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Influence of Two Plant Species (Flax and Tomato) on the Distribution of Nitrogen Dissimilative Abilities within Fluorescent *Pseudomonas* spp.

A. CLAYS-JOSSERAND,[1]* P. LEMANCEAU,[2] L. PHILIPPOT,[1] AND R. LENSI[1]

Laboratoire d'Ecologie Microbienne, Centre National de la Recherche Scientifique URA 1450, Université Lyon I, 69622 Villeurbanne Cedex,[1] and Laboratoire de Recherches sur la Flore Pathogène du Sol, Institut National de la Recherche Agronomique, 21034 Dijon Cedex,[2] France

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The distribution of nitrogen-dissimilative abilities among 317 isolates of fluorescent pseudomonads was studied. These strains were isolated from an uncultivated soil and from the rhizosphere, rhizoplane, and root tissue of two plant species (flax and tomato) cultivated on this same soil. The isolates were distributed into two species, *Pseudomonas fluorescens* (45.1%) and *Pseudomonas putida* (40.4%), plus an intermediate type (14.5%). *P. fluorescens* was the species with the greatest proportion of isolates in the root compartments and the greatest proportion of dissimilatory and denitrifying strains. According to their ability to dissimilate nitrogen, the isolates have been distributed into nondissimilatory and dissimilatory strains, nitrate reducers and true denitrifiers with or without N_2O reductase. The proportion of dissimilatory isolates was significantly enhanced in the compartments affected by flax and tomato roots (55% in uncultivated soil and 90 and 82% in the root tissue of flax and tomato, respectively). Among these strains, the proportion of denitrifiers gradually and significantly increased in the root vicinity of tomato (44, 68, 75, and 94% in uncultivated soil, rhizosphere, rhizoplane, and root tissue, respectively) and was higher in the flax rhizoplane (66%) than in the uncultivated soil. A higher proportion of N_2O reducers was also found in the root compartments. This result was particularly clear for tomato. It is hypothesized that denitrification could be a selective advantage for the denitrifiers in the root environment and that this process could contribute to modify the specific composition of the bacterial communities in the rhizosphere.

Denitrification is recognized as the main microbial activity producing gaseous nitrogen oxides and consequently contributes to modify global atmospheric chemistry, essentially through the greenhouse effect and ozone depletion (3, 20, 30).

Denitrification exhibits high spatial variability at the microscale (5, 16, 18, 22, 28, 29) and field or landscape scale (9, 10, 17, 23, 26). This makes the quantitative evaluation of this process difficult. Zones of high denitrifying activity predominantly occur in specific soil microsites (22) and in "activation sites" (soil influenced by roots or rehandled by fauna) (17).

It is well known that the rhizosphere is an important site providing denitrifying conditions. Growing plants stimulate denitrification in the root zone by reducing the oxygen tension and by providing carbon substrates in root exudates (12, 24, 25, 27, 35). Conversely, in some cases, plant roots can also inhibit denitrification, by consumption of nitrate or reduction of soil moisture content (1, 11, 32). Interpretation of these results is difficult because conflicting data were obtained from experiments with different plant species under widely different conditions.

Several hypotheses can be proposed to explain the positive effect of plant roots on denitrifying activity: increase in global heterotrophic microflora, including denitrifiers, specific selection of denitrifiers by plant roots, and stimulation of expression of denitrification.

Progress in the understanding of the microbial mechanisms of regulation of the denitrifying process is required to improve our knowledge on the quantitative influence of the activation sites.

Since denitrifiers are primarily aerobic heterotrophic bacteria, Tiedje (36) suggested that the major factor controlling the size of the denitrifying community would not be the ability to denitrify but rather the general ability of this community to compete for natural carbon substrates. In this case, the relative proportion of denitrifiers over the aerobic heterotrophic community would be constant. However, Linne von Berg and Bothe (19) suggested that this proportion can be increased by the presence of the plant.

The most common denitrifiers of temperate soils belong to the group of fluorescent *Pseudomonas* spp. (7), which also represents an abundant group in the rhizosphere (4). In the present study, the fluorescent *Pseudomonas* spp. were chosen as a model to assess the possible increase of organisms able to dissimilate nitrogen in the rhizosphere of two plant species, flax (*Linum usitatissinum* L.) and tomato (*Lycopersicon esculentum* Mill.). The proportion of nitrate reducers, denitrifiers, and N_2O reducers among a collection of *Pseudomonas* spp. isolated from uncultivated soil, rhizosphere, rhizoplane, and internal root tissues was determined.

MATERIALS AND METHODS

Isolation and characterization of fluorescent pseudomonads. Fluorescent pseudomonads were isolated either from an uncultivated silty-loamy soil or from the root environment of flax (*L. usitatissinum* L., cv. opaline) and of tomato (*L. esculentum* Mill., cv. H63.5). Plant cultivation and bacterial isolation were performed as described previously by Lemanceau et al. (15). Briefly, the same soil was either kept uncultivated or cultivated with flax or with tomato for five successive crops lasting 2 months each, alternating with 1-month uncultivated periods. Plants were harvested after 4 weeks of growth. Bacteria were isolated from four different compartments: rhizosphere (47 isolates from flax and 52

* Corresponding author. Mailing address: Université Lyon I, URA CNRS 1450, Ecologie Microbienne, Laboratoire de Microbiologie des Sols, Bâtiment 741, 43, Bvd du 11 nov. 1918, 69622 Villeurbanne Cedex, France. Phone: 33 72 43 13 78. Fax: 33 72 43 12 23.

TABLE 1. Distribution of isolates belonging to different species among the different compartments and analysis of this distribution by contingency tables and chi-square test[a]

Plant	Compartment	No. of isolates tested	*P. fluorescens*			Intermediate type			*P. putida*		
			No. of isolates		% of total	No. of isolates		% of total	No. of isolates		% of total
			Found	Expected		Found	Expected		Found	Expected	
Flax[b]	Uncultivated soil	49	9*	15.4	18	16*	9.24	33	24	24.36	49
	Rhizosphere	47	12	14.77	26	9	8.86	19	26	23.37	55
	Rhizoplane	50	25*	15.71	50	7	9.43	14	18*	24.86	36
	Root tissue	29	9	9.11	31	1*	5.47	3	19	14.42	66
	Total	175	55			33			87		
Tomato[c]	Uncultivated soil	49	9*	24.63	18	16*	7.95	33	24*	16.42	49
	Rhizosphere	52	25	26.14	48	8	8.44	15	19	17.42	37
	Rhizoplane	51	32*	25.63	63	6	8.28	12	13	17.09	25
	Root tissue	39	30*	19.6	77	1*	6.33	3	8	13.07	20
	Total	191	96			31			64		

[a] An asterisk indicates significant ($P = 0.95$) post hoc cell contributions.
[b] $\chi^2 = 21.544$, df = 6, $P = 0.0015$.
[c] $\chi^2 = 36.93$, df = 6, $P = 0.0001$.

isolates from tomato), rhizoplane (50 and 51 isolates), and root tissue (29 and 39 isolates) of flax and tomato plants as well as uncultivated soil (49 isolates). Ten independent samples were collected per compartment, consisting of 1 g of soil, one root system (rhizosphere and rhizoplane), or four root systems (root tissue). Rhizospheric suspensions resulted from gently washing root systems with adhering soil. The washed root systems were vigorously shaken to obtain the rhizoplane suspensions. Then, the washed root systems were surface sterilized and macerated with a sterile mortar and pestle to obtain the root tissue isolates. The absence of fluorescent pseudomonads on root systems was checked before maceration. All suspensions were prepared in 0.1 M $MgSO_4 \cdot 7H_2O$. Suspensions of the four compartment samples were dilution plated on modified KB medium (8) and incubated for 48 h at 25°C. Bacterial isolations from each compartment were always performed from samples diluted to the same level.

Species classification was done by the methods of Stanier et al. (33), Palleroni (21), and Digat and Gardan (6).

Tests for nitrogen dissimilation. One colony taken from solidified LB medium was inoculated into 5 ml of LB medium and incubated for 48 h. One hundred microliters of this culture was transferred into 15 ml of LB medium supplemented with KNO_3 (1 mM). The cultures were incubated aerobically on a shaker at 28°C. After 24 h, the optical density at 580 nm (OD_{580}) of the cultures was measured. Cells were centrifuged (15 min, 5,500 × *g*) and resuspended in fresh LB medium with KNO_3 (1 mM) in order to obtain an OD of 2. For each strain, 2 ml of culture was aseptically transferred into two 20-ml Vacutainer (Becton Dickinson) test tubes with a syringe through a rubber stopper. The atmosphere of one tube was previously evacuated and replaced with helium to ensure anaerobiosis. The atmosphere of the second tube was evacuated and replaced with a 90:10 He-C_2H_2 mixture to ensure anaerobiosis and inhibit N_2O reductase (37). All the test tubes were incubated for 6 days at 28°C on a shaker.

Gas samples were analyzed for N_2O with a Girdel 30 gas chromatograph (GC) equipped with a thermal conductivity detector. When no N_2O was detected by this technique, gas samples of the corresponding tubes were analyzed for N_2O on a Girdel 30 chromatograph with an electron capture detector. After gas analysis, the presence of NO_2^- and NO_3^- in the culture was determined colorimetrically with the Griess Illosway and Morgan reagents, respectively.

True denitrifiers were revealed by N_2O accumulation in the presence of acetylene (by thermal conductivity GC). Denitrifiers possessing N_2O reductase were revealed by no or significantly less N_2O accumulation in absence of acetylene. Among the nondenitrifiers, strains able to produce very low amounts of N_2O were distinguished from strains unable to produce any N_2O by measuring N_2O by electron capture detector GC.

Nitrate reducers were revealed by the presence of NO_2^- in the culture and the absence of significant amounts of N_2O. Total dissimilation was tested by the absence of NO_3^- in the culture. All the tests described were performed on the 317 isolates.

Several classes of microorganisms were distinguished according to their capacities to reduce NO_3^- into NO_2^- or gaseous products: (i) among all isolates, organisms unable to dissimilate NO_3^- in anaerobiosis (NDi) and organisms able to dissimilate NO_3^- in anaerobiosis, whatever the end products (Di); (ii) among the dissimilatory organisms, nitrate reducers only able to dissimilate NO_3^- to NO_2^- (NR) and denitrifying organisms able to transform NO_3^- into gaseous products (D); (iii) among the denitrifying organisms, strains able to reduce N_2O into N_2; and (iv) among the nondenitrifying organisms, organisms able to produce trace amounts of N_2O.

Statistical analysis. The percentage of isolates belonging to the different *Pseudomonas* species and dissimilating NO_3^- was analyzed by the chi-square test. All statistical analyses were performed with the StatView II program (34). The post hoc cell contributions are a form of standardized residual that indicates what each cell in the table contributes to the chi-square statistic.

RESULTS

Distribution of fluorescent *Pseudomonas* species in compartment samples. The isolates belonged to only two species, *P. fluorescens* (45.1%) and *P. putida* (40.4%), plus an intermediate type (14.5%) defined, according to Digat and Gardan (6), by their ability to hydrolyze gelatin or to utilize trehalose. The distribution of the bacterial isolates in the different *Pseudomonas* species was not aleatory and was affected by plant roots (Table 1). For both plant species, the proportion of *P. fluorescens* was higher in the compartments influenced by roots than in the uncultivated soil. This result was particularly obvious for tomato, with a clear gradient: root tissue > rhizoplane > rhizosphere > uncultivated soil. The proportion of isolates belonging to *P. putida* was lower in the compartments influenced by tomato roots (rhizosphere, rhizoplane, and root tissue) than in the uncultivated soil but was only lower in the flax rhizoplane compared with the uncultivated soil. The proportion of isolates belonging to the intermediate type was always lower in the root compartments than in the uncultivated soil (Table 1).

Distribution of dissimilative capacities among bacterial species. The *P. fluorescens* isolates included nondissimilative bacteria (6.3%) and nitrate reducers (3.5%); most of the isolates were denitrifying strains (90.2%). More than half of the *P. putida* strains were able to dissimilate nitrogen (54.7%), with only one denitrifying strain. The intermediate type constituted 56.5%, 23.9%, and 19.6% of nondissimilatory strains, nitrate reducers, and denitrifying strains, respectively.

Distribution of dissimilative capacities among bacterial isolates from different compartments. For both plant species, the proportion of bacteria able to dissimilate nitrate (Di) was significantly higher in the compartments affected by plants than in soil (Table 2). Among the dissimilative strains, the proportion of denitrifiers gradually and significantly increased with tomato root vicinity (44 and 94% in the uncultivated soil and in the root tissue, respectively; Table 3). The effect of plant roots on the distribution of denitrifiers versus nitrate reducers appeared less obvious for flax than for tomato, but the distribution of the

TABLE 2. Distribution of dissimilatory (Di) isolates in the different compartments and analysis of this distribution by contingency tables and chi-square test[a]

Compartment	Flax				Tomato			
	No. of isolates	No. Di		% Di	No. of isolates	No. Di		% Di
		Found	Expected			Found	Expected	
Uncultivated soil	49	27	32.2	55	49	27*	35.15	55
Rhizosphere	47	27	30.89	57	52	34	37.3	65
Rhizoplane	50	35	32.86	70	51	44*	36.58	86
Root tissue	29	26*	19.06	90	39	32	27.97	82
Total	175	115			191	137		

[a] Flax: $\chi^2 = 11.66$, df = 3, $P = 0.0086$. Tomato: $\chi^2 = 15.082$, df = 3, $P = 0.0017$. Also see Table 1, footnote *a*.

two classes of organisms was not aleatory (Table 3). Among the denitrifying organisms, the proportion of strains exhibiting N_2O reductase activity was much higher in the root compartments of both plant species than in the uncultivated soil (Table 4). A clear gradient between the different compartments, root tissue, rhizoplane, rhizosphere, and uncultivated soil, was recorded for flax (100% of N_2O reducers inside the roots), while for tomato, the differences between the compartments influenced by roots were less evident. However, the sample size was too small to perform statistical analysis on the distribution of N_2O reducers.

Forty-four percent of nondenitrifying strains exhibited the ability to produce trace amounts of N_2O. Among these strains, 85% belonged to the nitrate reducer group.

DISCUSSION

The present study shows that the proportions of nitrate-dissimilative strains, denitrifiers, nitrate reducers, and N_2O reducers inside the fluorescent *Pseudomonas* community are affected by plant roots.

In order to obtain valid results, bacterial cultures must be tested in nonlimiting conditions for the expression of their genetic ability to dissimilate nitrates. In this study, (i) the different culture steps were done in the presence of NO_3^- in order to ensure enzyme synthesis, (ii) tests were performed in total anaerobiosis to ensure optimal expression of enzymes, and (iii) tests were performed with low NO_3^- (1 mM) concentrations to avoid inhibition of dissimilative activities by nitrite (14).

From our results, the N_2O-producing strains could be distributed into two classes. The first class included strains able to convert at least 80% of the initial NO_3^- into N_2O in the presence of C_2H_2. These strains are listed as denitrifying strains. Such a conversion rate has been reported by Tiedje (36) as an important criterion to characterize denitrifying organisms. The second class included strains unable to convert more than 1% of the initial NO_3^- into N_2O in the presence of C_2H_2. As N_2O production by nondenitrifying strains has already been reported by Smith and Zimmerman (31), these strains are listed as nondenitrifying strains.

According to these two classes, only four isolates showed atypical N_2O production: three intermediate-type strains and one *P. putida* strain exhibited 13, 14, 30, and 20% conversion of initial nitrate into N_2O, respectively. Nevertheless, we have listed these strains as denitrifiers because (i) N_2O accumulation was similar after 6 and 10 days of incubation and (ii) NO_2^- was always detected in the culture. These results suggest that partial conversion of NO_3^- could be explained by the high NO_2^- sensitivity of these strains rather than by a low reduction rate.

As roots participate in the establishment of a concentration gradient of most of the denitrification regulating factors, interactions between denitrification and plants have been studied extensively in the past (2, 11, 12, 27, 32). Most of these works concentrated only on the influence of roots on denitrifying

TABLE 3. Distribution of nitrate reducers (NR) and denitrifiers (D) among the dissimilatory isolates (Di) in the different compartments and analysis of this distribution by contingency tables and chi-square test[a]

Plant	Compartment	No. of Di isolates	NR			D		
			No. of isolates		% of isolates	No. of isolates		% of isolates
			Found	Expected		Found	Expected	
Flax[b]	Uncultivated soil	27	15	14.56	56	12	12.44	44
	Rhizosphere	27	18	14.56	67	9	12.44	33
	Rhizoplane	35	12*	18.87	34	23*	16.13	66
	Root tissue	26	17	14.02	65	9	11.98	35
	Total	115	62			53		
Tomato[c]	Uncultivated soil	27	15*	7.69	56	12*	19.31	44
	Rhizosphere	34	11	9.68	32	23	24.32	68
	Rhizoplane	44	11	12.53	25	33	31.47	75
	Root tissue	32	2*	9.11	6	30*	22.89	94
	Total	137	39			98		

[a] See Table 1, footnote *a*.
[b] $\chi^2 = 8.6$, df = 3, $P = 0.0351$.
[c] $\chi^2 = 17.998$, df = 3, $P = 0.0004$.

TABLE 4. Distribution of N_2O reducers among the denitrifying isolates (D) in the different compartments

Compartment	No. of D isolates	% N_2O reducers
Uncultivated soil	12	17
Flax		
Rhizosphere	9	78
Rhizoplane	23	83
Root tissue	9	100
Tomato		
Rhizosphere	23	56
Rhizoplane	33	48
Root tissue	30	37

activity. A small number of studies reported by Knowles (13) deal with the microbial populations responsible for denitrification. These studies show that large populations of denitrifiers are usually present in the rhizosphere, their densities being 10 to 100 times higher than in the uncultivated soil.

To our knowledge, only Linne von Berg and Bothe (19), working on isolates of the total soil community, stressed that the ratio of denitrifiers to other heterotrophic organisms was increased near the roots. These authors, using DNA probes of dissimilatory NO_2^- and N_2O reductases, showed that the percentage of heterotrophic isolates giving strong or very strong hybridization signals was higher in the rhizospheric soil than in the corresponding bulk soil. However, they demonstrated that 25% of denitrifying isolates did not give any hybridization signal, indicating that the probes used were not universal and that this technique needs further improvements before being used extensively for denitrification studies.

Compared with the work of Linne von Berg and Bothe (19), we have limited our study to the fluorescent *Pseudomonas* community. Among this group, only two species and an intermediate type were present in our experimental conditions. This small number of species enabled us to work with a large number of isolates per species. The sample sizes allowed us to assess the root effect on the relationship between the species composition of the fluorescent *Pseudomonas* group and the distribution of dissimilative capacities. Moreover, in our work, we have taken into account the first step of the dissimilative pathway (NO_3^- into NO_2^-), which corresponds to the main energy-supplying transformation, and different compartments have been distinguished according to the distance from the roots.

The main objective of the present study was to assess the influence of plant roots on (i) the distribution of *Pseudomonas* species and (ii) the distribution of dissimilative abilities. To strengthen our conclusions, we have included two plant species. Although the isolation procedure was repeated several times in the space, the replicates have been pooled and the effect of tomato compared with flax has not been statistically analyzed.

The two species, *P. fluorescens* and *P. putida*, were almost equally represented among the isolates (about 45 and 40%, respectively). *P. fluorescens* was the species with the greatest proportion of isolates in the root compartments (Table 1) and the species with the greatest proportion of dissimilatory and denitrifying isolates. Tomato seemed to exert the strongest influence on the specific composition of the community (Table 1). However, it is not possible to ascribe these differences to differences in plant growth or to any specific interaction.

The distribution of nitrate-dissimilative strains, denitrifiers, and nitrate reducers was significantly affected by plants (Tables 2 and 3). Plants also seemed to be able to influence the distribution of N_2O reducers (Table 4), but the small sample size does not allow us to determine the significance of this result. Flax and tomato seemed to exert a similar influence on the distribution of dissimilatory bacteria (Table 2). However, the ratio of denitrifiers to nitrate reducers appears to be affected more by tomato than by flax (Table 3), while the proportion of isolates able to reduce N_2O appears to be more affected by flax than by tomato (Table 4).

The high proportion of trace-N_2O-producing strains among the nondenitrifiers was unexpected. This result could be of interest because these strains are potential contributors to emissions of nitrogen oxides. The ability of nondenitrifiers to produce N_2O seems to be largely correlated with the ability to reduce NO_3^- to NO_2^- (31). However, in our study, about 15% of the strains that were unable to dissimilate nitrate exhibited trace N_2O production.

Our results corroborate those obtained by Linne von Berg and Bothe (19), and we can conclude that the abundance of dissimilative organisms increased near the roots. As stated by these authors, "It is tempting to assume that denitrification is a selective advantage for the denitrifiers at the roots," or at least that the capacity to dissimilate NO_3^- could be a character that promotes root colonization and could contribute to explain the differences in the specific composition of the fluorescent *Pseudomonas* community between the different compartments. To assess this hypothesis, investigations at the population level involving introduction into the soil of isogenic mutants of representative denitrifiers lacking the ability to synthesize denitrifying reductases would be helpful.

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