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Nitrogen fertiliser rate affects the frequency of nitrate-dissimilating *Pseudomonas* spp. in the rhizosphere of *Lolium perenne* grown under elevated pCO_2 (Swiss FACE)

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Abstract

The effect of elevated pCO_2 (60 Pa) on the frequency of nitrate-dissimilating *Pseudomonas* (NDP) was investigated in the rhizosphere of fertilised *Lolium perenne* swards in the Swiss Free Air Carbon dioxide Enrichment (FACE) experiment. Numbers of cultivable root-associated *Pseudomonas* were greater under elevated (60 Pa) than under ambient (36 Pa) pCO_2 in both high and low N-fertilised swards. For both pCO_2 conditions, the NDP frequency decreased with closer root proximity to *L. perenne* roots in low fertilised swards. Anyway, in high N swards the NDP frequency was similar in root and soil fractions. Thus, N availability may be a major factor influencing NDP populations under elevated pCO_2, most likely due to increased competition for N between plant and nitrate-dissimilating bacteria.

Keywords: N availability; Nitrate dissimilation; *Pseudomonas*; Rhizosphere; Perennial grass; Rhizosphere; Elevated atmospheric carbon dioxide.

1. Introduction

There is uncertainty about the effects of increasing atmospheric concentrations of CO_2 (pCO_2) on denitrification in terrestrial ecosystems. Various studies have shown that potential denitrifying enzyme activity may be stable or decrease (Hungate et al., 1997; Barnard et al., 2004), whereas N_2O emissions may increase (Arnone and Bohlen, 1998; Baggs et al., 2003a,b) for herbaceous systems under elevated pCO_2. Little is known about the response of microorganisms involved in denitrification (Roussel-Delif et al., in press; Deiglmayr et al., 2004), partly because of the widespread ability to denitrify, and functional differences among denitrifying microorganisms make it difficult to relate denitrification activity to soil microbial communities (Cavigelli and Robertson, 2001). Roussel-Delif et al. (in press) recently showed that the frequency of root-associated nitrate-dissimilating *Pseudomonas* (NDP) in low fertilised (14 g N m^{-2} y^{-1}) *L. perenne* swards was increased under elevated pCO_2, suggesting that increased pCO_2 provided favourable conditions for the dissimilation of nitrate and related organisms. However, this is likely to be dependant on N fertilisation and competition with roots for available soil N.

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2. Material and methods

We investigated the abundance of culturable *Pseudomonas* and the frequency of NDP in the rhizosphere of *L. perenne* swards receiving different N fertilisation rates at the Swiss Free Air Carbon dioxide Enrichment (FACE) experiment. The hypothesis was that increased belowground C allocation under elevated pCO_2 would increase the number and frequency of nitrate-dissimilating *Pseudomonas* and that the extent of this response would vary with N application rate and proximity to *L. perenne* roots, due to competition with plants for

Table 1
Soil mineral N (mg N kg^{-1} dry soil) averaged over the 5 week re-growth period in low (14) and high (56 g N m^{-2} y^{-1}) N-fertilised *L. perenne* swards under ambient (36 Pa) and elevated (60 Pa) pCO_2

	Low N		High N	
	36 Pa	60 Pa	36 Pa	60 Pa
Available NH_4^+	39.9	24.7	30.8	46.5
(mg NH_4^+–N kg dry soil^{-1})	(±11.6)	(±6.1)	(±7.6)	(±6.1)
Available NO_3^-	35.9	51.3	190.6	135.1
(mg NO_3^-–N kg dry soil^{-1})	(±12.5)	(±15.6)	(±27.6)	(±20.5)

Values in parentheses are ± SEM. After Baggs and Blum (2004).

available soil nitrate. The FACE experiment at Eschikon (8°41′E, 47°27′N; 550 masl), Switzerland, consists of three elevated (60 Pa) and three ambient (36 Pa) CO_2 rings, established in an open field situation in 1993 (Hebeisen et al., 1997). The soil is a clay loam (sand 36%, silt 33%, clay 28%, organic matter 2.9–5.1%, pH 6.5–7.6) classified as an Eutric Cambisol (FAO classification). *L. perenne* L. (cv Bastion) swards were fertilised with NH_4NO_3 in solution at rates of 14 (low N) and 56 g N m^{-2} y^{-1} (high N) split between five swards regrowth periods. Three undisturbed plant-soil cores (20 cm depth) were sampled from each replicate plot on 15 July 2002, 3 weeks after application of 2.8 g N m^{-2} (low N) and 11.2 g N m^{-2} (high N), 1 week before the third cut to avoid any effect of defoliation on N availability (Gloser et al., 2000). Soil was sampled (0–25 cm) from each plot in July 2002 and mineral N determined colorimetrically after extraction in 1 M KCl (Baggs and Blum, 2004) (Table 1).

Non-rhizosphere soil (NRS), rhizosphere soil (RS) and root (noted RE for rhizosphere–endorhizoplane) crushed samples were diluted in phosphate sodium buffer 0.1 M, pH 7.0, and plated on selective mS1 medium. Thirty bacterial isolates were randomly selected for each sample and their affiliation to the *Pseudomonas* genus was checked by specific hybridization (Tarnawski et al., 2003). *Pseudomonas* counts were deduced from the proportions of PSM_G-hybridizing colonies to the total number of bacterial colonies growing on the *Pseudomonas*-selective mS1 medium. The ability of 680 *Pseudomonas* strains to dissimilate nitrate to nitrite and to gaseous compounds was tested during an in vitro assay, according to Roussel-Delif et al. (in press) and the strains were scored as non-dissimilators, nitrate reducers, or denitrifiers. The frequencies of nitrate -dissimilating (nitrate-reducing and denitrifying) strains were analysed using a generalised linear model (glm) with a logistic regression model. These frequencies were further compared using Tukey multiple comparison test (comparison between fractions), or Fisher LSD exact test (comparisons between treatments). Statistical analysis were performed using S-Plus 6 Statistical Software (Insightful Corporation, Seattle, Washington).

3. *Pseudomonas* numbers

N application rate had no consistent effect on *Pseudomonas* counts. *Pseudomonas* counts in both low and high-fertilised swards were higher in root-associated RE and RS fractions under elevated than under ambient pCO_2 (Fig. 1), confirming previous results obtained at this site using a 16S rDNA-based approach (Marilley et al., 1999). This was attributed to greater belowground C allocation associated with increased root biomass, turnover and exudation under elevated pCO_2 (van Kessel et al., 2000) favouring *Pseudomonas* populations that are known to be good heterotrophic competitors for C sources, irrespective of their denitrifying abilities (Tiedje, 1988). *Pseudomonas* can metabolize a wide range of organic compounds and use electron acceptors other than oxygen, which confers a competitive advantage to these organisms in nutrient-rich environments such as the rhizosphere (Latour and Lemanceau, 1997). In the same FACE experiment, *Pseudomonas* abundance varied temporally, but was always higher than 10^5 cfu g^{-1} root or soil dry weight (Tarnawski, S., 2004. Unpub. PhD thesis, University of Neuchâtel, Switzerland).

4. Frequency of nitrate dissimilating *Pseudomonas*

The frequencies of true denitrifiers (about 10% of all *Pseudomonas*) followed the same trends as for NDP (see below) in low N swards, with no distinct pattern in high N swards (denitrifying numbers were too low to perform statistical tests).

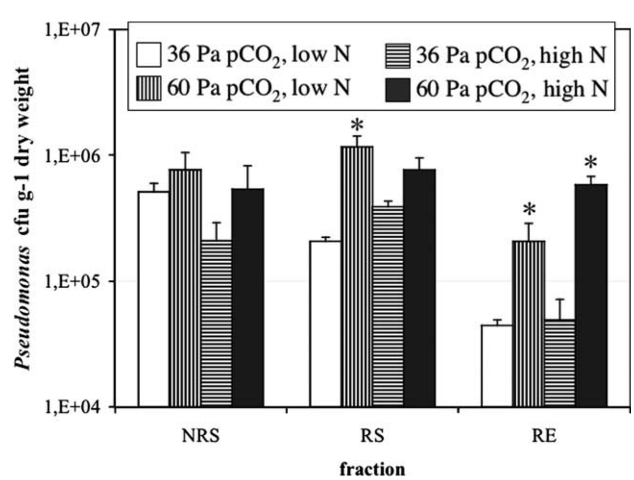

Fig. 1. *Pseudomonas* colony forming units (cfu) counts, NRS, non-rhizosphere soil; RS, rhizosphere soil; RE, rhizoplane–endorhizosphere (root). * Indicates a statistically significant difference ($P < 0.05$) in cfu counts between control (36 Pa) and CO_2-treated (60 Pa) plots for a given fraction.

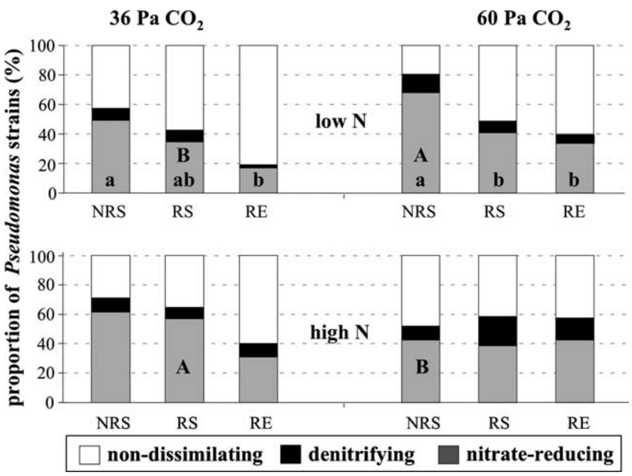

Fig. 2. Frequencies of nitrate-reducing, denitrifying and non-dissimilating *Pseudomonas* strains in the rhizosphere of low and high fertilised *L. perenne* under ambient and elevated pCO_2. Different letters indicate NDP frequencies that are statistically different ($P<0.05$). (a,b) for differences between fractions for a given CO_2 and N fertilisation treatment; (A,B) indicates differences between low and high N treatments for a given fraction and CO_2 condition.

NDP frequency was primarily influenced by the root proximity regarding the fraction considered ($P<0.05$) and by the N application rate ($P<0.01$), rather than by elevated pCO_2 ($P=0.2$) suggesting that N availability had a greater influence on NDP than pCO_2 treatment. In high N swards, the annual application of $56\ g\ N\ m^{-2}\ y^{-1}$ exceeded *L. perenne* requirements (estimated to be $29\ g\ N\ m^{-2}\ y^{-1}$ in these *Lolium* swards; M. Richter, unpub. PhD thesis, Swiss Federal Institute of Technology, 2003), resulting in availability of mineral N for nitrification and denitrification (Table 1). Accordingly, the NDP frequency did not decrease at root proximity.

In low N swards ($14\ g\ N\ m^{-2}\ y^{-1}$), nitrate was probably limiting for NDP in close proximity to roots because of plant uptake, and so competition would have arisen between NDP and *Lolium* plants for available N. Additionally, greater plant N demand (Daepp et al., 2000) or microbial immobilisation (Hungate et al., 1997) would have further reduced nitrate availability for NDP as indicated by lower available soil nitrate in the high N swards under elevated pCO_2 (Table 1).

The frequency of root-associated NDP tended to be higher under elevated pCO_2 (Fig. 2), as previously shown at this site (Roussel-Delif et al., in press), also it was not significant (95% confidence limit). This tendency towards higher NDP frequency under elevated pCO_2 may reflect favourable conditions provided in the rhizosphere for these bacteria. These conditions include increased belowground C allocation and subsequent increased microbial activity (van Ginkel et al., 2000; Williams et al., 2000), and increased water content under elevated pCO_2 (Hungate et al., 1997), which both result in lower pO_2, one of the main determinants of denitrification (Tiedje, 1988; Arnone and Bohlen, 1998). In accordance with this, Baggs et al. (2003a) measured increased emissions of denitrified N_2O from high-fertilised *Lolium* swards under elevated pCO_2 at this site, which were positively correlated with soil organic C (Baggs and Blum, 2004). The response of NDP can not be directly related to the denitrification activity measurements made in the field because other organisms could have been responsible for nitrate transformations, and also because N_2O emission can result from other processes (Baggs et al., 2003b; Müller et al., 2004). The influence of elevated pCO_2 on NDP frequency was not as strong as hypothesised. It is possible that the 9 y exposure to elevated pCO_2 resulted in an acclimation of the soil-plant system to function under these conditions (Rogers et al., 1998). Previous experiments have shown that the type of nitrate reductase enzyme carried by the NDP differed under ambient and elevated pCO_2 (Roussel-Delif et al., in press), suggesting that NDP populations may respond to elevated pCO_2 by altering both the enzyme type, and probably its activity.

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