Effects of nodular extracts of *Alnus glutinosa* (L.) Gaertn. on nitrogen fixation (Acetylene reduction assay) and denitrification in different soils

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Abstract

European alder (*Alnus glutinosa* [L.] Gaertn) nodules were maintained under conditions that allow their exudation (water stress). The effect of this nodular extracts colected on free nitrogen fixation (acetylene reduction assay [ARA]) and denitrification, was evaluated in three soils of significatively different physico-chemical characteristics. The comparisons with controls having different concentrations of glucose as carbon source suggest that, in the assayed conditions, the nodular european alder extracts activate ARA (up to 9.97% respect water control) and produces a partial inhibition of denitrification. The effects do not seem to be due neither to organic carbon nor to nitrate, but rather to specific activator(s) and inhibitor(s) of the processes considered.

Key words: *Alnus glutinosa* (L.) Gaertn., ARA, Denitrification, Exudates, nitrogen fixation, nodular extracts, water stress.

Resumen. *Efectos de los extractos nodulares de* Alnus glutinosa *(L.) Gaertn. Sobre la fijación de nitrógeno (ensayo de la reducción de acetileno) y desnitrificación en distintos suelos*

Nódulos radicales de aliso europeo [*Alnus glutinosa* (L.) Gaertn.] fueron mantenidos en condiciones que optimizan el proceso de exudación (estrés hídrico). Se evaluó el efecto de dichos extractos sobre la fijación libre de nitrógeno (ARA) y desnitrificación en tres suelos con diferencias significativas en sus características físico-químicas. Las comparaciones con diferentes controles, muestran que los extractos activan (en un 9.97%) el ARA y promueven una inhibición parcial de la desnitrificación. Los efectos no parecen causados por el aporte de carbono orgánico o de nitrato por parte de los exudados, sino por algún activador o inhibidor específico de los procesos considerados.

Palabras clave: *Alnus glutinosa* (L.) Gaertn., ARA, desnitrificación, exudados, fijación de nitrógeno, extractos nodulares, estrés hídrico.

Introduction

The rhizosphere is a region of intense microbial activity, where plant root exudates stimulate the development of a distinct rhizosphere community (Curl & Truelove,

1986; Whipps & Lynch, 1986). The rhizosphere microbial community transforms and selectively uses exudates, causing changes in residual carbon and nitrogen in the root environment. The role of the rhizosphere microorganisms in the processing and retention of nutrients in the plant-soil systems has been considered in general terms and in relation to broad attributes of ecosystem functioning (O'Neill & Reichele, 1980).

In the root exudates there are not only compounds like aminoacids, carbohydrates and lipids (Barber & Gunn, 1974; Vancura, 1967), but also a number of different substances that affect rhizosphere microorganisms: allelopatic compounds (Rice, 1984), phytoalexines (Ebel, 1986) or agglutinines (Mansfield & Brown, 1985).

Many authors have studied the effect of some diazotrophic plants on the nitrogen cycle microorganisms (Bollen & Lu, 1968; Tarrant & Trappe, 1971) in field conditions. Turner & Frantz (1985), identifyed four factors that may affect microorganisms: wash of leaves and stems, addition of organic matter by the litterfall, canopy effect, and excretion of different sustances by the roots.

The mediterranean alder riparian copse has two important characteristics during the dry season: letting down of water table (that implies exudation due to water stress) and more intensive plant growth. The aim of the present work is to study of the effect of nodular extracts (obtained under water stress conditions) of european alder (*Alnus glutinosa*) both on free-living nitrogen fixing and on denitrifying microorganisms, the main responsible agents of nitrogen inputs and outputs to the soil.

Materials and methods

Nodules were collected from a 20 years old *Alnus glutinosa* stand near Madrid. Surface of nodules was sterilized with NaClO (10 mg/l) and washed with sterile water. To obtain the exudates, collected nodules were stressed by keeping them in sterile conditions for 7 days. This conditions promove the maximal exudation due to water and photosintates stress (Klein et al., 1989). The nodules were then divided in three fractions of 100, 50 and 20 g of dry weight, each of wich were added to 1000 cm^3 of sterile water. After 12 h, the three aqueous fractions were filtred and stored at –20 °C; each fraction was the nodular extracts at 10%, 5% and 2%. Physico-chemical characteristics are represented in table 2.

To evaluate the effect of the nodular extracts on nitrogen fixiation (ARA) and denitrification, three soils of different geomorphological zones of Madrid were selected (table 1). These soils were collected near from the two dominant plants species of each zone, at 10–20 cm depth, taking 6 subsamples in each zone. The subsamples of each zone were mixed paired to obtain 3 replicates. All soils were dried at 25 °C, sieved through a 2 mm diameter sift, and stored at –4 °C. Soils shows different physico-chemical characteristics (table 2).

Total nitrogen was determined following Kjeldhal method (Bremner, 1965) using 10 g soils and 1 cm^3 of nodule extract. The organic carbon was determined following the Walkley & Black (1934) titulation method also using 10 g of soil and 1 cm^3 of nodule extracts. Nitrate was measured following Milham et al. (1970) method, in 5 g of soil and in 2 cm^3 of extract. This method uses a CRISON

digit 501 mv/pH meter with nitrate specific electrode (ORION 930700) and a ORION 900200 reference electrode. Ammonium was measured with a specific electrode ORION 951000 and the same mv/pH meter was used as in previous case; 5 g of soil and 2 cm^3 of extracts were also used. The pH was directly measured in the extracts using an pH meter BECKMAN Fi-12 with an electrode RADIOMETER GK 2401 C; the pH of soil was measured in destilled water (1:3). All measurements of the physico-chemical parameters were made in three replicates.

Nitrogen fixation was measured by the acetylene reduction assay (ARA), following Llinares et al. (1991) method: the different concentrations of nodular extracts were added at 100% Water Holding Capacity (WHC) of 100 g of each soil, in a flask of 375 cm^3 of volume. 10% of the flasks atmosphere was replaced by acetylene. Since the most important requirement of free-living nitrogen fixing bacteria is the source of organic carbon, four controls were made of each soil where extracts were replaced by the following: (1) sterile water at 100% WHC

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

	Parent material	Soil (USDA)	Texture Sand	(%) Clay	Silt	Dominant vegetation
Soil A	Arkose	Inceptisol	65	13	21	*Quercus rotundifolia* *Retama sphaerocarpa*
Soil B	Methaplutonic	Ustic	23	52	23	*Pinus sylvestris* *Cytisus purgans*
Soil C	Gypsum-Clays	Entisol	52	18	28	*Quercus coccifera* *Stipa tenacissama*

Table 2. Results of characterization of soils and nodular extracts (means ± standard error), and differences of the corresponding LSDs are representated by differents letters when there were statistical differences. Units: Organic C (%), Total N (mg/g), $N\text{-}NH_4^+$ (μg/g), $N\text{-}NO_3^-$ (μg/g).

	Soil A	Soil B	Soil C
pH	$7.30 \pm .01^a$	$4.30 \pm .00^b$	$8.01 \pm .02^c$
Organic C	$1.84 \pm .04^a$	$6.36 \pm .12^b$	$0.32 \pm .05^a$
Total N	$12.5 \pm .22^a$	$9.54 \pm .22^b$	$1.19 \pm .04^c$
$N\text{-}NH_4^+$	$2.68 \pm .31^a$	$4.71 \pm .31^b$	$0.78 \pm .09^c$
$N\text{-}NO_3^-$	$1.53 \pm .19^a$	$2.45 \pm .25^b$	$1.17 \pm .04^a$
	Extract 10%	**Extract 5%**	**Extract 2%**
pH	$4.66 \pm .01^a$	$5.20 \pm .00^b$	$5.51 \pm .00^c$
Organic C	$2.66 \pm .01^a$	$1.92 \pm .00^b$	$1.20 \pm .01^c$
Total N	$1.30 \pm .07^a$	$0.81 \pm .07^b$	$0.46 \pm .06^c$
$N\text{-}NH_4^+$	$0.44 \pm .00^a$	$0.16 \pm .00^b$	$0.10 \pm .00^b$
$N\text{-}NO_3^-$	$3.57 \pm .00^a$	$1.73 \pm .08^b$	$0.58 \pm .00^c$

(CW); (2) glucose in the same concentration of organic carbon found in the most concentrated extracts (10%) —that is 2.66%— plus sterile water at 100% WHC (CG); (3) twice the concentration of glucose in (2) —5.32%— (CDG); and (4) a control having both 2.66% of glucose and extracts at 100% WHC (CGE). The flasks were incubated 24 h at 25 °C . The ethylene produced was determinated by a KONIK 3000 HRGC gas chromatograph equipped with a flame ionization detector KNK 019-421, and PORAPAK R column, together with a KONIK 825-318 integrator. A control without acetylene was made to prove that spontaneous emission of ethylene did not occur. Three replicates of each treatment and control were made.

The potential of denitrification was estimated by the method of acetylene inhibition (Yoshinari & Knowles, 1976), in the conditions proposed by Llinares et al. (1991), that are at 100% WHC of soils. Different concentrations of nodular extracts were added to 375 cm^3 flasks, containing 50 g of soil. 10% of the headspace was replaced by acetylene. Controls identical to those for ARA were employed. After 96 h of incubation at 25°, the nitrous oxide produced was measured by a gas chromatograph KONIK 3000 HRGC with a termal conductivity detector KNK 019-501, and a CROMOSORB 101 column together with a KNK 825-318 integrator. Three replicates of each treatment and control were made.

The data were subjet to analysis of variance (ANOVA), two-way with three replicates in the experiments or one-way three replicates in the characterizations (Sokal & Rohlf, 1979). Differences between treatments were recognized by multiple comparisons employing the Least Significant Diference (LSD) procedure.

Results and discussion

The characterization of extracts and soils (Table 2) showed strong differences in the physico-chemical parameters. The extracts have an important amount of organic carbon and nitrate (2.66% and 3.57 μg/g respectively), in the most concentrated extracts (10%).

The results of nitrogen fixation are shown in Table 3. The ANOVA revealed significant differences between treatments and controls (6.42 d.f., $F = 310.6$, $p < 0.01$) and also between soils (2.42 d.f., $F = 8.941$, $p < 0.01$) but not for their interaction (12.42 d.f., $F = 1.229$). The fact that ARA increases both with the concentration of extracts and with that of glucose, suggests that this increment may have been caused by the source of organic carbon as pointed out by many (Child, 1981). However the existence of significant differences between control CDG and that CGE (60.33% of increase in the second case), indicates that the increase in ARA depends not only of the total amount of organic carbon because it is identical for both controls but it is only in form of glucose in CDG and in glucose plus exudate in the second one. These results point out that the nodular extracts of european alder have some specific activator(s) of freeliving nitrogen fixing bacteria. This is not in accordance with previous studies which show that the increase of the ARA in the rhizosphere is simply caused by the presence of organic matter (Beck & Gilmour, 1983; Bermúdez de Castro & Gutiérrez Mañero, 1987). Nevertheless, other authors pointed out that the ARA in soils increased with sustances like molybdenum, cofactor of nitrogenase (Grant & Binkley, 1987).

It stands to reason that the presence of such element (molybdenum) in the nodular extracts of *Alnus* which come from the nitrogenase of the endophyte *Frankia*, could be the cause of the increase observed in the ARA. Since it was found in the three soils assayed, the activator effect on the ARA must exist independently from the physico-chemical characteristics of the soil. Agreing with this, it must be pointed out that the interaction between soil and treatments is significative.

The results of denitrification appears in Table 3. The ANOVA showed significant differences between soils (2.42 d.f., $F = 170.1$, $p < 0.01$) as well as among treatments and controls (6.42 d.f., $F = 8.876$, $p < 0.01$), soil x treatment interaction was non significant (12.42 d.f., $F = 1.954$).The denitrification decreases with the increasing concentration of extracts, and increases with the concentration of glucose in the controls, except for CDG. The production of nitrous oxide in the treatments with extracts is higher than in the controls. Presumably the presence of the sustrate of reaction (nitrate) allows a higher activity than in the controls with glucose. The increase of denitrification with a source of carbon and nitrate has already been reported (Standford et al., 1975; Star & Parlange, 1975; Smith & Tiedje, 1979). On the other hand, when comparing the control CDG and CGE, the denitrification is significantly higher in the first than in the second one (43.87% plus). We think that this difference could be caused by the existence of a specific inhibitor(s) of the denitrifying microorganisms. Inhibitory effects on denitrification in soil contacting the roots have not been described, but it could answer to strategies similar to those pointed out in other processes (e.g. inhibition of nitrification, Moore & Waid, 1971; Baldwin et al., 1983). Besides this, it is possible that

Table 3. ARA and denitrification in the different soils and treatments (means ± standard error). Three soils mean for each treatment appears in each column (x). The LSD (treatments and controls) values appears as well. 10%, 5% and 2% nodular exudates at 10%, 5% and 2% respectively (assayed at 100% WHC); CW sterile water at 100% WHC; CG glucose at 2.66% plus sterile water at 100% WHC; CDG twice the concentration of glucose in CG —5.32%—; CGE control having both 2.66% of glucose and extracts at 100% WHC (CGE).

Treatments and controls							
	10%	**5%**	**2%**	**CW**	**CG**	**CDG**	**CGE**
	ARA (nmol ethylene g^{-1} h^{-1})						
Soil A	0.352 ±.045	0.056 ±.002	0.030 ±.005	0.077 ±.000	0.701 ±.088	1.383 ±.180	2.332 ±.166
Soil B	0.505 ±.155	0.049 ±.004	0.033 ±.005	0.017 ±.002	0.808 ±.004	1.501 ±.001	2.399 ±.019
Soil C	0.192 ±.034	0.030 ±.003	0.016 ±.001	0.016 ±.000	0.381 ±.009	1.326 ±.035	2.059 ±.016
x =	0.349	0.045	0.027	0.035	0.631	1.412	2.264
	$LSD_{0.01}$= 0.200		$LSD_{0.05}$= 0.145				
	Denitrification (nmol N_2O g^{-1} h^{-1})						
Soil A	4.133 ±.359	4.858 ±.262	5.889 ±.446	3.523 ±.160	3.877 ±.047	6.160 ±.051	4.070 ±.023
Soil B	2.976 ±.000	4.372 ±.030	4.636 ±.038	1.910 ±.067	2.230 ±.029	4.213 ±.023	3.080 ±.012
Soil C	0.223 ±.030	0.300 ±.006	1.277 ±.088	0.413 ±.021	0.490 ±.070	0.303 ±.007	0.270 ±.009
x =	2.444	3.176	3.884	1.949	2.199	3.558	2.473
	$LSD_{0.01}$= 1.031		$LSD_{0.05}$= 0.748				

exists a limitation in growth caused by other microbial groups. Nevertheless, the differences can not be due neither to organic carbon (present in both controls) nor to nitrate, which is more concentrated precisely in the control CGE.

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