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Effects of nodular extracts of *Alnus glutinosa* (L.) Gaertn. on ammonification, nitrification and CO_2 production in different soils

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Abstract. The effect of root nodule extracts from European alder (*Alnus glutinosa* [L.] Gaertn.) on the rates of ammonification, nitrification and CO_2 production was evaluated in three soils of different physico-chemical characteristics. The extracts decreased net ammonification rate (up to a 43% respect to water control), probably because of an increase of immobilization rather than an effect of a speciffc inhibitor. The rate of nitrification was increased by the extracts (up to 40% from water control) because of the ammonium present in the extracts. An increase in CO_2 production showed that the nodule extracts stimulate microbial activity. This depended not only on the amount of organic matter introduced by the extracts but also to some activator of microflora.

Resumen. Se evalúa el efecto de extractos nodulares del aliso europeo (*Alnus glutinosa* [L.] Gaertn.) sobre las tasas de amonificación, nitrificación y producción de CO_2 en tres suelos de características físico-químicas diferentes. Los extractos reducen la tasa de amonificación (un 43% menor que en el control con agua), probablemente debida a un aumento de la inmovilización más que a un inhibidor específico. La tasa de nitrificación crece por efecto de los extractos (un 40% respecto al control con agua) a consecuencia del amonio presente en éstos. El incremento de la producción de CO_2 muestra que los extractos estimulan la actividad microbiana. Esto no parece depender sólo del aporte extra de materia orgánica por los extractos, sino por algún activador específico.

Introduction

The exudation of organic compounds from roots or root nodules is a complex process involving several mechanisms. Organic substances pass from roots to the environment in many ways, as secretion and diffusion from epidermal cells. Root exudation includes the combined effects of all processes which donate substances to the root environment (Svenningsson *et al.* 1990).

Root exudates contain amino acids, lipids and carbohydrates (Vancura, 1967; Barber & Gunn, 1974), and various allelopathic compounds (Rice, 1984), phytoalexines (Ebel, 1986) or aglutinines (Mansfield & Brown, 1985) which affect

rhizosphere microorganisms. By changing the soil C/N ratio, exudates provide energy for the growth of different groups of rhizosphere microbes and should affect the microbial population of the rhizosphere. The rhizosphere microbial community in turn plays a critical role in accumulation and degradation of organic matter, in which plant nutrients are stored (O'Neill & Reichele, 1980).

A critical variable influencing the characteristics of exudates is the water availability (Klein *et al.*, 1989), though this has often been ignored in studies of the effects of diazotrophic plants on soil microorganisms of nitrogen cycle (Bollen & Lu, 1968; Tarrant & Trappe, 1971). Since exudation is strongly influenced by moisture stress (Martin, 1977) the objective of our work is to study the effects of nodules of european alder (*Alnus glutinosa*) extracts (obtained under water stress conditions) on ammonification and nitrification rates, and total microflora (measured as production of CO_2) in different soils.

Materials and Methods

Nodules were collected from a 20 years old *Alnus glutinosa* stand near Madrid. The surfaces of the nodules were sterilized with NaClO (10 mg/l) and washed with sterile water. The nodules were then stressed by keeping them in sterile conditions (laminar-flux) for 7 days. In these conditions exudation is at a maximum (Klein *et al.*, 1989; Martin, 1977). The nodules were then divided into three fractions of 100 g, 50 g and 20 g dry weight, each of which was added to 1000 cm^3 of sterile water. After 12 h, the three aqueous fractions were filtered and stored at −20°C.

To evaluate the effects of the nodular extracts on the ammonification, nitrification rates and CO_2 production, three soils near Madrid were selected (Table 1). These soils were collected from the two dominant species of plants rhizosphere of each zone, dried at 25°C, sieved through a 2 mm mesh and stored at 4°C. The three soils show different physico-chemical characteristics (Table 2).

Total nitrogen was determined by the Kjeldhal method (Bremner, 1965), using 10 g of soil and 1 cm^3 of the nodule extract. The organic carbon was determined

Table 1. Characteristics of the zones where soil were collected.

Soil	Parent Material	Soil (USDA)	Texture (%) Sand	Clay	Silt	Dominant Vegetation
A	Arkose	Anfisol	65	13	21	*Quercus rotundifolia* *Retama sphaerocarpa*
B	Plutonic	Inceptisol	23	52	23	*Pinus sylvestris* *Cytisus purgans*
C	Gypsiferans-Clays	Entisol	52	18	28	*Quercus coccifera* *Stipa gigantea*

by the Walkley & Black (1934) method also using 10 g of soil and 1 cm^3 of nodule extract. Nitrate was measured by the Milham *et al.* (1970) method in 5 g of soil and 2 cm^3 of extract. This method uses a CRISON digit 501 mv/pH meter with a nitrate specific electrode (ORION 930700) and a ORION 900200 reference electrode. Ammonium was measured with the same mv/pH meter and an ORION 951000 specific electrode; 5 g of soil and 2 cm^3 of extracts were used. pH was measured directly in the extracts using a BECKMAN fi 12 pH meter with an electrode RADIOMETER GK 2401 C; the pH of the soil was measured in distilled water (1:3).

Ammonification was measured by the method of Robertson & Vitousek (1981). Different concentrations of nodular extracts were added to 15 g of each soil. The volume of each solution was enough to bring them to 100% of water holding capacity (WHC). To each soil 0.09 g of asparagine, 0.15 cm^3 of lowconcentration-elements solution (Gutiérrez Mañero & Bermúdez de Castro, 1983) and 3.75 cm^3 of Winogradsky solution were added. As a source of mineralizable nitrogen is necessary for ammonification, two controls were made: (1) 0.09 g of asparagine and sterile water to 100% WHC (CW) and (2) 0.09 g of asparagine, the same concentration of mineralizable nitrogen as found in the most concentrated extracts —that is 10%—, and sterile water to 100% WHC (CMN). All were incubated at 21°C for 72 hours. Potential ammonification was calculated using the equation of Stanford & Smith (1972):

$$\log (Nt–No) = \log No^{-kt}$$

where No is the nitrogen mineralizable at time zero, Nt the nitrogen mineralizable at time t, and k the potential of ammonification. No and Nt (as $NH4^+$) were measured using the specific electrode and method described previously.

Potential nitrification was also determined by the method of Robertson & Vitousek (1981). Different concentrations of nodular extracts were added to 100 g of soil to give 100% WHC. 0.016 g ammonium sulphate was also added; this is

Table 2. Results of characterization of soils and nodular extracts (means ± standard error), and significance of the corresponding ANOVAs (**, p<00.1). Units: Organic C (%), Total N (mg/g), $N–NH_4^+$ (µg/g), $N–NO_3^-$ (µg/g). 10%, 5%, and 2%: different concentrations of nodular extracts assayed (for details see text).

	Soils				Extracts			
	A	B	C		10%	5%	2%	
pH	7.30 ±.01	4.30 ±.00	8.01 ±.02	**	4.66 ±.01	5.20 ±.00	5.51 ±.00	**
Organic C	1.84 ±.04	6.36 ±.12	0.32 ±.05	**	2.66 ±.01	1.92 ±.00	1.20 ±.01	**
Total N	12.5 ±.22	9.54 ±.22	1.19 ±.04	**	1.30 ±.07	0.81 ±.07	0.46 ±.06	**
$N-NH_4^+$	2.68 ±.31	4.71 ±.31	0.78 ±.09	**	0.44 ±.00	0.16 ±.00	0.10 ±.00	**
$N-NO_3^-$	1.53 ±.14	2.45 ±.25	1.17 ±.04	**	3.57 ±.00	1.73 ±.08	0.58 ±.00	**

enough to give a concentration of NH_4^+ between 10 ppm and 800 ppm, the limits within which nitrification occurs (Verstraete, 1981; Jones & Hedlin, 1970). Two controls were made: (1) with 0.016 g of ammonium sulphate and sterile water to 100% WHC (CN) and (2) with sterile water at 100% of WHC (CW). All were incubated at 21°C for 20 days, and nitrate was determined before and after. Potential nitrification was the difference between these measurements.

To determine the production of carbon dioxide, the different concentrations of nodular extracts were added to 100 g of each soil to give 100% WHC, in a 375 cm^3 flask. Four controls were made for each soil in which extracts were replaced by: (1) sterile water to 100% WHC (CW); (2) glucose at the same concentration of organic carbon found in the most concentrated extracts (i.e. 2.66%) plus sterile water to 100% WHC (CG); (3) similar to the second one but with a double concentration of glucose (5.32%) (CDG); and (4) a control having both 2.66% glucose and most concentrated extract (10%) to 100% WHC (CGE). All were incubated for 96 h at 25°C. The CO_2 produced was determinated by a KONIK 3000 HRGC gas chromatograph equipped with a KNK 019-501 thermal conductivity detector, a CROMOSORB 101 column, and a KONIK 825-318 integrator.

The data variance was analysed two-way ANOVA with three replicates or oneway with three replicates (Sokal and Rholf, 1979). Differences between treatments were evaluated by calculating the least significant differences (LSDs).

Results and Discussion

All the extracts and soils were significantly different in physico-chemical parameters (Table 2). In particular the extracts provided important amounts of organic carbon and ammonium.

Table 3. Ammonification (day^{-1}) in the different soils and treatments (means ± standard error). The means of three soils for each treatment appears in each column ($\bar{x}$). Five treatments and controls per soil appear in each row ($\bar{y}$). The LSD (treatments and controls, left and soils, right) are also given.

	Treatments and Controls					
Soil	10%	5%	2%	CW	CMN	
A	0.032 ±.005	0.093 ±.019	0.052 ±.002	0.094 ±.014	0.084 ±.009	0.077
B	0.042 ±.005	0.058 ±.012	0.039 ±.003	0.077 ±.025	0.025 ±.001	0.048
C	0.029 ±.001	0.058 ±.004	0.077 ±.005	0.066 ±.000	0.032 ±.001	0.051
	0.034	0.070	0.004	0.079	0.047	$\bar{x}$ $\bar{y}$

$LSD_{0\,01}$ = 0.0285 $LSD_{0\,01}$ = 0.0209
$LSD_{0\,05}$ = 0.0207 $LSD_{0\,05}$ = 0.0155

There were also significant differences in ammonification rates among treatments and controls (4,33 d.f., F=6700, p<0.01) and also among soils (2,33 d.f., F=8640, p<0.01) but not in soil-treatment interaction (8,33 d.f., F=1.672, p<0.01) (Table 3). The LSDs showed that the treatment with extracts at 10% was significantly different from the other treatments and controls, except the control CMN. However, the treatments with 2% and 5% extracts were not significantly different.

Only the nitrification results for the soils A and B were analysed statistically (Table 4), because the soil C variances were not homogeneous. There were significant differences among treatments and controls (4,29 d.f., F=5899, p<0.01), but neither between soils (1,29 d.f., F=1.702, p<0.01) nor in the soil-treatment interaction (8,29 d.f., F=1.271, p<0.01). The greatest nitrification potentials were found in the treatments with 10% extract and in control CN. Nitrification decreased with decreasing concentration of extracts, and the least activity was found in control CW, which was the only one to differ significantly from the other controls and treatments. The soil-treatment interaction in CW was also significantly different from the remainder, but in the soil B none of the interactions were significantly different.

There was a significant differece in of CO_2 production between soils (2,62 d.f., F=2330, p<0.01), in the soil-treatment interaction (8,62 d.f., F=181.6, p<0.01) and among treatments and controls (6,62 d.f., F=1238, p<0.01) (Table 5). Production of CO_2 increased with the concentration of extracts and with the content of organic carbon of the controls. There were differences in the processes between the soils, but the scheme of function is similar in each one, as shows the LSDs of interaction in each soil.

The method employed to obtain nodular extracts has limitations, but it is sought to maximize the exudation conditions (Klein *et al.*, 1989). Consequently our results are only approximations to the real effects in natural conditions. Differences

Table 4. Nitrification (ppm of NO_3^- produced) in the different soils and treatments (means ± standard error). The mean of three soils for each treatment appears in each column ($\bar{x}$). Five treatments and controls per soil appear in each row ($\bar{y}$). The LSD (treatments and controls, left and soils, right) are also given.

	Treatments and Controls					
Soil	10%	5%	2%	CW	CN	
A	0.370 ±.030	0.250 ±.016	0.140 ±.013	0.061 ±.004	0.370 ±.036	0.300
B	0.180 ±.010	0.210 ±.049	0.260 ±.044	0.150 ±.019	0.170 ±.048	0.194
C	0.003 ±.001	−0.018 ±.004	−0.005 ±.001	−0.006 ±.001	0.004 ±.011	0.003
	0.275	0.230	0.004	0.079	0.047	$\bar{x}$ $\bar{y}$

$LSD_{0.01}$ = 0.1281 $LSD_{0.01}$ = 0.2640
$LSD_{0.05}$ = 0.0903 $LSD_{0.05}$ = 0.1591

could be due to handing of soil (the inevitable disturbance of soil structure by sieving and homogenizing the samples), called «mixing effect», and the permanent moisture during incubation, but in any case are caused by the excreted products via root or nodule, which have been pointed out by many authors (Beck & Gilmour, 1983; Bhat, 1973; Gerlach, 1973; Purchase, 1974; Runge, 1983 and Svenningsson *et al.*, 1990). The above have suggest other methods of obtaining exudates and make biological assays, but all have simmilar limitations.

The moisture conditions employed in the present study did not limit nitrification, which is most intense if the moisture exceeds 65% WHC. On the other hand, 100% WHC is not optimum for ammonification (Miller & Johnson, 1964). Nevertheless, the moisture conditions did maximize diffusion of compounds in the extracts so that they reached all parts of the tested soil (Foster, 1986).

The different ammonification rates of the three soils could be explained by differences in their physico-chemical characteristics, specially pH and C/N ratio. The greater ammonification in soil A could result from its higher pH which would limit the development of the fungal microflora which immobilizes ammonium. On the other hand, the low activity or density of microorganisms in soil C, as indicated by its lower CO_2 production, could explain its low ammonification rate. The low C/N ratio of soil A might have increased its rate of ammonification (Barak *et al.*, 1983). Nevertheless our results are not in accordance with Delphin (1986) who pointed out that ammonification is greater in clay-soils than in sandy soils. We can not do more than speculate that pH is more important respect ammonification than texture.

Ammonification was lowest in the treatment with 10% extract (0.034 day^{-1}) and in the control CMN (0.047 day^{-1}); the first one provided a large amount of non-nitrogenous organic matter which the ammonifiers could use as an energy source, so that use of mineralizable nitrogen in the amino acids was unnecessary. This effect is also observable in CMN and CW (0.079 day^{-1}). The differences bet-

Table 5. CO_2 production (nmol CO_2 g^{-1} h^{-1}) in the different soils and treatments (means ± standard error). The mean of three soils for each treatment appears in each column ($\bar{x}$). Five treatments and controls per soil appear in each row ($\bar{y}$). The LSD (treatments and controls, left and soils, right) are also given.

	Treatments and Controls							
Soil	10%	5%	2%	CW	CG	CDG	CGE	
A	130.6 ± 5.8	119.8 ± 1.1	77.7 ± 8.0	68.0 ± 2.6	200.5 ± 1.6	305.1 ± 3.0	351.0 ± 1.4	179.1
B	177.7 ±11.0	125.4 ± 3.4	100.7 ± 4.2	82.2 ± 2.1	250.2 ± 1.0	254.4 ± 1.2	516.1 ± 2.4	215.2
C	46.1 ± 1.4	34.7 ± 1.0	22.9 ± 0.6	23.4 ± 0.6	50.2 ± 0.9	62.9 ± 1.2	120.1 ± 0.5	51.2
	117.5	93.3	67.1	58.8	116.9	207.5	329.4	$\bar{x}$ $\bar{y}$

$LSD_{0.01}$ = 11.29 $LSD_{0.01}$ = 6.842

$LSD_{0.05}$ = 8.194 $LSD_{0.05}$ = 5.114

ween 5% and 2% extracts (high ammonification) and 10% extracts (low ammonification) would suggest the presence of a specific inhibitor of the process. Chemical inhibition of various components of the ammonifying microflora by allelocompounds may ocur, specially by nodules of plants such as *Alnus sp.* which contain high concentrations of phenolics (Sprent, 1979) which are important allelochemicals (Rice, 1984). Nevertheless it is possible to rule out this hypothesis by following reasons: (i) the availability of carbon and nitrogen (amino acids) allows a great metabolization and immobilization, as observed by Barbarika & Sikora (1985) and Wardle & Greenfield (1991) studying *Alnus* nodules, (ii) the soil organic matter (N and C) affects strongly increasing ammonification (Powers, 1990), and (iii) a posible specific inhibitor would be detected in the production of CO_2 (as it is well known, the ammonifying microflora is the same as that of the heterotrophic) which was the case.

Our results support that the «rhizosphere effect» on ammonification from organic matter (coming from exudates) is positive in most cases (Schnurer & Roswall, 1987; Bakken, 1990), independently of the rhizosphere origin.

The significant difference in nitrification between soils A and B, could be explained by the lowest pH of the soil (Katial *et al.*,1988) as well as by the differences on clay contents (Cerna, 1967). On the other hand, the highest nitrification rates were found in CN and the 10% treatment (up to 40% with respct to CW), probably due to the supply of ammonia by the extracts and nitrification decreased with decreasing concentration of the extracts. This agrees with Montagnini & Buschbacher (1989) who pointed out that the substrate regulates the nitrification process. Also, the availability of organic carbon could affect it (Cresenzi *et al.*, 1988). Another factor affecting nitrifying activity could be phenolic compounds, which react with an intermediate nitrification product: nitrite, thus limiting formation of nitrate (Azhar *et al.*, 1986a and 1986b). However, our results do not show this effect which is important because phenolic compounds are very abundant in european alder.

The low nitrification rate observed in soil C is due to the low values of ammonium in this soil, was supplied only in the treatment with the most concentration of ammonium (that is 10% extracts) and CN. On the other hand, in this soil might also happen that with progress of succesion, nitrification is suppressed. This possibly could be explained by the presence of organic acids in soil via roots as reported by Rice & Pancholy (1972, 1973). In presence of a dominant climax vegetation, such as that of soil C sampling zone, nitrification process could be inhibited as Rice & Pancholy (1972, 1973) pointed out.

The production of CO_2 indicate the total metabolic activity of the microflora. The nodular extracts of european alder increased the metabolic activity or the growth of the soil microflora. The effects of treatments on CO_2 production were similar in the three soils, which showed only small differences related to the different amounts of carbon and nitrogen in the extracts. Nevertheless, the results obtained in the controls with organic carbon (CG and CDG) and the control CGE in the three soils suggest that the increase of CO_2 production was not only related

to organic matter. CGE was significantly higher than CDG, possibly because of the nitrogen matter of the extracts or to the presence of specific activators. The effects found on ammonification and nitrification related to nodular extracts seem directed to keep back nitrogen in soil. Similar strategies of self organization in the changes registred in mineral pools in different subsystems into which the soil may be divided, have been reported (Nannipieri *et al.*, 1985; Rapp, 1983).

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