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ORIGINAL PAPER

Nitrous oxide emissions from soil during soybean [(*Glycine max* (L.) Merrill] crop phenological stages and stubbles decomposition period

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Abstract The purpose of this study was to evaluate, during the phenological stages of inoculated soybean crop [*Glycine max* (L.) Merrill], the effect of different N fertilization levels and inoculation with *Bradyrhizobium japonicum* on N_2O emissions from the soil. Gas emissions were evaluated at field conditions by the static-chamber method. Nitrogen fertilization increased N_2O emissions significantly ($P<0.05$). The variable that best explained cumulative N_2O emissions during the whole soybean growing season was the soil nitrate level ($r^2=0.1899$; $P=0.0231$). Soil moisture presented a greater control on N_2O emissions between the grain-filling period and the crop commercial maturity ($r^2=0.5361$; $P<0.0001$), which coincided with a positive balance of the available soil N, as a consequence of the decrease in crop requirements and root and nodular decomposition. Only soil soluble carbon ($r^2=0.29$; $P=0.019$) and moisture ($r^2=0.24$; $P=0.039$) were correlated with N_2O emissions during the residue decomposition period. The relationship between soil variables and N_2O emissions depended on crop phenological or stubbles decomposition stages.

Keywords Denitrification · Nitrification · Greenhouse effect · Nitrogen

Introduction

Nitrous oxide, N_2O, is an important atmosphere component, which absorbs infrared radiation thus contributing to the greenhouse effect (Mosier et al. 1996), and is produced in soil mostly by nitrification and denitrification processes (Rochette et al. 2004). Agricultural systems are considered responsible for about 20 to 70% of the anthropogenic N_2O that is released to the atmosphere (Marinho et al. 2004). It has been considered that around 10 Tg of $N–N_2O$ is directly injected to the atmosphere every year only as result of fertilizers use (Watson 1992).

Several parameters were identified to affect the rate of N_2O production from agricultural systems, including N availability (Bouwman 1996; Brown et al. 2000; Maggiotto et al. 2000), temperature (Goodroad and Keeney 1984; Castaldi 2000), pH (Daum and Schenk 1998; Mogge et al. 1999), and soil moisture (Dobbie et al. 1999; Zheng et al. 2000). Mosier et al. (1996), which suggested that the conditions that increase N_2O production are high mineral N and organic C availability and middle-high moisture level that limits oxygen (O_2) diffusion.

The introduction of leguminous plants in agricultural systems can increase food production, but the biological nitrogen fixation can contribute to the N_2O emission in several ways (Yang and Cai 2005). Duxbury et al. (1982) suggested that leguminous plants may increase N_2O emissions two to three times with respect to nonfertilized soils. The *Bradyrhizobium japonicum* bacteria that are associated to the leguminous crop in radical nodules can fix atmospheric N but also carry out denitrification with production of N_2O (Mosier et al. 1996). The soybean [(*Glycine max* L.) Merrill] is one of the most important crop components in Argentina, occupying an area of about 15.2 million hectares with a grain production of 36.5 million tons (INDEC 2005). The soybean crop can affect N_2O emissions in different ways: by taking up water and NO_3^- from soil, thus reducing N_2O emissions, or by releasing soluble C and N with increase in microbial activity and

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consequent reduction in the rhizosphere O_2 concentration, thus increasing N_2O emissions.

The stubbles C/N ratio is inversely related to the initial decomposition rate of stubbles: therefore, crops with a low C/N ratio like soybean display high decomposition rates, with possible release of NO_3^-, and this may increases in N_2O emissions (Aulakh et al. 1991a). However, most of the published studies regarding N_2O emissions from soil under leguminous crops depended on the growing season (Aulakh et al. 1991a; Marinho et al. 2004; Yang and Cai 2005). At the present, there is little knowledge about N_2O emissions from the soil under soybean growing season and during stubbles decomposition. It has been hypothesized that N_2O emissions can increase because of inoculation with nitrogen-fixing bacteria and N fertilizers application, common practices for the soybean crop grown in the Argentinian Pampean Region (Ciampitti et al. 2005). The aim of this work was to study the effects of the inoculation of soybean with *B. japonicum* on N_2O evolution during all phenological stages and during the stubbles decomposition period in the presence of N fertilizers.

Materials and methods

Soil characteristics

The experiment was carried out in the experimental field of the Agronomy College of the Buenos Aires University, located in Buenos Aires city, Argentina. The soil is a silty clay loam (34% clay, 58% silt, and 8% sand) and was classified as a typic Argiudoll. At the beginning of the experiment, the soil presented the following chemical properties: $N-NO_3^-$ 4.08 mg kg^{-1}, content of organic C (Cox) 2.15%, total N (Nt) 0.20%; pH in a 1:2.5 soil/distilled water suspension 6.8, and extractable P 89 mg kg^{-1} (Bray and Kurtz 1945).

Field experiment, sampling, and analyses

The experimental field was divided in plots of 2×2 m, and the experimental design was a completely randomized design, with a factorial adjustment of three by three, with inoculation and fertilization as factors. Each treatment was replicated four times. Inoculation treatments were (1) plots sown with soybean inoculated with *B. japonicum* (P_i), (2) plots sown with soybean without inoculation (P_{ni}), and (3) without crop (P_0). The fertilization treatments were: without fertilization (N_0), and fertilization with 15 (N_1) and 30 (N_2) kg N ha^{-1}; the N fertilizer was urea, the most used N fertilizer in Argentina. Nitrogen fertilizers were only applied at the beginning of the experiment. Soybean seeds were inoculated with a liquid solution containing 10^8 number of microorganism per ml of inoculant. On December 28, 2004, a transgenic soybean with RR gene (gliphosate resistant), cultivar "Don Mario" 4800, maturity group IV, having an indeterminate growth habit, was sown on plots. On May 3, 2005, 126 days after sowing, the soybean crop was manually harvested. Weeds were controlled with gliphosate herbicide applications. The length of the experiment included the soybean growth and harvest and the stubbles decomposition period, which lasted 80 days after harvest.

N_2O measurements were done by the closed static-chamber method with PVC cylinders, 15 cm in length and 11 cm as internal diameter, according to Khera et al. (1999). The superior part of the cylinder was hermetically closed with a rubber septum cover through which the gaseous samples of the internal atmosphere were taken with a syringe. The cylinders were buried 8 cm into the soil and sealed carefully to prevent gaseous losses. Each measurement involved gas accumulation for 24 h into cylinders, to account for variations in daily temperature (Aulakh et al. 1991b) because it has been registered that the maximum can be five times higher than the minimum day flux (Williams et al. 1999). Three gaseous samples taken from the atmosphere were used as blanks. Once the samples were taken, they were immediately brought to the laboratory and analyzed by gas chromatography. The gas samples were taken every 2 weeks along the experiment and the sampling times corresponded to the following crop phenologic stages: sowing (S), one knot (V1), three developed knots (V3), flowering (R1), beginning of pods formation–fructification (R3), grain filling (R5.5), maximum grain size (R6.5), physiological maturity (R8), 17 days after harvest, the commercial maturity (MC), 59 days after harvest, the residues presence (R), and 80 days after harvest, the residues decomposition (RD). In case of rainfall, sampling frequency was altered so as to include these events and to obtain reliable measurements (Sexstone et al. 1985). We used a gas chromatograph 6890 Agilent with ECD detector and a hair column Carboplot, using helium as carrier gas; the work temperatures were 100°C for the oven and injector and 250°C for the detector.

On the same days of gas sampling, soil samples (0–20 cm) were taken from plots. Moist soils were used for measuring soil moisture and NO_3^- content and air-dried (at 40°C followed by sieving at 2 mm) soils for measuring soil pH, soil organic C, and water soluble carbon.

Soil pH was measured in a 1:2.5 soil/distilled water suspension using a precalibrated glass electrode (Thomas 1996). Soil organic C was evaluated by the Walkley–Black wet oxidation method (Nelson and Sommers 1982). Water-soluble C (Mazzarino et al. 1993) was extracted by stirring

soil samples with distilled water (solid phase/solution 1:50) for 24 h at room temperature. The suspension was centrifuged at 19,500×*g* for 10 min, and the supernatant was filtered through a 0.4-μm glass fiber container under vacuum filtering. Water-soluble C was determined by the dichromate oxidation method as reported for the determination of the organic C content. Soil moisture was calculated as the difference between fresh and dry weight (after drying at 105°C till constant weight). The $N-NO_3^-$ content of the moist superficial soil layer (0–20 cm) was determined by shaking 20 g of soil with 100 ml 0.25% $CuSO_4$+0.01 M BO_3H_3 solution: The suspension was filtered and NO_3^- determined by the hydrazine-reduction method (Carole and Scarigelli 1971).

Statistical analysis and calculations

We analyzed the fulfillment of the assumptions of variance homogeneity and normal distribution of the N_2O emissions, by log-transforming emissions when necessary. Cumulative N_2O emissions data were analyzed by standard two-way variance, with general linear procedures of the SAS Statistical Package (SAS Institute 1999) and with later separation of means by tests of multiple comparisons according to Duncan. Regression analyses between cumulative N_2O emissions and the measured soil properties were done by the SAS PROC REG procedure by considering either the whole experiment or each period.

Results and discussion

Evolution of nitrous oxide emissions

Nitrous oxide emissions were low and stable during 100 days after sowing and increased after the grain-filling step. The greatest values occurred between grain filling and commercial maturity, in all treatments, even in those without plants (P_0), and as an average, 1,348 μg $N–N_2O$ m^{-2} h^{-1} represented about 68% of total N_2O emissions (Fig. 1). The greatest emissions (5,516 μg $N–N_2O$ m^{-2} h^{-1}) were observed at commercial maturity, i.e., 142 days after crop sowing, in plots fertilized with the highest N rate and inoculated with N-fixing bacteria (N_2P_i; Fig. 1c). Yang and Cai (2005) also found out that about 94% of the total N_2O emissions were concentrated in the last stages of the soybean crop cycle. Therefore, it is possible to consider this period as the most critical one for N_2O emissions, during the soybean crop cycle (Ciampitti et al. 2005). During this period of high N_2O losses, which is about 20 days before and 20 days after the harvest, the trend in N_2O emissions was similar to that of $N-NO_3^-$ levels and soil moisture contents (Fig. 5).

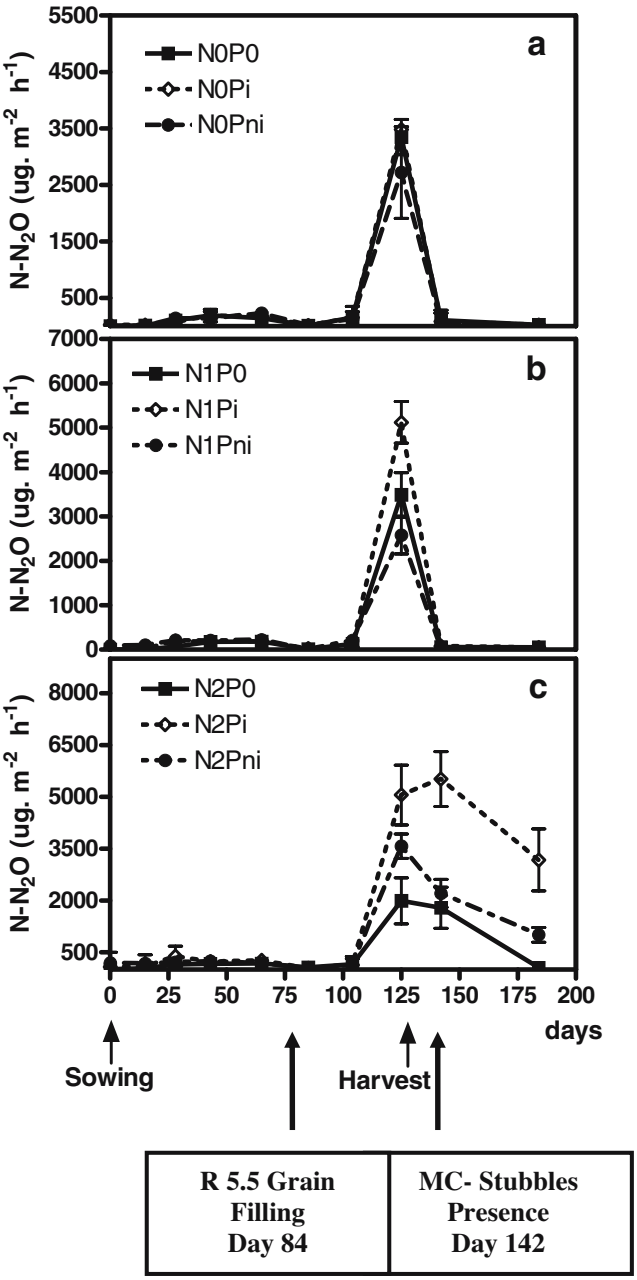

Fig. 1 Nitrous oxide emissions from plots without soybean plants (*P0*), with inoculated soybean (*Pi*), and with not inoculated plants (*Pni*), under the following treatments: not fertilized—*N0* (**a**), fertilized with 15 kg N ha^{-1}—*N1* (**b**), and fertilized with 30 kg N ha^{-1}—*N2* (**c**). Points are average of three replications for each treatment. *Error bars* represented standard deviation

Effects of fertilization and inoculation on nitrous oxide emissions

Significant effects of the interaction between N fertilization and inoculation (P=0.03) were detected on cumulative N_2O emissions during the crop cycle. When the plots were fertilized with liquid urea, cumulative N_2O emissions

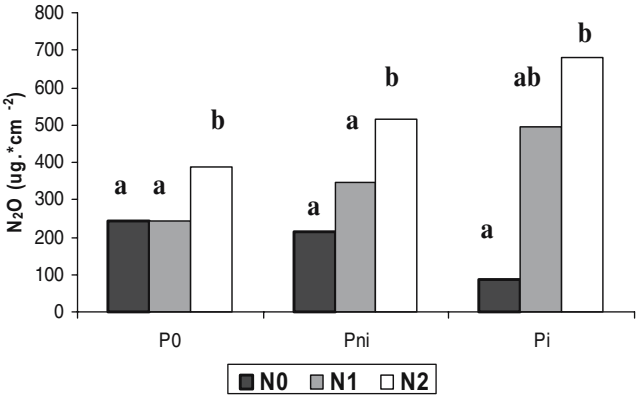

Fig. 2 Effect of the N fertilization (without fertilization—*N0*, 15 kg ha $^{-1}$—*N1*, and 30 kg ha $^{-1}$—*N2*) on cumulative N_2O emissions, from plots without plants (*P0*), with not inoculated soybean plants (*Pni*), and plants inoculated with *Bradyrhizobium japonicum* (*Pi*). The separation of treatments was made by tests of multiple comparisons according to DUNCAN. *Column with different letters* are significantly different

increased with soybean plants, especially if seeds were inoculated with microorganisms (P_i>P_{ni}>P_0), whereas in unfertilized plots, the crop presence decreased N_2O emissions (P_0>P_{ni}>P_i; Fig. 2). Nevertheless, the inoculation factor did not significantly affect N_2O emissions (P>0.05) at any fertilization level.

In the treatments with inoculated plants, nodulation was observed in the secondary soybean roots. The average number of nodules at flowering stage was 15 in the inoculated plants (P_i) and 6 in the noninoculated plots (P_{ni}), independently on the fertilization levels. Some authors suggest that biological N fixation is an important N_2O source (Mosier et al. 1996; Mosier 1998). Breitenbeck and Bremner (1989) suggested that although symbiotic fixing bacteria are able to denitrify NO_3^- under anaerobic conditions, the population of these microorganisms is very small to have any important influence on denitrification rate in the soil.

Nitrogen fertilization significantly increased cumulative N_2O emissions (Fig. 2), thus confirming what already reported (Kaiser et al. 1998; MacKenzie et al. 1998; Weitz et al. 2001; Ghosh et al. 2002). Fertilization treatments generated different N_2O emissions, which were greatest at inoculated soybean plants; fertilization at 30 kg N ha^{-1} (highest rate) increased N_2O emissions by 1.6, 2.4, and 7.8 times in treatments without crop and not inoculated and inoculated plants, respectively. The percentage of N fertilizer lost as N–N_2O during all experiment (about 7 months) was 0.55 and 1.97%, at fertilization rates of 15 and 30 kg N ha^{-1}, respectively. These values are close to those reported by Bouwman (1996) and adopted per year by the Interguvernamental Panel of Climate Change (Houghton et al. 2001), although the period of our study was shorter than 1 year. Henault et al. (1998) found the same emission factors at any fertilizer rate.

Plant tissues with a low C/N ratio (mean C/N=44, data not shown) such as soybean may stimulate microbial decomposition and release of easily available C and N, favoring N_2O emissions (Aulakh et al. 1991a). Highest emissions with a high level of N fertilization and with inoculated plants has been explained by Luciñski et al. (2002), as a result of the occurrence of "denitrification as a complementary process to nitrogenase"; indeed, high NO_3^- disemilatory reductase (NR) activity is characteristic of many symbiotic associations between legumes and rhizobia bacteria. In most species, this enzyme only occurs in nodule cytosol, but in soybean, dissimilatory NR activity has been also detected in bacteroids, where it accounts for 90% of total nodule NR activity (Hunter 1983). The synthesis of this enzyme is induced by NO_3^- inside of bacteroids, and it does not depend on the type of *Rhizobium* strain investigated (Arrese-Igor et al. 1990). Denitrification may be involved in detoxification when high-cytosol NR activity can accumulate nitrite (Heckman and Drevon 1987). It would be important to measure the contribution of inoculated fixing bacteria to N_2O emissions because if inoculated bacteria succeed in infecting the plant roots, the respective bacteriods may potentially be responsible of high N_2O losses as they are carbohydrate rich.

Soil controls of N_2O emissions during soybean crop-growing season

Fertilization effect may also be observed by the significant correlation between N_2O emissions and NO_3^- levels (r^2= 0.19; P=0.02) during the growing season (Fig. 3a), as urea is rapidly hydrolyzed and converted to NO_3^- in well-drained

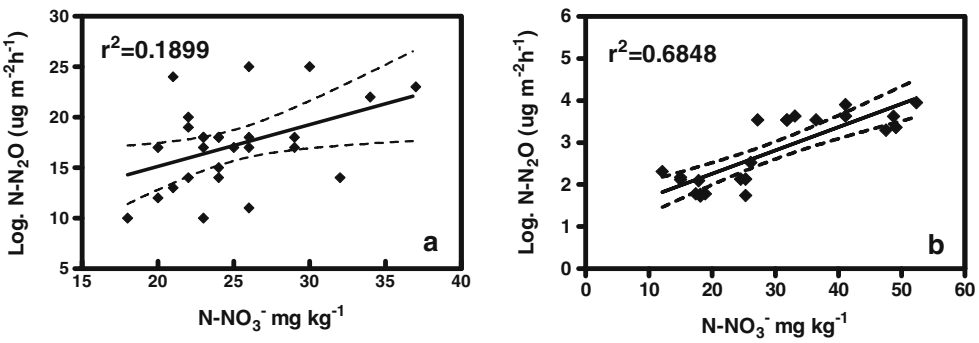

Fig. 3 Relationship between log transformed N–N_2O emissions and N – NO_3^- in all treatments (**a**), only for inoculated plants during the soybean crop cycle (**b**). *Dotted lines* represent 95% confidence intervals

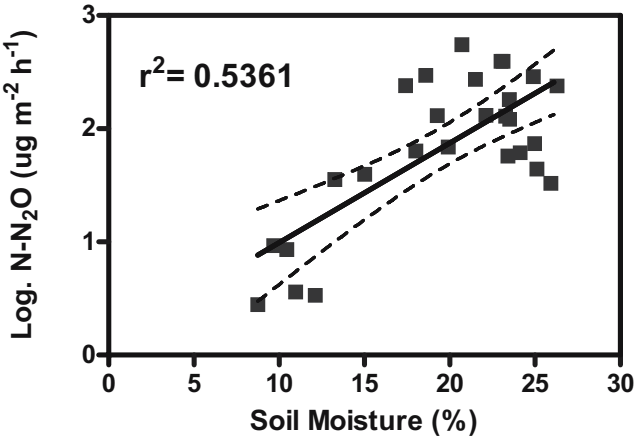

Fig. 4 Relationship between the log-transformed N–N_2O emissions with the soil moisture during the grain-filling period to commercial maturity of the soybean crop. *Dotted lines* represent 95% confidence intervals

soils (Dobbie and Smith 2003). Bremner (1997) stated that low NO_3^- concentrations could slow down N_2O reduction to dinitrogen (N_2), whereas high concentrations would almost completely inhibit this process (Stevens and Laughlin 1998). As NO_3^- is lost by denitrification, N_2, rather than N_2O, becomes the main produced gas (Stevens and Laughlin 1998) because NO_3^- is preferred with respect to N_2O as an electron acceptor (Schlegel 1992). The correlation between N_2O emission and NO_3^- levels is even more significant with inoculated plants (r^2=0.68; P<0.0001; Fig. 3b) probably because rhizodeposition and roots decomposition during the end of the growing season release C that stimulated growth of heterotrophic microorganisms, including denitrifying bacteria.

Soil moisture is considered one of the main factors regulating N gaseous emissions (McTaggart et al. 1997; Clayton et al. 1997; Dobbie et al. 1999) because it affects the soil redox status (Rowell 1981). However, in the present work, there was not a significant relationship between N_2O emissions and soil moisture, considering the whole soybean-growing season. Soil moisture was only significantly (r^2=0.53; P<0.0001) correlated with N_2O emissions (Figs. 4 and 5) between the grain-filling period and crop commercial maturity, when there was an increase in the amount of available N in the soil, probably because of a decrease in N uptake by the crop and decomposition of N-rich root and nodular debris (Yang and Cai 2005). The interplay between soil moisture and NO_3^- can explain the smaller N_2O emissions occurred from sowing to the beginning of fructification–pods formation (R3), when N_2O emissions decreased, soil moisture was high (30%), and NO_3^- content was low because of crop uptake (Fig. 5).

During the period of the highest N_2O emissions (R5-CM), the variables that were best correlated to these emissions were soil moisture and $N-NO_3^-$ contents; both

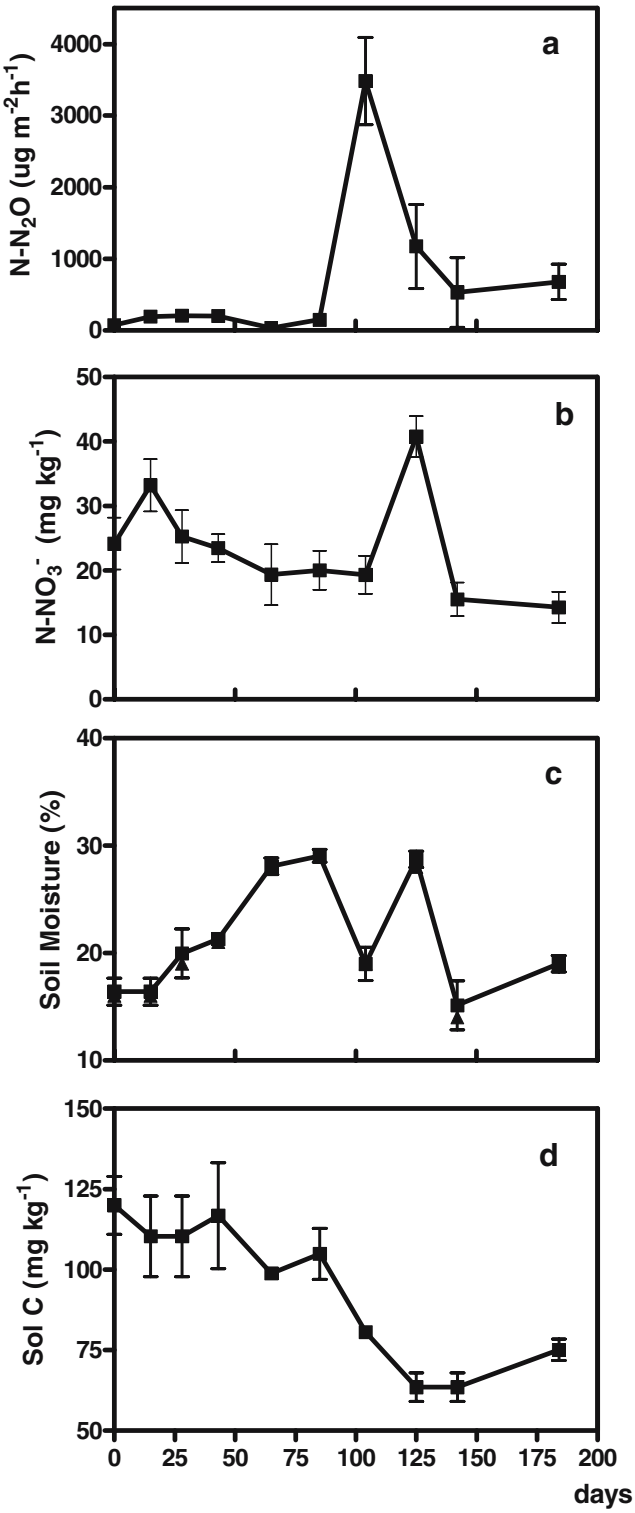

Fig. 5 Evolution of the N–N_2O emissions (**a**) and soil $N-NO_3^-$ contents (**b**), soil moisture (**c**), and soluble C content (**d**), during the soybean-growing season. Points are average from all treatments. *Error bars* represented standard deviation (SD)

variables explained the 47% of the emissions variability; pH values were not related to N_2O emissions, probably because these properties showed little changes. Multiple regression analyses were significant (P=0.0018; r^2=0.47);

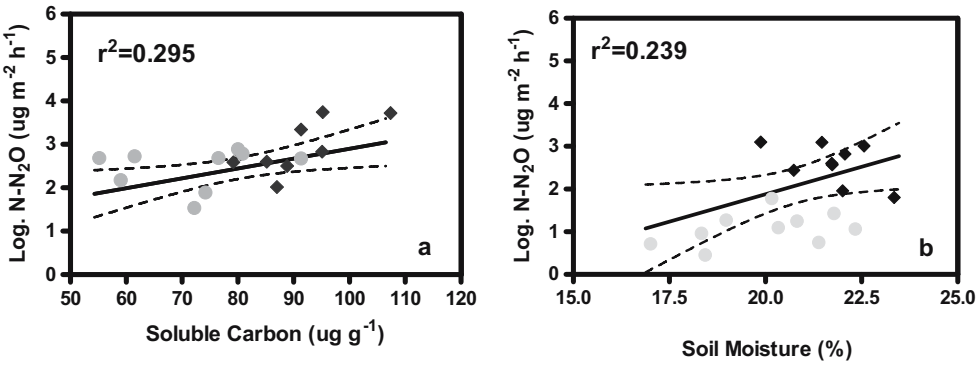

Fig. 6 Relationship between N_2O emissions with soluble C (**a**) and soil moisture (**b**) during the stubbles decomposition of the soybean crop. The treatments were separated in: inoculated (Pi, *black diamonds*) and not inoculated (Pni, *gray circles*). *Dotted lines* represent 95% confidence intervals

soil moisture (P=0.0008) and NO_3^- (P=0.05) positively and significantly correlated with N emissions. The best relationship explaining the N_2O emission during soybean-growing season was

$$N_2O \text{ emissions} = -2.18 + 0.09 \times \text{soil moisture (\%)} + 0.02 \times N - NO_3^- \left(\text{mg Nkg}^{-1}\right)\left(\mu\text{g N} - N_2O\ \text{m}^{-2}\text{h}^{-1}\right)$$

Period of stubbles decomposition

After commercial maturity, N_2O emissions decreased (Fig. 1). The postharvest period represented only 30% of the total experiment duration, and cumulative emissions, 1,068 μg N–N_2O m^{-2} h^{-1}, during this period constituted 28% of total N_2O emissions. Despite the important fluxes at the postharvest period, the highest fluxes occurred at the preharvest period (R5-MC) of the soybean crop. This contradicts that observed by Smith et al. (1998), who found that the highest fluxes were observed in the postharvest period of potato crops; these differences with our work may be due to different plant senescence dynamics, N leaf concentration, and their interactions with soil moisture. It was suggested that labile C from crop residues promoted sufficient aerobic respiration to induce the formation of anaerobic microsites. Indeed, we found a significant relationship between N_2O emissions and soluble C (r^2= 0.29; P=0.019) during stubbles decomposition (Fig. 6a); heterotrophic bacteria, including denitrifiers are controlled by C availability under aerobic conditions (Palma et al. 1997). In average, higher yields of inoculated plots, 3,207 kg ha^{-1}, with respect to that of noninoculated plants, 2,627 kg ha^{-1}, contributed with a great amount of stubbles, with a lower C/N ratio (data no shown).

Soil $N - NO_3^-$ levels were greater than 15 mg kg^{-1}, but they were not correlated with N_2O emissions during this time interval, probably because these values were not limiting the N_2O generating processes. Van Kessell et al. (1993) found that values lower than 10 mg kg^{-1} $N - NO_3^-$ concentrations were not limiting denitrification processes, unless favorable conditions for NO_3^- reduction were present Sainz Rozas et al. (2001) reported that concentrations lower than 40 mg kg^{-1} $N - NO_3^-$ were limiting for denitrification in typical Argiudoll and petrocalcic Paleudolls soils of the Argentinian Pampean Region. Soil moisture during the period of stubbles decomposition was significantly correlated with N_2O emissions (r^2=0.24; P=0.039; Fig. 6b).

To analyze the effect of stubbles decomposition, data from treatments without plants were excluded in the multiple regression analysis; the relationship was significant (r^2=0.50; P=0.046). Soil moisture (P=0.038) and soluble organic C (P=0.045) content were correlated positively and significantly with N_2O emissions, whereas soil NO_3^- content did not influence the magnitude of these losses during the process of stubbles decomposition, confirming the results of the simple linear regressions. The complete model that best explained the emissions during stubbles decomposition period was:

$$N_2O \text{ emissions} = 12.75 + 0.25 \times \text{moisture (\%)} + 0.03 \times \text{soluble C} \left(\text{mg kg}^{-1}\right)\left(\mu\text{g N} - N_2O\ \text{m}^{-2}\text{h}^{-1}\right)$$

Conclusion

Nitrous oxide emissions increased during the soybean growing season, with the highest accumulation from grain filling until commercial maturity, representing, as an average 1,348 μg N–N_2O m^{-2} h^{-1}, approximately 68% of total N_2O emissions. Nitrogen fertilization affected N_2O losses especially with inoculated soybean plants. Significant correlation was observed between N_2O emissions and soil NO_3^- contents with inoculated plants, suggesting that the main controlling variable of N_2O emissions was NO_3^- contents until harvest. Soil moisture and soluble C but not NO_3^- content was significantly correlated with the emissions during the stubbles decomposition period. The relationship between controlling factors, soil variables, and N_2O emissions depended on crop phenological or stubbles decomposition stages.

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