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Phenotypic structure of *Pseudomonas* populations is altered under elevated pCO_2 in the rhizosphere of perennial grasses

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Abstract

The increasing atmospheric CO_2 content (pCO_2) is likely to modify the ecosystem functioning including rhizosphere bacteria that are directly dependent on rhizodeposition. This may include alteration of *Pseudomonas* populations that display phenotypic traits in relation with plant fitness. In the present study, 1228 *Pseudomonas* strains were isolated from the non-rhizosphere soil, rhizosphere soil and root fractions of perennial grassland systems: *Lolium perenne* and *Molinia coerulea*. Both plants were grown under ambient (36 Pa) and elevated (60 Pa) pCO_2 in the Swiss Free Air CO_2 Enrichment (FACE) system. *Pseudomonas* spp. were tested for their ability to produce auxin, siderophores and hydrogen cyanide, and to dissimilate nitrate. No effect of root proximity and elevated pCO_2 was observed on the proportions of auxin producers. For *L. perenne* and *M. coerulea*, siderophore and hydrogen cyanide *Pseudomonas* producers were stimulated in the root fraction. In contrast lower frequencies of nitrate reducers were observed in the root fraction compared to non-rhizosphere soil. The frequencies of siderophore producers and nitrate dissimilating strains were higher, and those of hydrogen cyanide producers lower, under elevated pCO_2 for *L. perenne*. This alteration of the phenotypic structure of *Pseudomonas* guild in the root fraction is discussed in relation with the physico-biochemical modifications of the rhizosphere condition via rhizodeposition and environmental changes occurring under elevated pCO_2.

Keywords: Plant growth promoting rhizobacteria; PGPR; Siderophores; Hydrogen cyanide; Auxin; Nitrate reduction; Root colonisation; Rhizosphere competence

1. Introduction

Since the beginning of the industrial revolution, atmospheric CO_2 content (pCO_2) has been increasing significantly, changing the global climate and ecosystem functioning (Fuhrer, 2003). For example, the rate of net photosynthesis (Isopp et al., 2000) and C allocation to roots (Suter et al., 2002) are enhanced under elevated pCO_2. A significant part of this C is released by plants roots (rhizodeposition) into the rhizosphere, the region of soil in close proximity and under the influence of living plant roots (Lynch and Whipps, 1990). In the case of meadow plants, up to 30–50% of total assimilated carbon can be translocated to the soil (Kuzyakov, 2001; Gobat et al., 2004). Consequently, plants modify their surrounding soil and play a major role in regulating the rhizosphere microflora (Lynch and Whipps, 1990; Lemanceau et al., 1995; Paterson, 2003). Under elevated pCO_2, the C flow into the soil increases (Darrah, 1996; Suter et al., 2002), and its composition may be altered (Hodge et al., 1998). This might stimulate the growth and activities of soil microbiota feeding on plant-derived C sources (Weihong et al., 2000). Due to their metabolic versatility and high growth rate, bacteria belonging to the genus *Pseudomonas* are well adapted to colonizing the rhizosphere (Lemanceau et al., 1995; Lugtenberg and Dekkers, 1999; Rainey, 1999). Because of their competitivity for nutrients and for suitable niches on the root surface, they are able to respond rapidly to environmental modifications (Glick, 1995; Sørensen et al., 2001; Stres et al., 2004). Indeed, the dominance of 16S rDNA clones affiliated to the genus *Pseudomonas* was shown to increase in the rhizosphere of perennial ryegrass after 3 years under elevated pCO_2 (Marilley et al., 1999). Moreover, numerous *Pseudomonas* strains display plant growth promoting activities. Plant growth promoting rhizobacteria can act either directly (e.g. by enhancement of plant nutrient uptake or by production of phytohormones) or indirectly (e.g. by biological control of root pathogens and alteration of microbial populations balance) (Glick, 1995).

During the last decade, the effect of increases in pCO_2 on plant-microorganism interactions has drawn considerable interest. Changes in diversity and structure of microbial

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populations for plants grown under elevated pCO_2 have been described using both metabolic (Grayston et al., 1998; Hodge et al., 1998) and molecular approaches (Griffiths et al., 1998; Kandeler et al., 1998; Marilley et al., 1999; Montealegre et al., 2002). However, relatively little is known about the stimulation or repression by elevated pCO_2 of bacterial activities that interfere with plant fitness. In this study we investigated some well-known *Pseudomonas* activities involved in plant-bacteria interactions: (i) NO_3^- dissimilation, an anaerobic respiratory process during which NO_3^- is reduced (Ghiglione et al., 2000) and can therefore become unavailable for plant nutrition; (ii) production of auxin (IAA), an hormone involved in root elongation (Glick, 1995; Persello-Cartieaux et al., 2003); (iii) production of hydrogen cyanide (HCN) (Castric, 1977; Blumer and Haas, 2000) a secondary metabolite which can inhibit the growth of rhizosphere inhabiting bacteria and fungi, and sometime interfere with root growth (Bakker and Schippers, 1987); and (iv) production of siderophores, organic ligands that complex iron, providing a competitive advantage to producing organisms in the rhizosphere, an oxygen- and iron-depleted environment (Lugtenberg and Dekkers, 1999). We hypothesized that environmental modifications induced by elevated pCO_2 in the rhizosphere via the plant could alter the structure of *Pseudomonas* populations with particular traits related to plant fitness. Two perennial grasses with different trophic requirements (Vázquez de Aldana and Berendse, 1997) were used: the oligonitrophilic *Molinia coerulea*, and the nitrophilic *Lolium perenne*. Culturable *Pseudomonas* strains were isolated during two growing seasons and tested for their ability to produce auxin, siderophore and hydrogen cyanide and to dissimilate nitrate.

2. Materials and methods

2.1. Study site and plant material

L. perenne and *M. coerulea* were grown under ambient (36 Pa, control plots) and elevated pCO_2 (60 Pa, treated plots) in the Free Air CO_2 Enrichment (FACE) facility, Eschikon, Switzerland (Hebeisen et al., 1997). The treated plots were enriched with CO_2 during daylight between March and December. *L. perenne* cv *Bastion* was grown as a monoculture on three control and three CO_2-treated replicate plots since May 1993. The plants were cultivated in the local autochthonous fertile, Eutric Cambisol soil. Shoots were harvested four times a year. The control and treated plots received $14\ \mathrm{g\ m^{-2}\ year^{-1}}$ N as NH_4NO_3 (at the beginning of the season, and then after each cut, except the last). *M. coerulea* plants originated from a littoral meadow located at the southern shore of Lake Neuchâtel (Cudrefin, Switzerland). The local soil is a Gleysol, typic Haplaquoll with low N content (Hamelin et al., 2002). Plants with undisturbed root systems were taken with their surrounding and underlying soil and transferred to the FACE facility in September 1999. About $0.7\ m^2$ of littoral meadow with reconstructed soil profile below the root horizon (total depth: 35 cm) was installed in one control plot and one CO_2-treated plot. *M. coerulea* plants were neither cut nor fertilized.

2.2. Sampling plan

For *L. perenne*, the three replicates for both control and treated plots were sampled in June, July, and October 2000, as well as in July and December 2001. During the growth season, sampling of *L. perenne* was always performed before a cut in order to avoid the effect of cutting on plant N demand (Daepp et al., 2000). For *M. coerulea*, both the control and the treated plots were sampled in June 2000, then in July and December 2001. From each sampled plot, three soil cores (about 3 cm diameter, 10–12 cm depth), including dense root systems, were taken and pooled for analysis. The soil were sampled and analysed the same day and three fractions were recovered: non-rhizosphere soil (NRS) for soil devoid of roots (corresponds to soil which comes off the roots after shaking: it is probably influenced by roots in the case of grassland that display dense root systems, but to a lesser extent than other fractions); rhizosphere soil (RS) for root-adhering soil (recovered by washing the roots with its adhering soil twice in 20 ml sodium phosphate buffer 0.1 M, pH 7.0 (SPB) under agitation during 30 min), and rhizoplane–endorhizosphere (RE) for thoroughly SPB washed roots. For *M. coerulea*, as the rhizosphere fraction was not easy to recover, only the two fractions NRS and RE were analysed.

2.3. Pseudomonas *counts and isolation*

Counting and isolation of *Pseudomonas* strains was performed as previously described (Tarnawski et al., 2003). Briefly, approximately 1 g of fresh weight root or soil was crushed in 10 ml SPB. The root and soil suspensions were ten-fold serially diluted in SPB and spread on mS1 medium, which is selective for *Pseudomonas* (Tarnawski et al., 2003). Colony forming units (CFU) were determined after 72 h of incubation at 24 °C. Twenty (June 2000), 12 (July, October 2000 and July 2001), and 15 bacterial colonies (December 2001) were randomly picked from appropriate dilution plates (between 20 and 200 colonies per Petri dish) and checked for purity on 10-fold diluted Tryptic Soy Agar (Difco). The affiliation of mS1 isolates to the genus *Pseudomonas* was ensured by hybridisation with PSM_G probe (Marilley et al., 1999). This method was previously applied to screen soil *Pseudomonas* from *M. coerulea* (Locatelli et al., 2002).

2.4. Auxin production assay

For the indole-3-acetic acid (IAA) production assay, fresh cultures were inoculated in Nuclon microtitre plates (Nagel Nunc International, NY, USA) containing 100 µl Angle liquid medium per well (Angle et al., 1991) supplemented with L-tryptophan (final concentration 5 mM), a metabolic precursor of auxin (Bric et al., 1991). After incubation for 72 h at 24 °C in the dark, the supernatant of each culture was transferred to a new microtitre plate. The presence of IAA in the supernatant was detected by a colorimetric technique specific for the detection of indolic compounds (Bric et al., 1991; Glickmann and Dessaux, 1995). Hundred microliter of Salkowski reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid) were added to each well, and the absorbance at 550 nm was measured after 30 min at room temperature. The accuracy of the test was checked by including on each plate the IAA-producing *Pseudomonas fluorescens* strain CHA0 and the non-producing *P. fluorescens* strain ATCC 17400 as controls. Addition of the Salkowski reagent result in a faint pink coloration when auxin compounds are produced in the medium, and no visually detectable colour reaction is produced with non-auxin-producing strains. Strains positive for auxin production were noted *iaa+*.

2.5. Siderophores production assay

The ability of *Pseudomonas* strains to synthesize and excrete siderophores when grown under Fe^{3+}-limiting conditions was evaluated during a plate assay, as described by Schwyn and Neilands (1987). Fresh cultures were plated onto blue-agar CAS plates, using Angle medium (Angle et al., 1991), buffered with 0.1 M piperazin-1,4-bisethanesulfonic acid (PIPES) (Acros Organics, Geel, Belgium) and containing 10 μM $FeCl_3$ (Fluka, Buchs, Switzerland) complexed to 10 μM chrome azurol S (CAS, Fluka) in the presence of 0.2 mM hexadecyltrimethylammonium bromide (HDTMA, Merck AG, Dietikon, Switzerland). When the Fe^{3+} is removed from the chrome azurol S complex by high-affinity siderophores, its colour changes from blue to orange. The occurrence of orange halos around colonies was determined after 48 h at 24 °C and strains were noted *sid+* when positive. *Pseudomonas fluorescens* strains CHA0 and *Enterococcus cloacae* strain NEU 1027 (bacterial strain collection, University of Neuchâtel) were used as positive and as negative references, respectively.

2.6. Hydrogen cyanide production assay

Each *Pseudomonas* strain was tested for the production of hydrogen cyanide (HCN) as described by Castric and Castric (1983) in 96 wells Nuclon microtitre trays. Each well was filled with 100 μl of a synthetic medium (Castric, 1977) consisted in (final concentrations): L-glutamate (20 mM), glycine (12.5 mM), DL-methionine (5 mM), K_2HPO_4 (5 mM), NaH_2PO_4 (5 mM), $MgSO_4$ (2 mM), ferric citrate (0.02 mM), tris(hydroxymethyl)aminomethane (50 mM), and 13 g l^{-1} agar. Glutamate, glycine and methionine were added as precursors of hydrogen cyanide (Castric, 1983). The pH of the medium was adjusted to 7.5. Wells were inoculated and covered with an indicator paper consisting of Whatman 3MM paper (Amersham Pharmacia Biotech, Buckinghamshire, England) soaked in 5 mg ml^{-1} copper(II)ethylacetoacetate (Strem chemicals, Newburyport, MA) and 5 mg ml^{-1} 4,4-methylenebis-*N,N*-dimethylaniline (Fluka) in chloroform, and air-dried. Production of cyanide caused the indicator on the paper to turn blue. *Pseudomonas fluorescens* strains CHA0 and *Enterococcus cloacae* strain NEU 1027 were used as positive and negative references, respectively. After 48 h at 24 °C, strains positive for hydrogen cyanide production were noted *hcn+*.

2.7. Nitrate reduction assay

Nitrate-dissimilative activity was tested as described by Roussel-Delif et al. (2005). Briefly each strain was cultivated in tubes containing 6 ml Nutrient Broth (devoid of fermentable substrate) (Merck) supplemented with 2 g l^{-1} KNO_3. A small inverted Durham tube was added to each tube in order to detect gas formation. The cultures were incubated for 7 days at 24 °C. The presence of nitrate and nitrite in tubes after incubation was evaluated using standard Griess reagent A (sulfanilic acid 0.8% w/v), reagent B (*N,N* dimethyl-1-naphthylamine 0.6% v/v) and powdered zinc to reduce the remaining nitrate to nitrite. Strains that were able to reduce nitrate, were noted *nr+* and included strains reducing nitrate to nitrite or to gaseous compounds (putative denitrifiers).

2.8. Statistical analysis

iaa+, *sid+*, *hcn+* and *nr+* frequencies were compared between *Pseudomonas* populations from *L. perenne* and *M. coerulea* using Khi-2 test analysis. The frequencies of *iaa+*, *sid+*, *hcn+* and *nr+* strains were analysed using a generalised linear model (*glm*) with a logistic regression model. This analysis was used to compute the probabilities corresponding to the effects of the descriptors: pCO_2 treatment, fractions, sampling dates, and plots, on the frequencies of *iaa+*, *sid+*, *hcn+* and *nr+* strains (i.e. *glm* analysis can show whether the variability of a frequency is significantly explained by one or more descriptors). The probability that the *iaa+*, *sid+*, *hcn+* and *nr+* *Pseudomonas* frequencies are different between fractions for each pCO_2 conditions or between pCO_2 conditions for a given fraction was computed using multiple comparison tests. Tukey multiple comparison test (for *L. perenne* data) or Fisher LSD exact test (for *M. coerulea* data) were used. Data were analyzed after grouping the different sampling dates and plots, taking into account the variability due to the sampling dates and plots evidenced by *glm* analysis. For all statistical analyses, the null hypothesis (similar frequencies) was rejected and the observed differences were considered as either significant for $P \leq 0.05$ or highly significant for $P \leq 0.01$. The tests were performed using S-plus 6 Statistical Software (Insightful Corporation, Seattle, Washington).

Links between characters were displayed using an unconstrained ordination method: Correspondence analysis (CA) was used to plot the four *iaa+*, *sid+*, *hcn+* and *nr+* characters regarding the proportions of the combined 16 possible phenotypes among the isolated *Pseudomonas* strains. Calculations were done with R 1.9.1 (R Development Core Team, 2004).

3. Results

3.1. *Pseudomonas population density*

Pseudomonas densities in the rhizosphere of *L. perenne* and *M. coerulea* grown under both ambient and elevated pCO_2 were assessed by CFU counting on the *Pseudomonas*-selective mS1 medium. *Pseudomonas* densities were highly variable and no consistent trend was observed regarding the influence of sampling date (data not shown), root proximity, or pCO_2 treatment (Fig. 1). After checking their affiliation, 1228 *Pseudomonas* isolates were analysed for the four phenotypic characters: siderophore (*sid*+), hydrogen cyanide (*hcn*+), auxin (*iaa*+) production and nitrate dissimilation (*nr*+). The frequencies of *sid*+, *hcn*+, *iaa*+ and *nr*+ strains were compared regarding the fraction they originated (soil, NRS; rhizosphere soil, RS; and root, RE) and the pCO_2 conditions (36 vs. 60 Pa) (Fig. 2).

3.2. *Frequency of auxin producers*

Twenty seven percent of the strains associated with *L. perenne* and 25% of the strains associated with *M. coerulea* were *iaa*+ ($P = 0.63$). For both plants species, global *glm* analysis allowed only a significant effect of sampling date on *iaa*+ frequencies to be detected ($P < 0.001$, data not shown). No significant effect of soil fraction or pCO_2 condition on auxin production was observed (Fig. 2).

3.3. *Frequency of siderophore producers*

Of the *Pseudomonas* strains associated with *L. perenne* 70% were *sid*+ compared to only 44% for *M. coerulea* ($P < 0.001$). For *L. perenne*, the frequencies of *sid*+ strains were different according to the sampling date ($P < 0.01$), the soil/root fraction sampled ($P < 0.001$), and the pCO_2 condition ($P < 0.001$). Higher *sid*+ frequencies were retrieved in root (RE) compared to soil (RS and NRS) fractions for both ambient and elevated pCO_2 (Fig. 2). These frequencies increased significantly from RS to RE fractions by 17 and 22% under ambient and elevated pCO_2, respectively. Moreover, *sid*+ frequencies were significantly higher under elevated pCO_2 compared to ambient pCO_2 (+16 and +8% for NRS and RE fractions, respectively, Fig. 2). For *M. coerulea*, the *sid*+ frequencies varied according to the root/soil fraction sampled ($P < 0.01$) and the pCO_2 conditions ($P < 0.05$). In contrast with the results obtained for *L. perenne*, *sid*+ frequencies were significantly lower under elevated than under ambient pCO_2 for the NRS fraction (Fig. 2). Finally, as for *L. perenne*, higher frequencies of *sid*+ strains were retrieved in RE (53%) compared to NRS (20%) under elevated pCO_2 only ($P < 0.001$).

3.4. *Frequency of hydrogen cyanide producers*

Of the *Pseudomonas* strains isolated from *L. perenne* and *M. coerulea* 23 and 29%, respectively, were *hcn*+ ($P = 0.06$). The frequencies of *hcn*+*Pseudomonas* differed according to plots ($P < 0.05$), to sampling dates ($P < 0.01$), and to root proximity ($P < 0.001$). For both plants and pCO_2 conditions, significantly higher proportions of *hcn*+ were retrieved in root compared to soil fractions, *hcn*+ frequencies being similar for *L. perenne* in RS and NRS fractions. From NRS to RE fractions, significantly higher frequencies (+42 and +18% for *L. perenne* and +41 and +49% for *M. coerulea*) were observed for ambient and elevated pCO_2, respectively (Fig. 2).

Regarding the influence of pCO_2, *hcn*+ frequencies were 20% lower in RE fraction for *L. perenne* ($P < 0.001$) grown under elevated pCO_2, and tended to be higher under elevated pCO_2 for *M. coerulea* ($P < 0.05$ in June 2000).

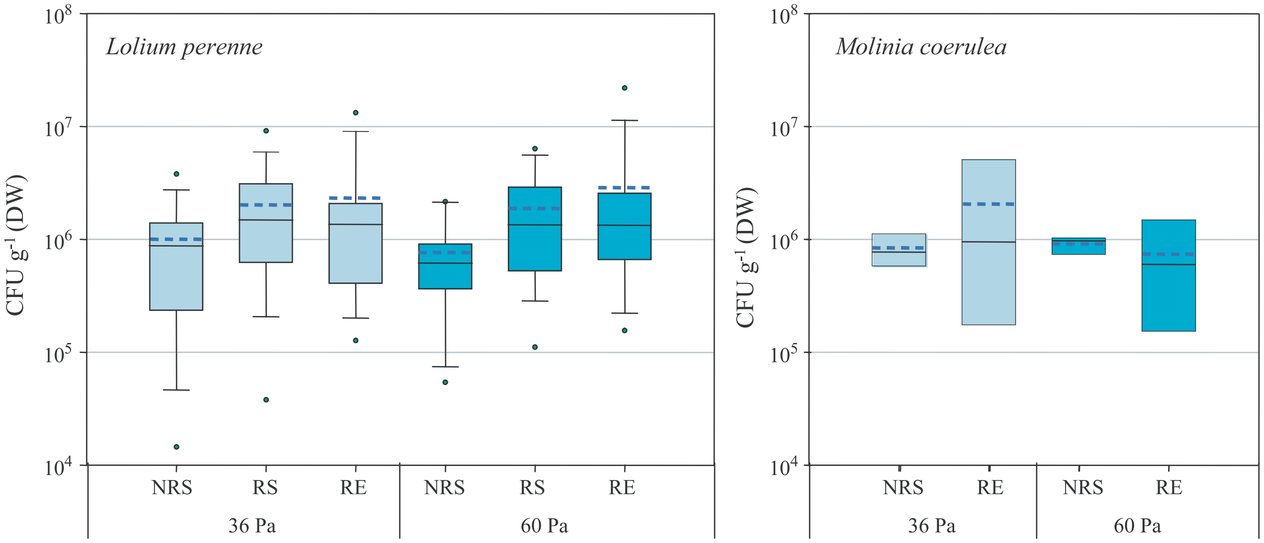

Fig. 1. Variation of colony forming units (CFU) per gram of dry weight counted on mS1 *Pseudomonas* selective medium for NRS, non-rhizosphere soil; RS, rhizosphere soil; RE, root fraction; from data of the 5 and 3 sampling dates for *Lolium perenne* and *Molinia coerulea*, respectively. Dash line indicates the mean; full line indicates the median; each box represents dispersion of the values between 25 and 75 percentiles; circle indicates outlier points.

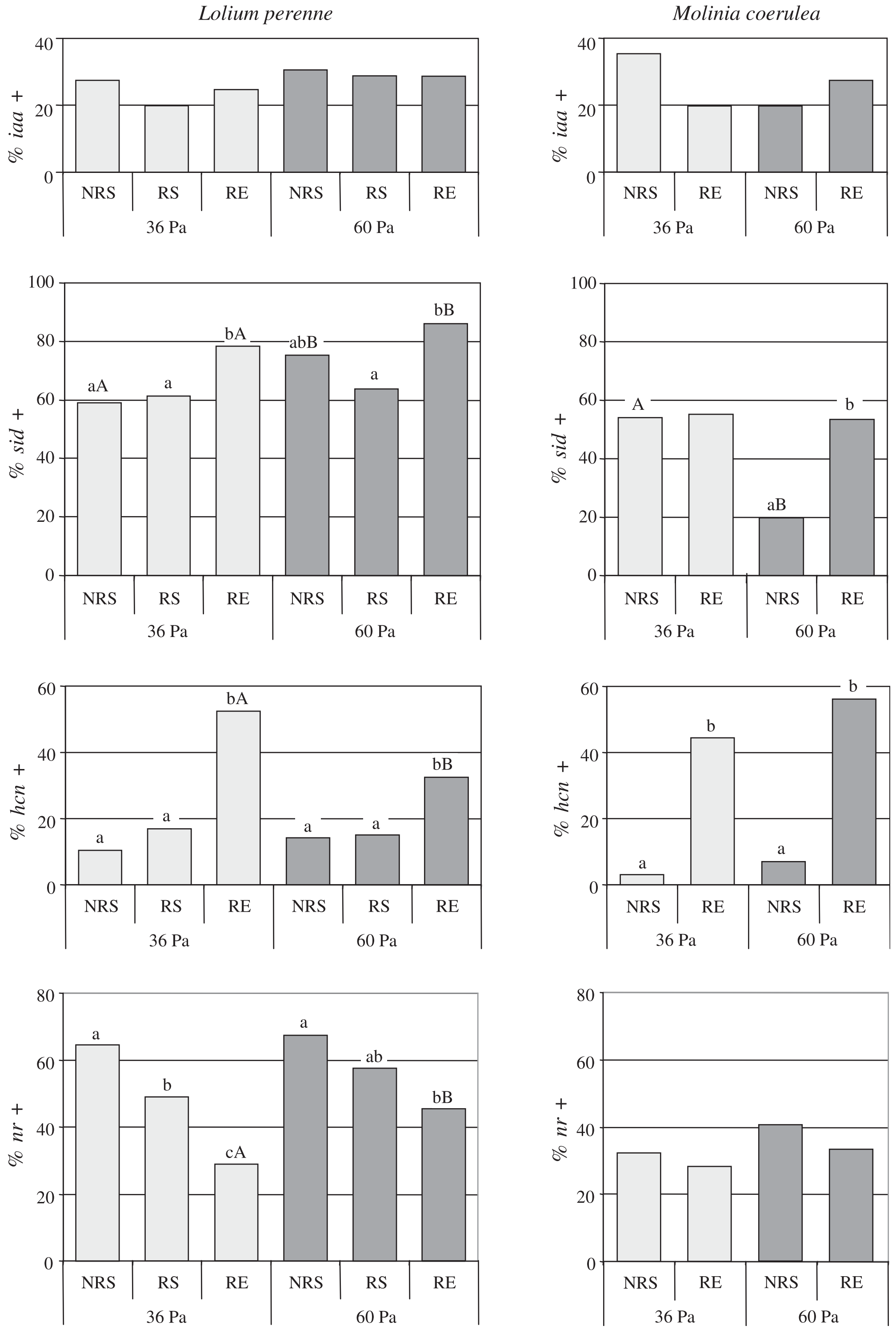

Fig. 2. Frequencies (number of positive strains among total tested strains) of auxin (*iaa*+), siderophore (*sid*+) hydrogen cyanide (*hcn*+) producing and nitrate reducing strains (*nr*+) associated with *L. perenne* and *M. coerulea* under ambient (36 Pa) and elevated (60 Pa) pCO_2 growing conditions; result for 5 (*L. perenne*) and 3 (*M. coerulea*) sampling dates. NRS, non-rhizosphere soil; RS, rhizosphere soil; and RE, root fraction; A, B, statistical differences between pCO_2 conditions within a fraction; a, b, c, statistical differences between fractions within a pCO_2 condition. Similar values are not indicated. Number of strains tested for *L. perenne*: $n=369$ for NRS, $n=287$ for RS, and $n=291$ for RE. Number of strains tested for *M. coerulea*: $n=136$ for NRS, $n=145$ for RE.

3.5. *Frequency of nitrate-dissimilating* pseudomonas

The data related to nitrate-dissimilating *Pseudomonas* were detailed in Roussel-Delif et al. (2005), and are summarised in Fig. 2. In general, 52% of *Pseudomonas* strains from *L. perenne* plots were able to reduce nitrate, compared to 32% from *M. coerulea* ($P<0.001$). For *L. perenne*, the frequencies of *nr*+ *Pseudomonas* differed according to the fraction sampled

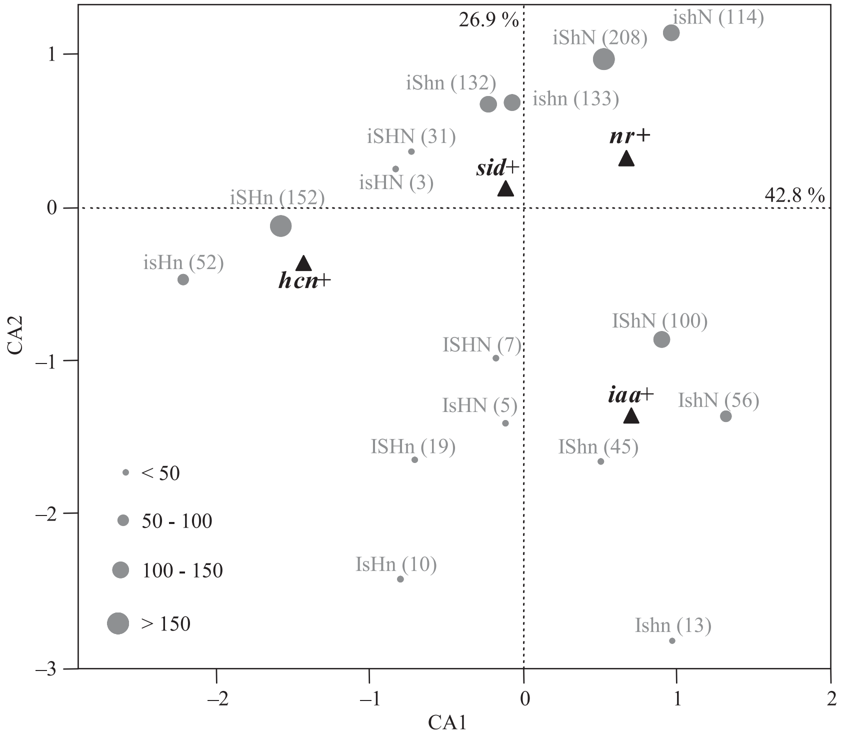

Fig. 3. Correspondence analysis (CA) based on the physiological test *iaa*+, *sid*+, *hcn*+ and *nr*+ data. The four characters (triangles) were plotted according to the proportions of the 16 different phenotypes (circles) obtained by testing 1080 strains isolated from *Lolium perenne* and *Molinia coerulea*. The phenotype notation used is: I/i, for *iaa*+/*iaa*−; S/s, for *sid*+/*sid*−; H/h, for *hcn*+/*hcn*−; N/n, for *nr*+/*nr*−. Numbers of strains within phenotypes were indicated between brackets, and circle size is proportional to this number. Total inertia of the matrix was 0.7817. Opposite direction of triangles, according to the origin of the graph, corresponded to negatively linked characters.

($P<0.001$), the pCO_2 content ($P<0.01$), the plots ($P<0.001$) and the sampling dates ($P<0.001$). For ambient and elevated pCO_2, significantly higher proportions of *nr*+ were retrieved in the soil fraction (NRS) compared to the root fraction (RS and RE) (Fig. 2), *nr*+ proportions being similar in RS and RE fractions under elevated pCO_2. The pCO_2 influence was observed only in RE fractions (higher *nr*+ frequencies under elevated pCO_2). For *M. coerulea*, proportions of *nr*+*Pseudomonas* were neither significantly influenced by elevated pCO_2 nor by root proximity.

3.6. Link between phenotypic characters

The link between *iaa*+, *sid*+, *hcn*+ and *nr*+ phenotypic traits in *Pseudomonas* strains is shown by the correspondence analysis presented in Fig. 3. The *nr*+ and *hcn*+ traits were negatively related and the (*hcn*+ *nr*+) phenotype was present only in 4.3% of the tested strains. The opposite effect between *nr*+ and *hcn*+ is displayed along the CA1 axis which account for 42.8%. The *iaa*+ trait behaved independently from the *nr*+ and *hcn*+ traits.

4. Discussion

4.1. Response of pseudomonas *populations to elevated* pCO_2

Culturable *Pseudomonas* associated with the soil and the rhizosphere of *L. perenne* and *M. coerulea* grown under ambient and elevated pCO_2 content were investigated. *Pseudomonas* densities were not consistently influenced by pCO_2 treatment after 8 years of exposure to elevated CO_2 concentrations (Fig. 1). In contrast, with a cloning sequencing approach, Marilley et al. (1999) showed a stimulation of *Pseudomonas* in the rhizosphere of *L. perenne* after 3 years of elevated pCO_2. Such a difference could be explained by their different methodological approach (molecular vs. cultural). A possible resilience of *Pseudomonas* populations during long-term pCO_2 enrichment may also be possible. There is a lack of information on microbial responses to CO_2-induced changes in natural mature ecosystems, as most of the published results have been obtained from short-term studies or from investigations using high-nutrient conditions (Hu et al., 1999).

A total of 1228 isolated strains were tested in vitro for their potential ability to produce auxin (*iaa*+), hydrogen cyanide (*hcn*+), siderophore (*sid*+) and to dissimilate nitrate (*nr*+). The results do not presume the actual in situ activity in the rhizosphere, but give insight about the potential functionality of *Pseudomonas* spp. in the rhizosphere. Gross results showed that elevated pCO_2 influenced the phenotypic structure (*sid*+, *hcn*+, and *nr*+ frequencies) of *Pseudomonas* associated mainly with the root fraction of *L. perenne*.

4.2. Elevated pCO_2 *did not influenced IAA producing* pseudomonas

IAA production is a widespread trait among rhizobacteria (Forlani et al., 1995; Persello-Cartieaux et al., 2003). Bacterial synthesis of IAA in the rhizosphere may affect the development of root system either positively (production of root hairs and lateral roots) at very low concentrations (Arshad and Frankenberg, 1991) or negatively at higher concentrations. For the plant, it can result in an increase of ion uptake (Persello-Cartieaux et al., 2003). A stimulation of root hair and lateral root production would be all the more important under elevated pCO_2 where plants need more nutrients for their increased growth (Daepp et al., 2000). In return, the advantage for root-associated bacteria would be the access to a rich supply of nutrients due to rhizodeposition (Patten and Glick, 2002). Of the *Pseudomonas* isolates 25% were auxin producers whatever the sampling date (data not shown), soil fraction, or the pCO_2 content for both soil–plant systems. Other studies have shown that the auxin producing strain frequencies were influenced by root proximity (Lebuhn et al., 1997) and sampling season (Halda-Alija, 2003). Nevertheless additional information about the level of auxin production by individual strains could also be important (Khalid et al., 2004) as it may reveal differences in potential auxin level that cannot be reflected by *iaa*+ frequencies.

4.3. Siderophore producing pseudomonas *responded differently to elevated pCO_2 for L. perenne and M. coerulea*

Siderophore production confers a competitive advantage in rhizosphere competence and root colonization (Lugtenberg and Dekkers, 1999). The ecological significance of siderophore production by *Pseudomonas* strains in root colonization and plant growth promotion but also in phyto-pathogenesis is well documented (Kloepper et al., 1980; O'Sullivan and O'Gara, 1992; Glick, 1995; Lugtenberg and Dekkers, 1999; Rainey, 1999; Sørensen et al., 2001). In the rhizosphere, a generally carbon rich and iron limited environment, production of siderophores that complex Fe^{3+} may result in growth inhibition of other microorganisms whose affinity for iron is lower (Elad and Baker, 1985; Lemanceau et al., 1993). Competition for iron would be strengthened under elevated pCO_2 condition because of the higher carbon input into the soil and the mineral limitation due to increased plant growth. In our experiment, both perennial grassland systems responded differently to the pCO_2 increase regarding the frequency of siderophore producing *Pseudomonas* (decrease for *M. coerulea*, increase for *L. perenne*). However, for both plant species, the siderophore producing *Pseudomonas* populations benefited generally from the root influence.

4.4. HCN producing pseudomonas *were mainly influenced by root proximity*

HCN is a potent inhibitor of cytochrome *c* oxidase and of several other metalloenzymes. No role is known for HCN in primary bacterial metabolism, and it is generally considered as a secondary metabolite (Blumer and Haas, 2000). HCN producing bacteria can help plants in their defence against fungal pathogens (Voisard et al., 1989; Blumer and Haas, 2000). This property was predominantly described among *Pseudomonas* strains (Kremer and Souissi, et al., 2001). In the present study, about 50% of root associated *Pseudomonas* isolates were potential HCN producers for both *L. perenne* and *M. coerulea*, as previously shown in the potato and wheat rhizosphere (Bakker and Schippers, 1987). These frequencies were higher in root compared to soil fractions for both plants (Fig. 2). The physico-chemical conditions prevailing in the rhizosphere could explain this stimulation of HCN producing bacteria. Indeed, Castric (1983) showed that the bacterial ability to produce hydrogen cyanide was enhanced under microoxic conditions. Oxygen consumption by roots and respiration of rhizosphere microbial populations creates a decreasing gradient of oxygen from the soil to the root (Højberg et al., 1999). This might provide favourable conditions for HCN producing bacteria, as shown in this study.

4.5. Root proximity and elevated pCO_2 influenced nitrate dissimilating pseudomonas

Denitrification is an anaerobic nitrate respiration process in soil environments (Gamble et al., 1977). The ability to use nitrate as an alternative electron acceptor could be a competitive advantage for bacteria in the rhizosphere where carbon sources are abundant and oxygen pressure is low (Ghiglione et al., 2000). However, our results showed lower frequencies of nitrate dissimilating *Pseudomonas* in the root compared to soil fractions for both plants (Roussel-Delif et al., 2005). This result contrasts with those of several studies showing that nitrate-dissimilating heterotrophic bacteria (e.g. Linne von Berg and Bothe, 1992), which includes *Pseudomonas* (Clays-Josserand et al., 1999), were more frequently detected in root-associated habitats than in non-rhizosphere soil. In the two plant–soil systems studied nitrogen availability was clearly limiting (*L. perenne* cultivated with a low N supply and *M. coerulea* growing on its native oligotrophic soil; Daepp et al., 2000; Hamelin et al., 2002). A low availability of nitrate could explain the lower proportions of nitrate-dissimilating strains in root-associated fractions, where N depletion is increased by plant uptake (Fromin et al., 2005).

4.6. Rhizosphere environment under elevated pCO_2 altered pseudomonas *populations*

Hydrogen cyanide production and nitrate reduction are two physiological activities which are energetically dependent on the availability of organic carbon sources and low oxygen pressure; i.e. conditions which commonly prevail in the rhizosphere. Interestingly, the frequencies of hydrogen cyanide producers and nitrate reducers responded differently to root proximity (increase for *hcn*+ and decrease for *nr*+, Fig. 2). Correspondence analysis investigating the statistical link between these two characters confirmed that *nr*+ strains were generally not *hcn*+. These results suggest that phenotypically different *Pseudomonas* guilds inhabited soil and root environments, and that *nr*+ and *hcn*+ characters may be involved in selection or counter-selection of *Pseudomonas* strains in the rhizosphere.

Root respiration and rhizodeposition (consisting mostly of low molecular weight organic carbon compounds, Darrah, 1996) increased under elevated pCO_2 for *L. perenne* (Suter et al., 2002). Oxygen limitation as well as an increased availability in organic carbon can be expected to occur near the roots and under elevated pCO_2. Consequently, we expected both nitrate reducing and HCN producing bacteria to be favoured under elevated pCO_2. This was the case for nitrate reducing *Pseudomonas*, which were generally more frequent under elevated pCO_2 for both plants (see also Roussel-Delif et al., 2005). However the *hcn*+ frequencies were lower under elevated pCO_2 in the root fraction for *L. perenne*. The significance of this result is unclear, but it could indicate an alteration of rhizodeposit composition under elevated pCO_2 (Hodge et al., 1998; Darrah, 1996), as the type of amino-acid present can influence the HCN biosynthesis (Castric, 1983).

4.7. Conclusions

This study showed that *Pseudomonas* associated with different plant/soil systems may respond differently to elevated pCO_2. This differential response could be explained by the contrasting ecology of the two plants used as models in this study (Vàzquez de Aldana and Berendse, 1997) or by the different exposure time to elevated pCO_2 (2–3 years for *M. coerulea* and 7–8 years for *L. perenne*). As pCO_2 influence was mostly visible for *L. perenne* associated *Pseudomonas*, a possible long-term selection of the best adapted strains to the plant rhizosphere under raising pCO_2 could be evoked. We hypothesize that this modification of *Pseudomonas* guilds under elevated pCO_2 will exert a feedback effect on plant fitness.

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