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Behaviour of Bacterial Populations in Response to Amoebal Predation in the Rhizosphere*

P. GAMARD,[1] C. STEINBERG[1] and G. FAURIE

Université C. Bernard-Lyon 1, Laboratoire de Microbiologie des Sols, URA CNRS 697 Ecologie Microbienne – I.A.S.B.S.E. – bat 741, 43 Bd du 11 novembre 1918, F. 69622 Villeurbanne Cedex.

Abstract

The population dynamics of an introduced bacterial population, of the indigenous microflora, and of the indigenous amoebal community were compared in a rhizospheric and a non rhizospheric soil. The classical rhizospheric stimulating effect was observed for the indigenous soil bacterial populations and for the amoebal community too: the plant by means of the various exudates was able to stimulate the bacteria, then amoebae were developing on this microflora. The introduced population decreased because of the predators whether the soil was planted or not.

It was concluded that the behaviour of microbial populations in response to amoebal predation was dependent on the localization of the bacteria within or outside the soil aggregates. Thus, the predation regulating mechanisms will act in the rhizospheric soil as in the non rhizospheric soil.

Introduction

Colonization of the soil rhizosphere by indigenous or introduced bacterial populations is important in determining the extent of benefits for crop yield. This implies particular bacterial population dynamics with their regulation mechanisms. Indeed, rhizosphere soil is characterized by a marked increase in the numbers of bacteria compared with non rhizospheric soil (Gamard et al., 1987, Ramirez and Alexander 1980), this increase being mainly due to plant exudate releases (Martin and Kemp 1980, Bottner and Billes 1987) or to physical and chemical changes of the soil by the roots themselves (O2 partial pressure, pH, soil structure) (Nye 1981).

Conversely, evidence is now accumulated on the view that the size and the activity

*Reviewed

1. Present address:
INRA Flore pathogène dans le sol
B.V. 1540, 21034 Dijon Cedex, France

of microbial populations are strongly determined by protozoan predation (Danso and Alexander 1975, Habte and Alexander 1977, Heynen et al., 1988, Steinberg et al., 1987). In the soil, predation is mainly due to the amoebae which seem to be the single most important group of soil protozoa, they can reach 95% of the total protozoan biomass (Elliott and Coleman 1977, Gupta and Germida 1988), certainly because they are the best adaptated to life in the soil (Clarholm 1981): naked amoebae are more flexible and need a thinner water film to move (to creep) in mineral soil than ciliates which are rather swimming protozoa, better adaptated to aquaeous medium. Their importance will be larger in wet organic soils where there is enough space for their feeding activities. Flagellates could be of quantitative equal importance as amoebae, nevertheless, their feeding impact on the bacterial populations will be much less owing to their 20 times smaller size.

A paradoxal situation will come into view from these two points: bacteria will increase in the rhizosphere because of the root exudates, resulting in an activation of the amoebae which will in turn graze on the bacteria and reduce their populations.

In fact, the role of protozoa in the rhizosphere was rather studied through their impact on the availability of bacterial nitrogen to plants due to higher bacterial N turn over *via* microbial predation (Clarholm 1985, Kuikman and Van Veen 1989, Woods et al., 1982). Barsdate et al., (1974), Elliott et al., (1979), have demonstrated a higher release in C and P in presence of predators. More generally, protozoa enhance mineralization of nutrient immobilized in the microbial biomass by grazing on bacteria and excreting excess nutrients (Stout 1973).

Nevertheless, very few authors reported population dynamics studies of both bacteria and amoebae in the rhizosphere (Darbyshire and Greaves 1973).

The present study was designed to gain further informations about the mechanism of protozoan predation on the rhizosphere microflora using an introduced bacterial population as a biological tracer in a rhizospheric and a non-rhizospheric soil.

Materials and Methods

Soil. The soil used was a silt loam (from the region of Lyon, France) exhibiting no extreme characteristics and whose properties were: 32.2% sand, 31.4% clay, 36.4% silt with a pH (1:2 in water) of 6.2, 2.64% organic C, a C/N ratio of 7.6 and a water holding capacity of 40%. Field moist soil was sieved at 2 mm and it was preincubated at 28°C eight days before the beginning of the experiment in order to allow the indigenous microflora to reach an equilibrium state because of the temperature, avoiding a thermic artefact at time zero of the experiment.

Bacterial strain. The *Azospirillum brasilense* strain 245 nr-(nitrate reductase negative) isolated in Brazil from surface sterilized wheat roots (Boddey et al., 1986) was grown in nutrient broth (Difco) (NB). The culture was incubated at 28°C on a rotary incubator.

Plant. Seeds of wheat (biovar PF 839197) were surface sterilized by immersion in concentrated sodium hypochlorite for 10 mn. The seeds were then rinsed with sterile distilled water and immersed in commercial Milton solution (stabilized sodium hypochlorite 2%) for 30 mn. The seeds were finally rinsed three times with sterile distilled water and allowed to germinate on presterilized moistened papers in the dark.

Preparation of the microcosms. Microcolumns consisting in 8 g of soil (dw) in a sterile syringe which was in turn placed in a test tube containing sterile distilled water were prepared as described by Steinberg et al., (1989).

Four ranges of microcolumns were prepared:

(i) unplanted soil inoculated with *Azospirillum* (treatment 1)
(ii) wheat planted soil inoculated with *Azospirillum* (treatment 2)
(iii) non inoculated unplanted soil (treatment 3)
(iv) non inoculated wheat planted soil (treatment 4)

24 hours before the inoculation, pregerminated wheat seeds were placed in microcosms for planted treatments then all the devices were preincubated in a climate room with temperature regulated to $22° \pm 2°C$ and a light period of 16 hours per day.

Bacterial cells in liquid culture were collected by centrifugation at 7000 g for 15 mn then washed twice using sterile phosphate buffer saline solution (PBS). The cells were resuspended in PBS and the bacterial concentration was adjusted to 8×10^7 cells ml^{-1} by optical density evaluation.

Each microcolumn was inoculated with 1 ml of the bacterial suspension to provide 10^7 cells g^{-1} of dry soil. One ml of sterile PBS was added to the non inoculated microcosms.

The microcolumns were then replaced in the climate room. Every 2 or 3 days, 3 microcolumns from treatments 1 and 2 and 1 microcolumn from treatments 3 anc 4 were removed and assayed for the counts of indigenous amoeba, of *Azospirillum*, and of indigenous bacteria. There was no replicate for treatments 3 and 4 because they were considered as control.

Extraction of the microorganisms. The whole sample (soil + roots) was shaken for 20 mn in 40 ml of sterile distilled water on a magnetic shaker. 5 ml were removed for the amoebal counts. 65 ml of sterile distilled water were then added to the soil suspension which was in turn used for the extraction of the bacteria. This suspension was blending for 2 mn in a waring blender (Eberbach corp.). One ml was immediately removed for the counts of indigenous bacteria. The remaining suspension was finally assayed for the extraction of *Azospirillum* using the flocculating method of Bezdicek and Donaldson (1980).

Counts. The amoebal counts were done using the rings method (Singh 1946) and calculations of the Most Probable Number of amoebae in the samples were done using the table VIII 2 from Fisher and Yates (1943). The ability for the amoebae to feed on *A. brasilense* has been tested in Petri dish using a strain of *Acanthamoebae*

previously isolated from the soil. This was a prerequisite for the rings method since each ring was filled with a saline non nutrient agar (Page 1976) covered with a layer of *A. brasilense*.

The indigenous bacterial microflora of the soil was counted on nutrient agar (Biomérieux-F 69160 Charbonnières) by the plate count method (3 plates per dilution level) (Pochon et Tardieu 1962). The counts were done twice after 3 and after 6 days of incubation at 22°C.

The *A. brasilense* strain was specifically counted by using the indirect fluorescent antibody technique (Schmidt 1974). No cross reaction with the indigenous bacteria was detected prior to the inoculation of the *Azospirillum* strain. Counts were made on Nuclepore black polycarbonate membranes (0.4 μm).

Results

In the non planted soil, inoculated with *A. brasilense* (treatment 1), the number of indigenous amoebae increase from 3.4×10^4 to 1.3×10^5 amoebae g^{-1} dry soil consequently to the inoculation of *A. brasilense* whose density decreased from 2.5×10^7 to 3.0×10^4 bacteria g^{-1} dry soil (Fig. 1). Conversely, the treatment 3 (non inoculated, non planted soil) showed a quantitative stability of the amoebal community near 2×10^4 amoebae g^{-1} dry soil (Fig. 1).

In the treatment 1, the indigenous bacterial microflora increased from 3.8×10^7 to 2.4×10^8, then oscillated around 2.0×10^8 bacteria g^{-1} dry soil. In the treatment 3, the indigenous microflora remained established at about 3.0×10^8 bacteria g^{-1} dry soil. (Fig. 1).

In the planted soil (Fig. 2) inoculated with *A. brasilense* (treatment 2), the number of amoebae increased from 3.4×10^4 to 2.2×10^5 amoebae g^{-1} rhizospheric dry soil in 14 days then slightly decreased. As in unplanted soil (treatment 1, Fig. 1), the *A. brasilense* population fastly decreased from the inoculum value (2.5×10^7 bacteria g^{-1} rhizospheric dry soil) to 2.0×10^4 bacteria g^{-1} rhizospheric dry soil. On the contrary, the indigenous bacterial microflora fastly increased from 3.8×10^7 to 1.4×10^9 bacteria g^{-1} rhizospheric dry soil in 4 days, decreasing then to 4.4×10^8 bacteria g^{-1} rhizospheric dry soil and remaining around this high level until the end of the experiment.

In the treatment 4 (non inoculated planted soil, Fig. 2) the number of amoebae increased as in the treatment 2 from 3.4×10^4 amoebae g^{-1} rhizospheric dry soil to reach the same final value (around 2.3×10^5 amoebae g^{-1} rhizospheric dry soil). As well, the indigenous bacterial microflora exhibit the same pattern as in the inoculated soil (treatment 2).

Discussion

The R/S ratios found (Table 1) in this study are always lower than those found by

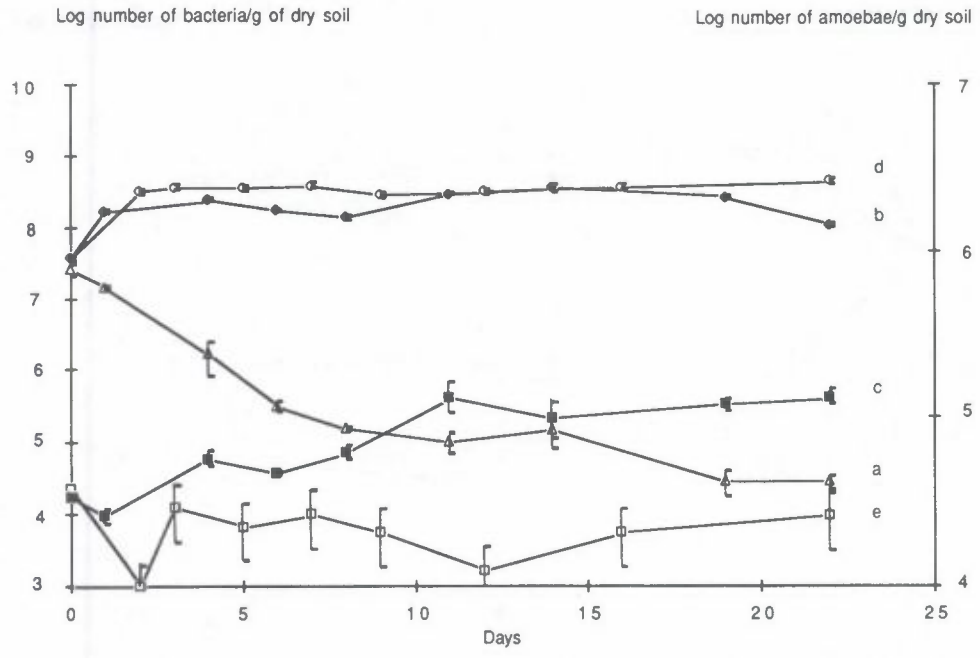

Figure 1. Population dynamics in non planted soil:
treatment 1. (a) introduced *A. brasilense* population; (b) indigenous bacterial microflora in inoculated soil; (c) indigenous amoebae in inoculated soil.
treatment 3. (d) indigenous bacterial microflora in non inoculated soil; (e) indigenous amoebae in non inoculated soil. Bars represent the standard deviation of the mean.

other authors (Darbyshire and Greaves 1973, Clarholm 1981) because in our case, the whole sample (soil + roots) was shaken and blended for the counts of microorganisms while generally, samples of the rhizospheric soil was done taking the soil fraction very close to the root surface. So, the number of rhizospheric microorganisms was diminished by our method. Indeed, the number of microorganisms decrease rapidly beyond few mm from the rhizoplane (Papavizas and Davey 1961).

When incubated at 22°C, the indigenous bacteria rapidly reached their maximal density in the soil. This density corresponds to the equilibrium state of the population according to abiotic factors (water holding capacity in this case) and biotic factors (predation and competition). Ramirez and Alexander (1980) have already noticed that the first days after planting, the total number of bacteria was increased by two orders of magnitude and then remained constant.

The inoculation of *Azospirillum* in treatment 1 (non planted soil) resulted in a strong reduction in the size of this population. The decrease coincided with amoebal growth. Such a growth was not observed in the treatment 3.

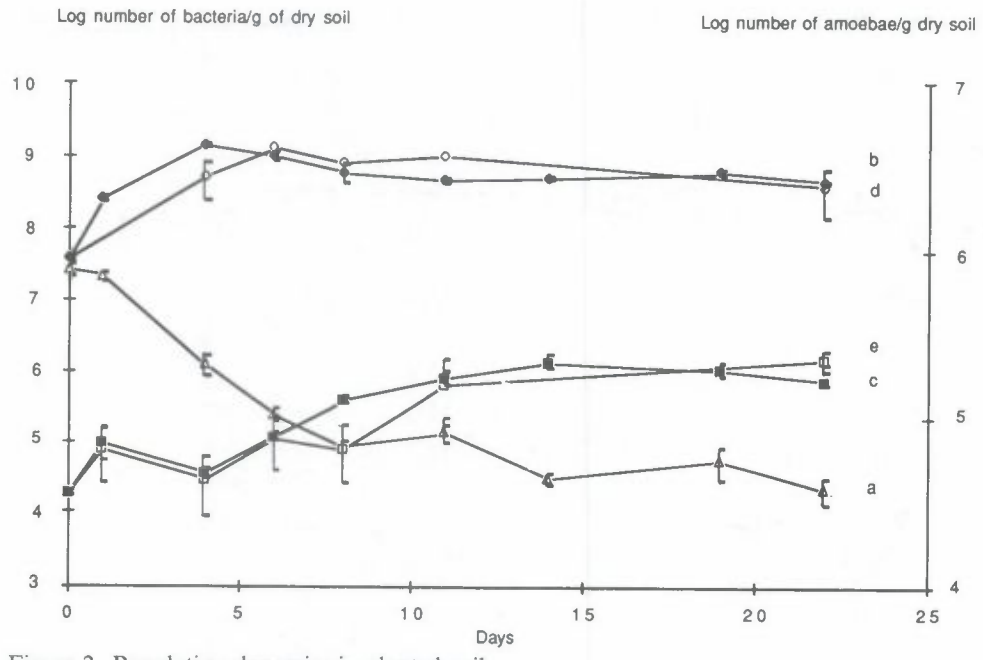

Figure 2. Population dynamics in planted soil:
treatment 2. (a) introduced *A. brasilense* population; (b) indigenous bacterial microflora in inoculated soil; (c) indigenous amoebae in inoculated soil.
treatment 4. (d) indigenous bacterial microflora in non inoculated soil; (e) indigenous amoebae in non inoculated soil. Bars represent the standard deviation of the mean.

These results are in agreement with previous works (Danso and Alexander 1975, Habte and Alexander 1977, Steinberg et al., 1987) who have also reported a regulation of introduced bacterial population due to protozoan predation. The increase in amoebal number in the soil must then be interpreted as the result of their predatory activity on bacteria. Nematodes can be bacterivorous and we found some within rings used for the counts of the amoebae. Nevertheless, they were sparse and we think that they were rather feeding on the protozoa according to Elliott et al., (1980), but we have no evidence for that. These authors have shown that the trophic relation structure was rather of that way: "nematodes-protozoa-bacteria".

The indigenous bacterial microflora did not seem to be affected either by the inoculation of *A. brasilense* or by the protozoa. Steinberg et al., (1987) suggested that if the indigenous bacterial microflora was essentially located in the internal sites within the soil aggregates, they would be less sensitive to predation. Indeed, Vargas and Hattori (1986) demonstrated that a population of protozoa introduced into a sterile soil inoculated with a bacterial population was unable to graze on the bacteria of the internal fraction as it was defined by the washing and sonication technique of

Table 1. R/S ratios (number of microorganisms counted in planted samples compared with number of microorganisms counted in unplanted samples) calculated at each sampling time for the introduced population, the indigenous microflora and the indigenous amoebae. The mean R/S ratio was calculated in each case ± the standard deviation of the mean

	Inoculated soil (treatment 2/1)			Non inoculated soil (treatment 4/3)	
Day	Amoebae	Microflora	*Azospirillum*	Amoebae	Microflora
0	1	1	1	1	1
1	1.83	1.47	1.43	6.7	ND
4	0.79	5.6	0.8	1.81	1.45
6	1.68	5.64	0.81	2.8	3.65
8	2.1	4.2	0.54	3.1	2.78
11	1.35	1.55	1.36	13.1	3.13
14	2.24	1.41	0.19	ND	ND
19	1.65	2.34	1.84	ND	ND
22	1.31	3.98	0.73	8.9	0.88
x ± SDM	1.62 ± 0.06	3.47 ± 0.26	0.962 ± 0.07	4.66 ± 0.75	2.38 ± 0.29

Nishio et al. (1968). Even if the fact that the protozoa used by these authors were ciliates, and therefore less flexible than amoebae, our results and previous ones are consistent with the results of Vargas and Hattori. In the same way, Heynen et al. (1988) found that bentonite clay could induce the formation of microniches conferring partial protection against protozoan predation. By contrast, the introduced bacterial population may be located in the soil solution at first and thus will be more accessible to the grazers. In this case, the *Azospirillum* population is grazed by the amoebae and the rapid decrease of the population is followed by a steady state around 10^4cells g^{-1} at this level, the number of bacteria became too low to allow further predation, predation being a density dependant mechanism (Alexander 1981).

In planted soil, the decrease in *Azospirillum* numbers was of the same order as in unplanted soil. The R/S ratio for this strain was surprisingly of about 1 (0.96 ± 0.02). Indeed, a rhizospheric effect of the wheat rhizosphere on inoculated *A. brasilense* sp 245 has been demonstrated elsewhere (Baldani et al., 1986, Boddey et al., 1986). The behaviour of the *A. brasilense* population in this planted soil could result primarily from predation on this introduced population before any rhizospheric effect within the time period of this experiment. The number of protozoa increased nearly two times higher than in the treatment 1 (Mean R/S ratio = 1.62 ± 0.06). So, if we assume that the bacterial biomass ingested per protozoa is constant to allow them either to encyst or to reproduce, the amoebae must have grazed on indigenous bacteria in addition to the *A. brasilense* population.

The number of bacteria increased in the rhizospheric soil from 10^7 to more than

10^9 cells g^{-1} dry soil (the mean R/S ratio being of 3.27). Then, amoebal number was increasing. Most of these indigenous bacteria were probably growing within the internal sites of the aggregates but it is likely that some of these bacterial colonies were growing out of the aggregates and were liable to predation in the same way as the introduced bacterial population. More over, the bacterial density and the growth rate of the indigenous bacteria were high owing to the root effect. In such circumstances, the bacteria are able to replicate and compensate for killing. Then the effect on the prey density was few apparent.

The present findings are consistent with the view that growth of protozoa in the rhizosphere depends not only on the *Azospirillum* population, but also on the density of other bacteria. Ramirez and Alexander (1980) have shown that adding a simple carbon source would stimulate the populations of rhizobia, protozoa and total bacteria in a fashion similar to that observed when seeds are planted in the soil.

Therefore, the three following points could be stated as a conclusion.

– The indigenous bacteria would be principally located within soil aggregates and protected from protozoan predation. The rhizospheric influence involved an increase in bacterial number. These additional bacteria will grow out of the internal sites and then became more exposed to predatory protozoa.

– The introduced *A. brasilense* population was susceptible to predators because of its location on the outside of the aggregates. The predatory activity was thus acting on this sensitive bacterial population immediately after its inoculation into the soil before the expected stimulatory effect of roots.

– In a rhizospheric soil, the population dynamics of amoebae, was dependent on a supply of accessible bacteria as it was in a non rhizospheric soil. This supply could arise either from an inoculation of bacteria in a non planted soil, or from an increase in indigenous bacteria due to the rhizospheric effect.

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REFERENCES

Alexander, M. 1981. Why microbial predators and parasites do not eliminate their prey and host. *Ann. Rev. Microbiol.* **35:** 267–279.

Baldani, V.L.D., Alvarez, M.A. de B., Baldani, J.J., and Dobereiner, J. 1986. Establishment of inoculated *Azospirillum* spp. in the rhizosphere and in roots of field grown wheat and sorghum. *Plant and Soil.* **90:** 35–46.

Barsdate, R.J., Prentki, R.T., and Fenchel, T. 1974. Phosphorus cycle of model

ecosystems: significance for decomposer food chain and effect of bacterial grazers. *Oïkos.* **25:** 239–251.

Bezdicek, D.F. and Donaldson, M.D. 1980. Flocculation of *Rhizobium* from soil colloids for enumeration by immunofluorescence. In: Microbial adhesion to surfaces. R.C.W. Berkeley, J.M. Lynch, J.M. Melling, P.R. Rutter and B. Vincent, eds. University of Reading. E. Horwod, Chichester, pp 297–309.

Boddey, R.M., Baldani, V.L.D., Baldani, J.I., and Dobereiner, J. 1986. Effect of inoculation of *Azospirillum* spp. on nitrogen accumulation by field-grown wheat. *Plant and Soil.* **95:** 109–121.

Bottner, P. and Billes, G. 1987. La rhizosphère: site d'interactions biologiques. *Rev. Ecol. Biol. Sol.* **24:** 369–388.

Clarholm, M. 1981. Protozoan grazing of bacteria in soil: impact and importance. *Microb. Ecol.* **7:** 343–350.

Clarholm, M. 1985. Interactions of bacteria, protozoa and plants leading to mineralization of soil nitrogen. *Soil Biol. Biochem.* **17:** 181–187.

Danso, S.K.A. and Alexander, M. 1975. Regulation of predation by prey density: the protozoan-rhizobium relationship. *Appl. Microbiol.* **29:** 515–521.

Darbyshire, J.F. and Greaves, 1973. Bacteria and protozoa in the rhizosphere. *Pestic. Sci.* **4:** 349–360.

Elliott, E.T., Anderson, R.V., Coleman, D.C., and Cole, C.V. 1980. Habitable pore space and microbial trophic interactions. *Oïkos.* **35:** 327–335.

Elliott, E.T. and Coleman, D.C. 1977. Soil protozoan dynamics in a shortgrass prairie. *Soil Biol. Biochem.* **9:** 113–118.

Elliott, E.T., Coleman, D.C., and Cole, C.V. 1979. The influence of amoebae in the uptake of nitrogen by plants in gnotobiotic soil. In: The soil-root interface. J. Harley and R.S. Russel, eds. Academic press London, pp. 221–229.

Fisher, R.A. and Yates, F. 1943. Statistical tables for biological agricultural and medical research. Oliver and Boyd, eds. Edinburgh.

Gamard, P., Laguerre, G., and Bardin, R. 1987. Dénombrement d'*Azospirillum lipoferum* dans le sol et la rhizosphère par la méthode des anticorps fluorescents. *Rev. Ecol. Biol. Sol.* **24:** 389–402.

Gupta, V.V.S.R. and Germida, J.J. 1988. Population of predatory protozoa in field soils after 5 years of elemental S fertilizer application. *Soil Biol. Biochem.* **20:** 787–792.

Habte, M. and Alexander, M. 1977. Further evidence for the regulation of bacterial populations in soil by protozoa. *Arch. Microbiol.* **113:** 181–183.

Heynen, C.E., van Elsas, J.D. and Kuikman, P.J. 1988. Dynamics of *Rhizobium leguminosarum* biovar *trifolii* introduced into soil: the effect of bentonite clay on predation by protozoa. *Soil Biol. Biochem.* **20:** 483–488.

Kuikman, P.J. and van Veen, J.A. 1989. The impact of protozoa on the availability of bacterial nitrogen to plants. *Biol. Fertil. Soil.* **8:** 13–18.

Martin, J.K. and Kemp, R.J. 1980. Carbon loss from roots of wheat cultivars. *Soil Biol. Biochem.* **12:** 551–554.

Nishio, M., Hattori, T., and Furusaka, C. 1968. The growth of bacteria in sterilized soil aggregates. *Rep. Inst. Agr. Res.* Tohoku Univ. **19:** 37–43.

Nye, P.H. 1981. Changes of pH across the rhizosphere induced by roots. *Plant and Soil.* **61:** 7–26.

Papavizas, G.C. and Davey, C.B. 1961. Extent and nature of the rhizosphere. *Plant and Soil.* **13:** 384–390.

Pochon, J. and Tardieu, P. 1962. Technique d'analyse en microbiologie du sol. Editions de la Tourelle. St Mandé, France, pp 24–33.

Ramirez, C. and Alexander, M. 1980. Evidence suggesting protozoan predation on *Rhizobium* associated with germinated seeds and in the rhizosphere of beans (*P. vulgaris*). *Appl. Environ. Microbiol.* **40:** 492–499.

Schmidt, E.L. 1974. Quantitative autecological study of microorganisms in soil by immunofluorescence. *Soil Sci.* **118:** 141–149.

Singh, B.N. 1946. A method of estimating the number of soil protozoa, especially amoebae based on their differential feeding on bacteria. *Ann. Appl. Biol.* **33:** 112–119.

Steinberg, C., Faurie, G., Zegerman, M. and Pave, A. 1987. Régulation par les protozoaires d'une population bactérienne introduite dans le sol. Modélisation mathématique de la relation prédateur-proie. *Rev. Ecol. Biol. Sol.* **24:** 49–62.

Steinberg, C., Gamard, P., Faurie, G., and Lensi, R. 1989. Survival and potential denitrifying activity of *Azospirillum lipoferum* and *Bradyrhizobium japonicum* inoculated into sterilized soil. *Biol. Fertil. Soil.* **7:** 101–107.

Stout, J.D. 1973. The relationship between protozoan populations and biological activity in soils. *Amer. Zool.* **13:** 193–201.

Vargas, R. and Hattori, T. 1986. Protozoan predation of bacterial cells in soil aggregates. FEMS *Microbiol. Ecol.* **38:** 233–242.

Wood, L.E., Cole, C.V., Elliott, E.T, Anderson, R.V., and Coleman, D.C. 1982. Nitrogen transformations in soil as affected by bacterial microfaunal interactions. *Soil Biol. Biochem.* **14:** 93–98.