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Protease and deaminase activities in wheat rhizosphere and their relation to bacterial and protozoan populations

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Abstract Protease and deaminase activities and population dynamics of bacteria and protozoa were measured in the rhizosphere of wheat to study their interactions with the mineralization of nitrogen. The experimental design allowed the separation of roots and soil material by means of a gauze. The most pronounced "rhizosphere effect" was detected for all the measured variables in the soil closest to the gauze. The number of bacteria was significantly higher in the presence than in the absence of plants up to 4 mm away from the soil-root interface and the closer to this interface the higher the number. Protozoan and bacterial population dynamics were positively correlated; generally, populations of flagellates and amoebae were comparable and their sum accounted for the population of total protozoa. For both enzyme activities the rhizosphere effect extended up to 2 mm away from the soil-root interface. The histidinase activity was of bacterial origin, while it is likely that bacteria, protozoa and root hair all contributed to the overall caseinase activity. Decomposition of root exudates and native organic matter in the rhizosphere, reflected by a growing microbial population, is associated with nitrogen mineralization through increases in casein-hydrolysing and L-histidine-deaminating activities. The adopted soil-plant microcosm is suitable for the study of the rhizosphere effect over time of incubation and distance gradient from the soil-root interface.

Key words Rhizosphere effect · Protease activity · Deaminase activity · Bacteria · Protozoa · Nitrogen mineralization

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Introduction

Nitrogen mineralization in the rhizosphere is increased by protozoan grazing (Clarholm 1985) and the release of ammonium derived from assimilated bacteria and/or a stimulation of the turnover of soil organic matter through the microbial biomass (Kuikman and van Veen 1989; Rutherford and Juma 1992). Conversion of organic N to ammonium (ammonification) is carried out through biochemical transformations mediated exclusively by heterotrophic microorganisms (Ladd and Jackson 1982). Little information is available about how bacterial growth induced by root-released organic C and N in the rhizosphere and successive protozoan grazing affects the activity of soil enzymes involved in the ammonification process, such as proteases and deaminases.

Plant roots stimulate acid and alkaline phosphatases in their vicinity when growing through the soil (Tarafdar and Jungk 1987). The increase in phosphatase activity was related to depletion of organic P and depended on plant age, plant species and soil type.

The amount of rhizosphere soil obtained after careful separation from roots is often poorly defined for extensive investigation of biochemical (e.g. soil respiration, enzymatic activities) and microbiological parameters (e.g. microbial biomass, bacterial and fungal counts). Soil-plant microcosms used to study a well-defined rhizosphere soil in more detail are generally complicated and time-consuming (Helal and Sauerbeck 1983; James et al. 1985; Martin and Foster 1985).

Kuchembuch and Jungk (1982) successfully evaluated exchangeable potassium concentrations very close to an induced soil-root interface. They grew *Brassica napus* L. plants on a nylon gauze to separate soil from roots, with only root hair penetrating the gauze (clear mesh of 30 μm); the soil was incubated on a porous ceramic plate to maintain a constant water content. After freezing soil by liquid nitrogen, thin soil slices were prepared. Dijkstra et al. (1987) substituted the plate for a sandy water tension table and studied bacterial distribution as a function of the distance from the root-soil interface with young *Triticum*

aestivum L. plants. Neither study monitored the dynamics of the "rhizosphere effect" during plant growth. Klemedtsson et al. (1987) did not find any significant change in bacteria, flagellates or naked amoeba numbers up to 20 mm away from roots and for 5 weeks of growth in a soil-plant microcosm with barley.

Kuikman et al. (1990) showed that a bacterial population grazed by protozoa, even though smaller in number, can be more active in mineralizing soil nitrogen measured as N plant uptake. Thus, the measurement of population only may be misleading if not accompanied by independent estimations of microbial activity, such as enzyme activities.

The aim of this work was to: (1) provide an easily reproducible soil-plant microcosm to study the "rhizosphere effect" in time and over a distance gradient from the soil-root interface and (2) determine whether the rhizosphere affects the activity of enzymes involved in mineralization of organic N, such as casein hydrolase and L-histidine-deaminase, and how these enzyme activities are related to bacterial and protozoan population dynamics around the roots of growing wheat plants.

Materials and methods

The soil used (loamy sand) was collected from an A horizon on arable land near Ede (NL) and had the following chemical properties: organic C 2.0%, total N 0.13%, pH_{KCl} 6.2, CEC 9 meq 100 g^{-1}, $CaCO_3$ 0.1%. It was sieved (mesh size <4 mm) and fertilized with 4.41 g $K_2HPO_4 \cdot 3\ H_2O$, 2.60 g KH_2PO_4 and 0.44 g NH_4Cl kg^{-1} dry soil prior to the experiments.

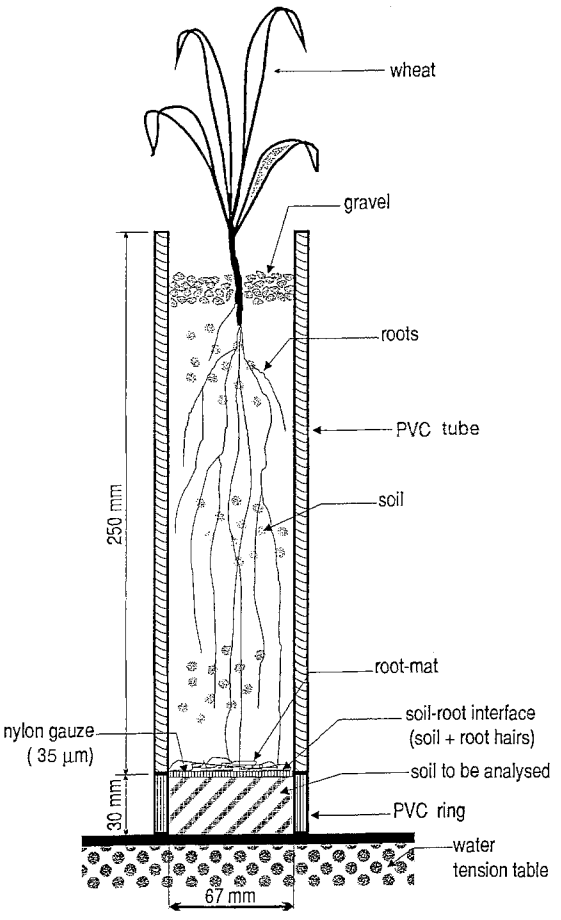

Fig. 1 Schematic representation of the soil-plant microcosm

Soil-plant microcosm

The soil-plant microcosm used in this work (Fig. 1) consisted of the following parts: (1) PVC rings (inner diameter 6.7 cm, height 3.0 cm) filled with freshly sieved field soil (bulk density and water content on a dry weight basis 1.20 g cm^{-3} and 9%, respectively; (2) PVC tubes (inner diameter 6.7 cm, height 25 cm) filled with 1.1 kg soil (15% as water content) for about 23 cm; (3) an artificial sandy water tension table (particle size 73 µm; Eijkelkamp, NL) used to regulate the soil water content; the height of the water column was fixed at –40 cm with a pF of 2.2; and (4) 4-day-old pregerminated wheat seeds (*Triticum aestivum* L. cv. Sicco).

Each PVC tube was closed at the bottom by a nylon gauze (mesh 35 µm, Swiss Silk Bolting Cloth, Zürich) glued with silicone. Before placing the soil column onto the soil ring, the latter was slowly rewetted by gently pressing it on the tension table. The water bottle was raised to the same height of the water tension table. Thirty seconds were sufficient to completely cover the tension table by a very thin water layer and 2 min to wet the soil ring by capillarity. In this way, maximum contact between the tension table and the soil ring was established. Then the water bottle was lowered to –40 cm and the rings were left undisturbed for 1 day before determining the initial soil conditions (time zero). Six soil columns were planted with four pregerminated wheat seeds and four columns were left unplanted; all were covered with 50 g gravel to preserve soil humidity and placed on ten soil rings (Fig. 1). Soil-plant microcosms were kept in a growth cabinet for 33 days at 20°C (day) and 15°C (night), with an air relative humidity of 60% and a photoperiod of 14 h, supplying approximately 240 µE m^{-2} s^{-1}. Every 2, at the latest 3 days, soil columns were weighed and water lost by evapotranspiration was supplied on top. The weight of shoots and roots gained by plant C fixation was estimated from results from previous experiments.

About 10 days after planting, roots reached the nylon gauze and started to penetrate it with root hair. By regular examination of the bottom of the soil column during watering while it was raised from the respective soil ring, it was possible to evaluate the amount of soil covering the gauze under the soil column and held by root hair. This "gauze soil" was considered to be the real rhizosphere soil. The distance from the nylon gauze was considered as the distance from the soil-root interface (Fig. 1). Both harvesting and soil sampling were carried out when approximately 50% and 100% of the gauze surface was covered by soil, at 21 and 33 days after seed planting into soil columns, respectively. At each harvest (21 and 33 days), three planted and two unplanted microcosms were destructively sampled.

Soil samples were taken at measurable distances from the induced soil-root interface by sequentially slicing the 3-cm-high smooth soil cylinder contained in the PVC ring by a sharp steel blade. After carefully detaching the ring from the sandy table, the soil cylinder was pushed out from the bottom by a full PVC cylinder onto which PVC rings of various thicknesses were slid and whose diameter was equal to the inner diameter of the ring containing the soil; the thickness of the sequential soil slices (i.e. distance from the root-soil interface) was predetermined by sequentially sliding off from the full PVC cylinder the PVC ring of the same thickness of soil slice to be sampled. Thus, every soil cylinder progression was followed by a slicing. Only the soil contained in the 3-cm-tall ring was analysed since only in this case could an unequivocal distance from the roots be determined. A 2-mm-thick soil slice furnished sufficient soil to permit both microbiological and biochemical analyses (about 10 g moist soil). In the case of gauze soil, i.e. the 0- to 1-mm layer attached to the root hair, the amount of soil obtained was smaller (about 4.0 g) but was enough to perform all the analyses.

Analyses

Soil analyses were carried out immediately after sampling to avoid storage problems (Nannipieri et al. 1990). Soil slices were kept in aluminium foil for 1–2 h at most to prevent water losses.

The number of bacteria and protozoa were determined in the same soil suspension obtained by extracting 4 g fresh soil (2 g for gauze soil) in 40 ml (20 ml for gauze soil) 0.1% sodium pyrophosphate on a rotary shaker (10 min, 200 rpm). For bacteria a tenfold dilution series in sterile demineralized water was prepared and appropriate dilutions were plated (in triplicate) on 1:2 tryptone soya agar. After 48 h at 29°C, the total number of colony-forming units per plate was determined (Kuikman et al. 1990). For protozoa a modified most probable number method (Rowe et al. 1977) was used: fourfold dilutions in eight replicated series of the previous soil suspension together with *Pseudomonas fluorescens* and *Enterobacter cloacae* as food source were prepared. The microtiter plates were incubated at 12°C in the dark and scanned for the presence of protozoa after 1, 2 and 4 weeks (Kuikman et al. 1990).

After cutting off the shoots, roots were separated from the soil and washed on a fine sieve (0.5 mm). Shoot and root yield were obtained by weighing the plant material after drying at 80°C for 48 h.

Protease and deaminase activities were assayed by using casein and L-histidine as substrates, respectively. The methods of Ladd and Butler (1972) and Frankenberger and Johanson (1982) were used with the following modifications: 0.5 g fresh soil and 0.25 g fresh soil from the gauze were incubated at 50°C for 1 h after addition of 1.25 ml and 0.625 ml, respectively, of 0.1% casein dissolved in 0.1 *M* TRIS-HCl buffer at pH 8.1 (protease assay); in the case of the deaminase assay the same amounts of soil were added with 0.9 and 0.45 ml TRIS-H_2SO_4 buffer 0.1 *M* pH 9.0, 0.1 and 0.05 ml toluene and 0.1 ml 0.5 *M* L-histidine; then samples were incubated at 37°C for 48 h. Ammonium released after L-histidine deamination was exchanged by KCl and measured by continuous flow analysis using Nessler's reagent (Van Ginkel and Sinnaeve 1980).

Soil moisture content was determined gravimetrically by the water lost after drying for 24 h at 105°C.

Statistics

The data reported are mean values from three planted and two unplanted replicated soil microcosms, respectively, and from different layers at two harvests. The data were analysed by ANOVA (analysis of variance) for the factors "distance from the gauze" and "days of incubation"; LSD (least significant difference) was calculated at the 95% confidence level and is indicated in the graphs. Linear regression analysis was applied to correlate enzyme activities and microbial counts. The mean water content of the different soil layers was correlated to its respective average distance from the soil-root interface (1, 5, 15 and 25 mm).

Results

Regardless of incubation time, the closer to the gauze the lower the water content of soil in both unplanted ($r>0.960$, $P<0.05$, $n=4$) and planted ($r>0.934$, $P<0.1$, $n=4$) microcosms. No significant change in moisture was observed in the unplanted soil rings over time, whereas it decreased slightly in the planted soil rings (Fig. 2).

Shoot, root and total plant dry weights per microcosm more than doubled from day 21 to day 33 of growth, while the shoot to root ratio did not change significantly (Table 1).

With regard to microbial counts, high variances relative to the means were found, particularly in planted microcosms at the second harvest and nearest to the soil-root interface (Fig. 3). In the absence of plants at 21 and 33 days after initiation, numbers of aerobic bacteria in the 0- to 2- and 2- to 4-mm soil layers were 4 times and 2 times, respectively, those at time zero. At 21 days the number of bacteria was the highest, regardless of depth of soil layer (Fig. 3). In the presence of plants, the number of bacteria was significantly higher than without plants up to 4 mm away from the soil-root interface, and the closer to the interface (nylon gauze) the higher the number. Bacterial numbers in gauze soil were 5.5 and 10 times higher than in the unplanted 0- to 2-mm slice after 21 and 33 days, respectively, of plant growth (Fig. 3).

At the first harvest in planted microcosms the mean numbers (thousands g^{-1} dry soil) of total protozoa, flagellates and amoebae at the gauze level were 46, 17 and 7, and in the 0- to 2-mm soil layer 23, 9 and 9, respectively. At the second harvest the corresponding numbers were 99, 67 and 30, and 38, 22 and 13, respectively. Generally, populations of flagellates and amoebae were comparable and their sum accounted for the population of total protozoa (Fig. 3). No significant difference was observed in unplanted microcosms between soil layers or harvests.

A clear rhizosphere effect was detected for both enzyme activities at the gauze level, and up to 2 mm away from the soil-root interface for protease activity at both

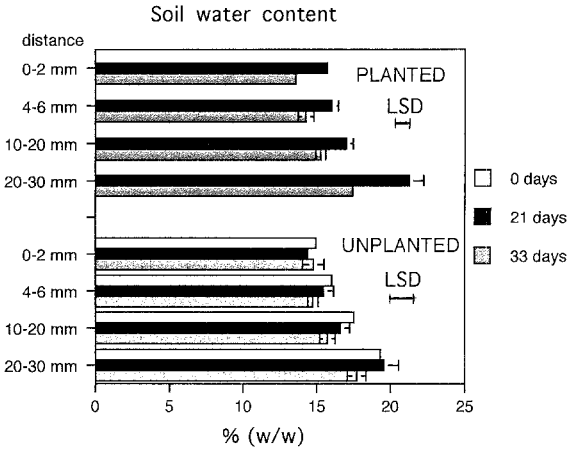

Fig. 2 Soil water content (g water 100 g^{-1} dry soil) in the unplanted and planted soil cylinder as a function of distance from the soil root-interface and after 0, 21 and 33 days of incubation. *Bars* indicate standard deviations. *LSD*, least significant difference, was calculated by a two-factor ANOVA (distance and days) at $P=0.05$

Table 1 Plant mass and shoot:root ratio (SRR) after 21 and 33 days of incubation. Values indicate an average of three replicates (±SD) and are expressed as grams dry matter (48 h at 80 °C) per microcosm

Measure	Growth period (days)		LSD[a]
	21	33	
Shoots	1.12±0.14	2.61±0.06	0.24
Roots	0.74±0.06	1.83±0.15	0.26
Total	1.86±0.20	4.44±0.15	0.40
SRR	1.51±0.20	1.43±0.14	0.38

[a] Least significant difference between 21 and 33 days at $P=0.05$

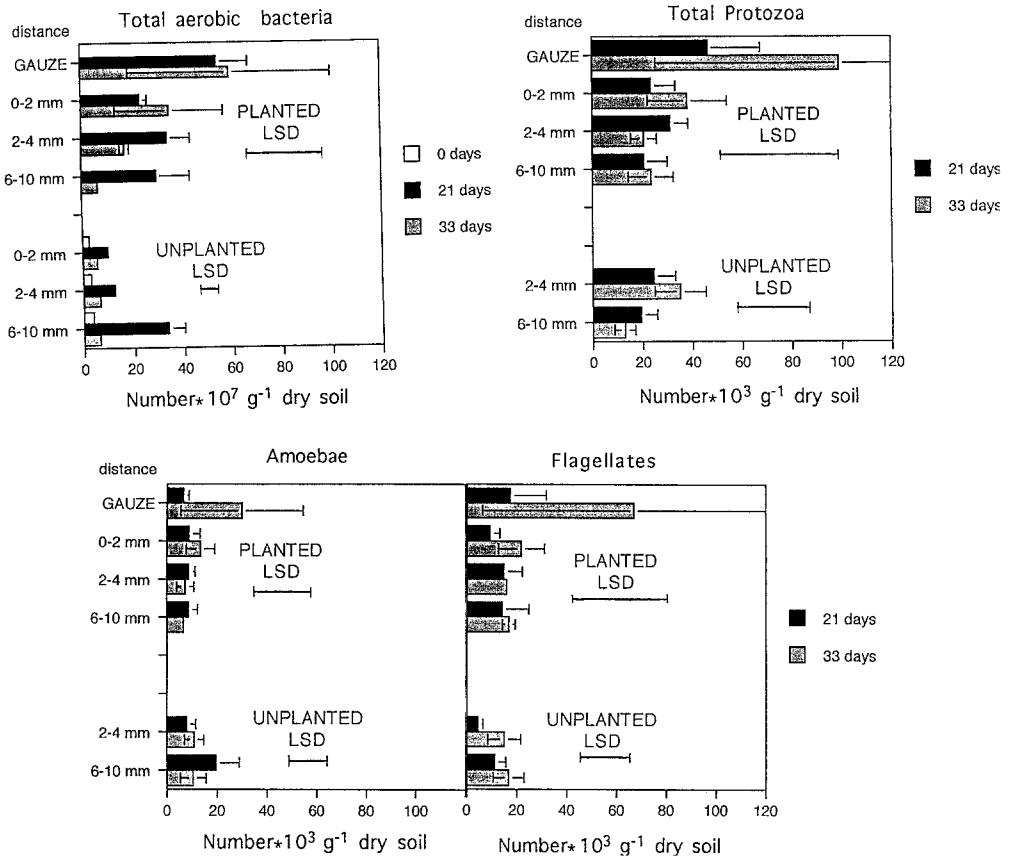

Fig. 3 Numbers of total aerobic bacteria on plate counts, total protozoa, amoebae and flagellates g^{-1} dry soil in the unplanted and planted soil cylinder after 0, 21 and 33 days of incubation as a function of distance from the soil-root interface. *Bars* indicate standard deviations. *LSD*, least significant difference, was calculated by a two-factor ANOVA (distance and days) at $P=0.05$

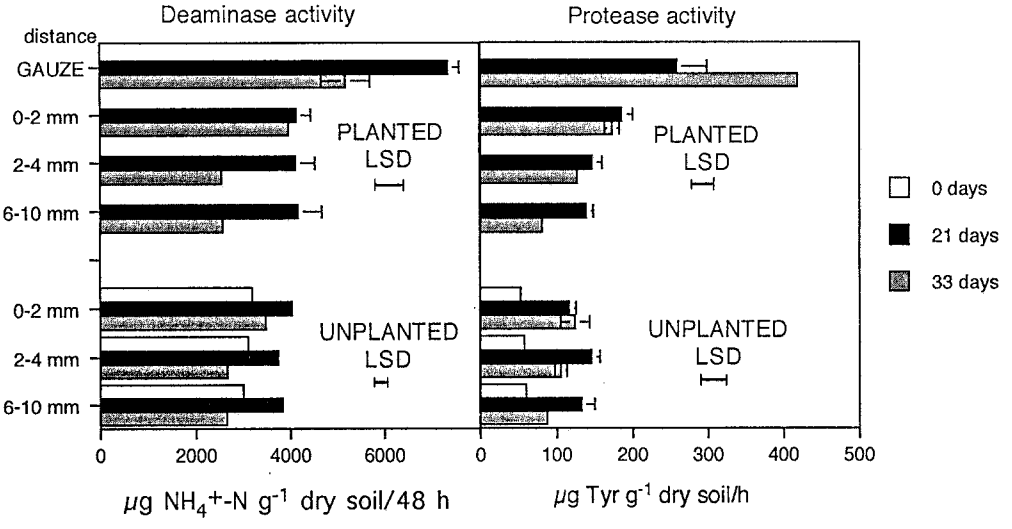

Fig. 4 Enzyme activity (L-histidine-deaminating and casein-hydrolysing activity g^{-1} dry soil) in the unplanted and planted soil cylinder as a function of distance from the soil-root interface and after 0, 21 and 33 days of incubation. *Bars* indicate standard deviations. *LSD*, least significant difference, was calculated by a two-factor ANOVA (distance and days) at $P=0.05$

harvests, while for deaminase activity this was only observed at 33 days. Both enzyme activities were higher at 21 days than at 33 days, the only exception in the gauze soil being for protease activity (Fig. 4).

As shown in Table 2, mean values of enzyme activities, total bacteria and protozoa were significantly correlated, the only exception being total protozoa versus deaminase activity; if only planted microcosms were considered, the correlation between protease and deaminase activities was not significant.

Discussion

The sandy table used in our soil-plant microcosm provided a continuous supply of water to the ring soil; however, it could not replenish all the water lost through evapotranspiration by the plants. The soil in the columns delivered water to the plants as indicated by weight losses detected during weighing and the growth of the plants. Also the ring provided water as is evident from the decrease in soil moisture content close to the gauze (Fig. 1) and the pres-

Table 2 Matrix of linear correlation coefficients (r) between mean values of protease activity (*PR*), deaminase activity (*DE*), total aerobic bacteria (*BACT*) and total protozoa (*PROT*) by considering either all planted and unplanted (*top value*) or only planted (*bottom value*) microcosms. Mean values of BACT and PROT were divided by 10^7 and 10^3, respectively. The number of observations was 17 (4 soil layers and 2 harvests for planted microcosms, 3 layers and 3 harvests for unplanted microcosms) or 12 (with regard to PROT unplanted microcosms: 2 layers and 2 harvests) for top value and always 8 for bottom value

	PR	DE	BACT	PROT
DE	0.719[b] 0.651 NS			
BACT	0.877[a] 0.879[b]	0.817[a] 0.859[b]		
PROT	0.927[a] 0.949[b]	0.525 NS 0.501 NS	0.723[b] 0.804[c]	

[a]Significant at $P<0.001$, [b]significant at $P<0.01$, [c]significant at $P<0.05$, *NS* not significant

ence of root hair. Thus, an influence of roots in the upper layers of the ring soil could be expected.

The increase and the successive decline of the aerobic bacterial population in unplanted soil may be due to exhaustion of substrates and nutrients, probably made available through disturbance and mixing of soil during the potting of the rings. A water shortage was not considered as a likely cause of this decline even though the water level dropped significantly.

Plant roots stimulated bacterial growth as shown by the higher bacterial numbers in the soil up to 4 mm away from the soil-root interface at both harvests. Dijkstra et al. (1987) found that 10-day-old spring wheat plants positively influenced the dynamics of *Enterobacter cloacae* up to 2 mm from the soil-root interface, using a soil-plant microcosm similar to that of Kuchembuch and Jungk (1982).

Foster and Dormaar (1991) have shown by electron microscopy the digestion and breakdown of soil bacteria by amoebae in the rhizosphere soil. In our microcosm a significant "rhizosphere effect" in regard to protozoan counts was observed only in the gauze soil. The absolute protozoan counts and the ratio of the number of protozoa in the rhizosphere (R) to non-rhizosphere soil (S) of 2:1 in our system corresponded well to those reported by Darbyshire and Greaves (1973). Most of the protozoa were amoebae and flagellates while ciliates were hardly detectable in our experiment, which is quite common (Clarholm 1994). Usually, in soil without plants where bacterial production is low, flagellates are much more numerous than amoebae since they need to graze numbers of bacteria an order of magnitude lower to divide (Clarholm 1994). Since amoebic and flagellate populations were comparable in the unplanted soil layers, the production of bacteria seemed sufficient to sustain grazing by amoebae. This is indirectly confirmed by the significant correlation between number of bacteria and total protozoa (Table 2).

The inefficacy of toluene in inhibiting soil microbial activity and proliferation (Frankenberger and Johanson 1986) could partially explain the high ammonium production during the 48-h soil deaminase activity assay in our experiment. Burton and McGill (1989) concluded that soil histidase activity declines in the absence of microbial growth. Frankenberger and Johanson (1982) originally, in the presence of toluene, observed a linear and not exponential accumulation of ammonium for at least 168 h, thus excluding any interference by microbial growth. They chose a 48-h incubation assay since the ammonium released after 12–24 h was too low. Shorter incubations than 48 h were discarded in this work.

The significant correlation between aerobic bacterial numbers and L-histidine-deaminating activity in both planted and unplanted soil slices (Table 2) may be taken as evidence that the measured histidase activity largely depends on the actual intracellular enzyme production rather than on the extracellular enzymes already present in the soil and associated with colloids (Burns 1982). The relatively long incubation time used in the deaminase assay may have caused microbial growth in soil samples and this may have reduced differences in enzyme activity between the gauze soil and deeper layer soils. According to the correlation study (Table 2), protozoa did not seem to contribute to the deaminating activity of the soil.

The highest casein-hydrolysing activity was found in the gauze soil after 33 days, when 100% of the nylon gauze was covered by the soil held back by root hair, whereas the highest deaminase activity was observed at day 21 (Fig. 4). Since wheat root hair is able to pass through nylon gauze and grow into the soil as far as 2 mm from the soil-root interface (James et al. 1985), it seems logical to assume a contribution of root hair enzymes to the protease activity at the soil-root interface after 33 days. The caseinase activity was significantly positively correlated to both bacteria and protozoa numbers (Table 2); it was less affected by microbial growth during the assay than the deaminase activity due to the shorter incubation at higher temperature (1 h at 50°C). The microbial synthesis of casein-hydrolysing enzymes may have been stimulated by root exudates. Positive correlations between soil protease activity and bacterial number have been observed in short-term incubation assays with glucose amendment (Nannipieri et al. 1983; Asmar et al. 1992) to mimick the rhizosphere environment.

Recently, Asmar et al. (1995) and Fritz et al. (1994) showed that the rhizosphere extended from 2 to 6–8 mm away from an artificial soil-root interface, respectively, employing a microcosm like that used here. In the latter case the rhizosphere effect may have been larger than in our experiment because of a more sensitive X-ray technique combined with a higher soil spatial resolution due to a sample size of 2–20 mg.

The major advantage of the soil-plant microcosm presented here is its suitability to study the "rhizosphere effect" over time of incubation and distance gradient from the soil-root interface. Stimulation of ammonification by protozoan grazing, which inter alia induces bacteria to produce more enzymes, has been widely documented (Woods et al. 1982; Kuikman and van Veen 1989). Our work

shows that bacterial and protozoan growth occurring in the rhizosphere soil is associated with increases in casein-hydrolysing and L-histidine-deaminating activities, both of which are involved in the N mineralization process (Ladd and Jackson 1982). Whereas the histidinase activity was of bacterial origin, protozoa and root hair also most likely contributed to the overall caseinase activity. In conclusion, our approach points to the need for further mechanistic studies into soil-plant interactions and root-induced N-mineralization.

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