterial copy numbers was not significantly correlated to increased N-fertilization in either leaves (r = -0.11) or roots (r = -0.30) (Table 2). Furthermore to test for interactions between leaves and roots correlation test was done for different variables. In this test only *nif*H quantity roots was positively correlated (0.512**) to the *nif*H quantity in leaves among other variables.

Table 2. The impact of N fertilization other studied variables

Variables	Leaves (r values)	Roots (r values)
Dry weight	+ (0.72**)	+ (0.46**)
Nitrogen content	+ (0.86**)	0 (0.31)
*nif*H quantity	0 (-0.10)	0 (-0.15)
*nif*H percentage	0 (-0.11)	0 (-0.30)

Keys: + Positive correlations; - Negative correlations; 0 insignificant correlations; * Correlation is significant at the 0.05 level; ** Correlation is significant at the 0.01 level.

4. Discussion

The unexpected insignificant differences between means and correlation coefficients found between *nif*H, *nif*H proportion in relation to N-fertilization could be explained by the fact that most diazotrophic bacteria have multiple plant growth promoting functions in associated plants, and BNF is one of those (Yuan et al., 2012; Fernando & Linderman, 1995; Xu et al., 2004). Other functions around the plants include phosphate transformer genes, siderophore genes as wells as phytohormone production genes and of course these functions vary between strains (Witzel et al., 2012). These other functions can contribute to a selective advantage unrelated to nitrogen fixation for plants colonized by diazotrophic bacteria. In this model, the absence of the need for the BNF does not necessarily lead to the loss of diazotrophs from the plant as they still provide other benefits to the plant. It is worth noting that presence of *nif*H gene-containing microorganisms does not invariably indicate ongoing BNF, especially when there is mineral nitrogen available to the plant (Fritzsche & Niemann, 1990). Fritzsche and Niemann (1990) also documented a decrease in BNF activity with provision of increased NH_4Cl but did not

report the impact on the nitrogen fixing bacterial population.

The regulation of diazotrophic activities seems very complex. In this study, provision of sufficient ammonium nitrate was ensured by adding the compound above the documented optimum requirements in the literature (Carmen et al., 2007). From the Fritzsche and Niemann (1990) findings, it appears that BNF in the rhizosphere does not solely depend on total absence of mineral nitrogen sources. Similar findings recorded by Lovell (2000) indicate that there was still BNF activity in open marine water that had abundant ammonium availability. In addition some findings indicated that high level of mineral nitrogen supply might inhibit N_2 fixation in some diazotrophic species but not in all (Rosado, 1998; Lovell, 2000; Ruppel & Merbach, 1995). Lovell (2000) even reported an increase in nitrogenase expression with increase in mineral N availability and concluded that short term manipulation in nitrogen supply does not have significant impact on the diazotrophic communities. Similarly Juvaera et al. (2006) reported that although there were physiological changes in plants due to N fertilization these did not cause significant changes to *nif*H gene abundance or to the diazotrophic culturable organisms around the plants.

5. Conclusion

These results revealed that increased NH_4NO_3 fertilization had no significant impact on diazotrophs abundance in rhizosphere and in the rhizosphere of the studied kohlrabi plants (*Brassica oleracea)*, despite the increased nitrogen content observed within plant tissues particularly in the phylosphere. However, these results highlight the potential importance of the phyllosphere in BNF, since diazotrophs abundance (*nif*H gene copy numbers) were higher in leaves than in roots. Finally there is a need for more extensive investigations in both phyllosphere and rhizosphere to determine if the *nif*H genes were being expressed or not in the diazotrophic communities under adequate supply of mineral nitrogen.

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